CONFIDENTIAL

Laub BioChem

HUMATE SAFETY, PHARMACOKINETICS, AND EFFICACY

1401 Quail Street, Suite 121, Newport Beach, CA 92660

tel (888) 664 - 8642 fax (949) 476 - 9885

www.LaubBioChem.com

TABLE OF CONTENTS

What Is Humic Acid?

Overview of Nutriceutical Substances Derived from Humic Acid

Safety

(6) M. E. Botes, J. Dekker, and C. E. J. van Rensburg. Phase I trial with oral oxihumate in HIV-infected patients. *Drug Devel. Res.* **2002**, *57*, 34-39.

humic acid administered to thirty-seven antiretroviral-naïve adults in a randomized placebo-controlled double-blind study was well tolerated and showed an excellent safety profile when tested over 2 weeks (study was too short to establish drug efficacy, optimal dosage levels)

(7) U. N. Riede and B. Seubert. Huminates, process and uses. **US** 5,284,651 (February 8, 1994).

large-molecule as well as small-molecule synthetic humic acids exhibit minimal toxicity while still exhibiting pharmacological activity

Pharmacokinetics

(8) J. Hampl, I. Herzig, and J. Vlcek. Pharmacokinetics of sodium humate in chickens. *Vet. Med. (Praha),* **1994**, *39*(6), 305-313.

free as well as liposome-encapsulated humates were administered to chickens intracardially, orally or subcutaneously; pharmacokinetic parameters were then determined, including elimination half-life, steady-state distribution volume, blood clearance, maximal drug concentration, time required for appearance of maximal drug concentration, area under the curve, and bioavailability

Skin Penetration Kinetics

(9) W. Wohlrab, B. Helbig, R. Kloecking, and M. Sprossig. Penetration kinetics of potential virustatics into the human skin. *Pharmazie* **1984**, *39*(8), 562-564.

a synthetic humate penetrated skin quickly from a 1% water-oil emulsion, and formed a reservoir in the horny layer; concentrations of 1-3% of the externally-applied amount were achieved

(10) H. Wagner. Topical agent for treatment of vesicular rash caused by herpes virus. **DE** 3,830,333 C1 (15 March 1990).

German patent on a humate-based topical lotion for treating herpes-induced rashes

(11) J. R. Snyman, J. Dekker, S. C. K. Malfeld, and C. E. J. van Rensburg. Pilot study to evaluate the safety and therapeutic efficacy of topical oxifulvic acid in atopic volunteers. *Drug Devel. Res.* **2002**, *57*, 40-43.

double-blind randomized crossover safety and efficacy studies of topically-applied 4.5% to 9% concentrations of humic acid (cream base) with 23 healthy volunteers; no significant effects on any safety parameters were found, nor did the creams induce sensitization when applied on the skin

Efficacy Against Skin Diseases

(12) X. Dong, A. Wumanjiang, B. Wen, Y. Zhang, L. Fan, and L. Ma. Ointment containing conjugated oleic acid zinc salt and humic acid sodium salt. **CN** 1,372,926 (October 9, 2002).

humate ointment used to treat skin diseases, including eczema, psoriasis, dermatitis, and skin neoplasm

(13) F. L. Jordan. Transdermal delivery system. **US** 6,946,144 (September 20, 2005).

highly effective transdermal delivery system that can deliver high molecular weight pharmaceuticals such as humates to subdermal cells for the treatment of skin diseases

Efficacy Against Herpes Viruses

(14) V. F. Schiller, R. Kloecking, P. Wutzler, and I. Farber. Topical ammonium humate treatment of human HSV infections–prelimninary clinical results. *Dermatol. Monatsschr*. **1979**, *165*, 505-509.

topical application of a 1% solution of natural-product ammonium humate was found to be efficacious in treating herpes simplex in 70 out of 78 cases tested

(15) R. Kloecking and B. Helbig. Interaction of humic acids and humic-acid-like polymers with herpes simplex virus type 1. In *Humic Substances in the Aquatic and Terrestrial Environment;* Berlin: Springer-Verlag, **1991**; 407-412.

synthetic and natural-product humates were found to be efficacious against herpes simplex type 1 at concentrations of 2-20 μg/mL

(16) R. Kloecking, B. Helbig, G. Schotz, M. Schacke, and P. Wutzler. Anti-HSV-1 activity of synthetic humic acid-like polymers derived from p-diphenolic starting compounds. *Antivir. Chem. Chemother*. **2002**, *13*, 241-249.

natural-product and 10 synthetic humates were found to be efficacious against herpes simplex type 1 at concentrations of <1-350 μg/mL

(17) K. D. Thiel, R. Kloecking, H. Schweizer, and M. Sprossig. In vitro studies of the antiviral activity of ammonium humate against herpes simplex virus type 1 and type 2. *Z. Bakt. Parasit. Infekt. Hyg. Abt. Orig. A. Med. Mikrobiol. Parasit.* **1977**, *239*(3), 304-321.

inhibition of the replication of herpes simplex virus types 1 and 2 was observed in all cell systems at concentrations of <1-20 μg/mL*; viral fusion inhibition was the predominant mechanism of drug action; prophylactic effects of humates were observed as well at >10* μg/mL

Efficacy Against Hepatitis Viruses

(18) L. M. Stepchenko, L. V. Zhorina, and L. V. Kravtsova. The effect of sodium humate on metabolism and resistance of highly productive poultry. *Biol. Nauk. (Moscow)* **1991**, (10), 90-95.

feed supplementation improved the resistance of broiler chicks to hepatitis

(19) D. I. Kuz'menko, G. N. Sidorenko, E. F. Levitskii, B. I. Laptev, and E. I. Dzhuraeva. A comparative evaluation of the effect of different types of sapropel on dynamic liver function in intact rats and in the modelling of toxic heptatitis. *Vopr. Kurort. Fizioter. Lech. Fizich. Kult.* **1998**, (2), 37-38.

natural-product humates were found to be efficacious in restoring hepatic function in rats infected with toxic hepatitis

Efficacy Against Influenza Viruses

(20) J. Hils, A. May, M. Sperber, R. Klocking, B. Helbig, and M. Sprossig. Inhibition of several strains of influenza virus type A and B by phenolic polymers. *Biomed. Biochim. Acta* **1986**, *45*(9), 1173-1179.

two natural-product and 10 synthetic humates were found in vitro to be efficacious against several strains of influenza A and B

(21) F. J. Lu, S. N. Tseng, M. L. Li, and S. R. Shih. In vitro anti-influenza virus activity of synthetic humate analogues derived from protocatechuic acid. *Arch. Virol.* **2002**, *147*(2), 273-284.

natural-product and synthetic humates were found to be efficacious against influenza A at concentrations of 45-55 and 6-55 μg/mL, *respectively*

(22) A. Jankowski, B. Nienartowicz, B. Polanska, and A. Lewandowicz-Uszynska. A randomized, double-blind study of the efficacy of Tolpa Torf Preparation (TTP) in the treatment of recurrent respiratory tract infections. *Arch. Immunol. Ther. Exp. (Warsz)* **1993**, *41*(1), 95-97.

a natural-product humate was found in a randomized double-blind placebo-controlled study with 39 patients to be efficacious when administered orally against recurrent respiratory tract infections

Efficacy Against AIDS

(23) J. Schneider, R. Weis, C. Manner, B, Kary, A. Werner, B. J. Seubert, and U. N. Riede. Inhibition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: mechanism of inhibition. *Virology* **1996**, *218*(2), 389-395.

a synthetic humate was found in vitro to prevent HIV viral fusion by interfering with a V3 loop-mediated step of virus entry

(24) G. K. Joone, J. Dekker, and C. E. J. van Rensburg. Investigation of the immunostimulatory properties of oxihumate. *Z. Naturforsch.* **2003**, *58C*, 263-267.

4 g per day of a modified natural-product humate (molecular weight 57,000-70,000 Da) was administered in vivo to a number of individuals for 2 weeks, and was found inter alia to strongly stimulate the proliferative response of human lymphocytes in HIV-infected patients; the material was therefore said to exhibit both immunostimulatory and anti-viral activity

(25) G. Kornilaeva, A. Bercovich, T. Pavlova, and E. Karamov. New humc acid derivative as a potent inhibitor of HIV-1 replication. *Med. Gen. Med.* **2004**, *6*(3), A10360.

inhibitory concentrations in vitro of a modified natural-product humate were found to range from 0.85 to 3.5 μg*/mL; the combination of humate with AZT increased the antiviral activity 30-100 times*

Proceedings of the

California Association of Chemistry Teachers

Cornelius Steelink
University of Arizona
Tucson

What Is Humic Acid?

It was probably Berzelius who first asked the question: "What is humic acid?" more than 150 years ago. Since that time, hundreds of investigators have sought for an answer to this question. Although the literature is full of references to humic acid, precious little is known about the chemical structure of this ubiquitous soil constituent. Current investigations in a dozen research laboratories throughout the world are occupied with such problems as the isolation, structure, origin, and function of this dark-brown, amorphous substance.

Why is there such persistent scientific interest in a material which has yielded so little information, despite the many fruitless years of effort spent to probe its structure? No doubt, because it has been associated with soil fertility and agricultural productivity. Perhaps it may be one of the key substances in man's ceaseless search for a more abundant food supply. Perhaps the frustrating difficulties associated with its characterization constitute a sufficient challenge to keep stubborn investigators busy in their laboratories.

Humic acid is certainly no ordinary natural product. One does not simply extract it from a convenient reproducible source, crystallize it, determine its physical properties and empirical formula, and then proceed to determine its structure by the methods of modern organic chemistry. Like lignin or tannins, there exists no simple unique "humic acid." It is usually defined as that fraction of the soil organic matter which is soluble in dilute base but insoluble in mineral acid and alcohol. Like lignin and tannin, it is described in terms of its environment and its extraction procedure. For example, a typical sample would be described as "podzol humic acid, from a B soil horizon, extracted with 0.5 *M* NaOH under nitrogen." When one obtains a sample of humic acid by basic extraction from the soil, he is aware that the material may have been significantly modified by the extraction procedure; this is also a characteristic phenomenon in lignin chemistry.

Thus, when an organic chemist looks at humic acid, he must bear in mind that this substance may best be defined in terms of its origin and soil environment, rather than in rigid terms of chemical properties. In fact, criticism has been leveled (*1*) at chemists for oversimplifying the structural problems. Some investigators have claimed to have synthesized humic acids (brown, polymeric, base-soluble materials) by cooking carbohydrates in concentrated hydrochloric acid for prolonged periods of time (*2*) or by carrying out browning reactions between monosaccharides and amines (*3*). From these reactions, they have inferred that carbohydrates are the prime builders of humic acid. However, these experiments, as well as others, have been carried out under conditions alien to ordinary soil processes; and they have been justifiably criticized on this basis.

Although this article will be primarily devoted to a review of the chemical investigations of soil humic acid, the reader should understand that a complete characterization of this substance will depend also on the results of microbiological and pedological studies.

Origin

Humic acid occurs in rotting vegetable matter and can be detected in the black slime of an ordinary compost pit in a home garden. It is found in the brown organic matter of a variety of soils, as well as in peats, lignite, and brown coals. It is apparently formed by the bacterial and chemical degradation of plant tissue, but in soils it is most likely formed by certain secondary processes: polymerization of polyphenols leached by rain from surface leaf litter and condensation of phenols, quinones, and proteins which are provided by the action of soil micro-organisms and small animals on soil carbohydrates.

There is some evidence (*4*) that humic acid may be present in the plant, due to the microbiological decomposition of cell wall tissue, in the form of a soluble phosphate complex. Figure 1 summarizes the possible sources of humic acid (*5*).

Definition

As mentioned earlier, the commonly accepted definition of humic acid is "that portion of the soil organic matter which is soluble in base and insoluble in mineral acid and alcohol." Certain non-humic substances,

This article appears as a guest contribution to the CACT section at the invitation of the CACT Editor. Dr. Steelink was at one time associated with the laboratory of Prof. Norman Kharasch at the University of Southern California.

such as polyuronic acids and complexed proteins, may also be separated from the soil by basic extraction procedures, as well as numerous metallic ions coordinated with humic acid. The use of strong base is objectionable on the ground that serious chemical transformations may take place during the separation procedure.

Conventional extraction techniques, therefore, employ dilute bases, such as cold 0.5 *M* NaOH, NaF or $Na_4P_2O_7$ in an atmosphere of nitrogen. Hydrogen fluoride has even been used, because of its ability to break humatesilica complexes, and thus put humic acid into solution.[1] Usually the soil is first treated with an alcohol/benzene solution to remove polyphenols, resins, and waxes. Ion exchange or dialysis techniques separate the major part of the inorganic constituents. Some investigators (*6*) prefer to use mild acid hydrolysis in order to remove the carbohydrate and protein fraction.

The definition of humic acid rests on an operational scheme. This may cause some concern among investigators who prefer the more precise language of organic chemistry (e.g., "white, crystalline solid, mp 51°C, etc.). However, reference to common descriptions of lignin (e.g., "Braun's native black spruce lignin; Western hemlock sulfuric acid lignin, etc.") are also imprecise in terms of classical chemical language. Nevertheless, this has not prevented the development of a considerable body of respectable chemical knowledge of the structure of lignin.

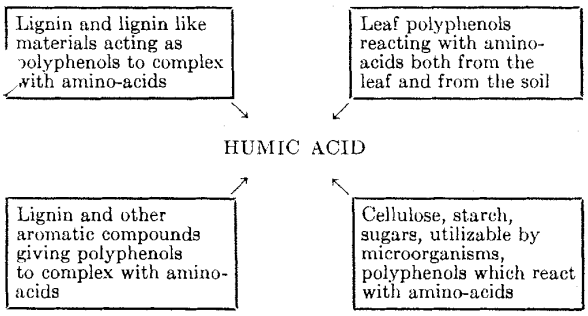


Figure 1. Humic acid formation in the soil (*5*).

Properties

Although the properties of humic acid will vary somewhat with soil origin and environment, there are certain characteristic features common to all specimens. The dark-brown material is a macromolecule and a polyelectrolyte, whose molecular weight has been estimated to range from 800 to 500,000; but whose most commonly cited molecular weight appears to be in the range $2.0–5.0 \times 10^4$ (*7*).

Of particular interest to soil scientists is the base exchange capacity and the complexing ability of humic acids. These properties are important in relation to soil stability and state of aggregation, transport of metal ions in the soil and through plant tissue, and stabilization of soil organic matter against microbiological attack.

The base exchange capacity, as measured by calcium acetate, ranges from 400 to 600 milliequivalents/100 grams of humic acid (*1, 8*). Evidence of chelate formation with metals of the transition group has been presented by Martin and Reeve (*9*). The property of complexing metals has been known for a long time; most soil humic acids have high ash contents, and are difficult to separate from these mineral constituents. Experiments with artificial soil mixtures have been carried out by Bloomfield (*10*) to illustrate the role of chelation in the formation of soil horizons. Thus, an insoluble iron-humate layer was formed several inches below the surface, when aqueous leaf extracts were poured over an iron oxide-celite mixture (Figure 2). The polyphenol solution reduced the iron(III) to iron (II) and the latter formed of dark-colored complex.

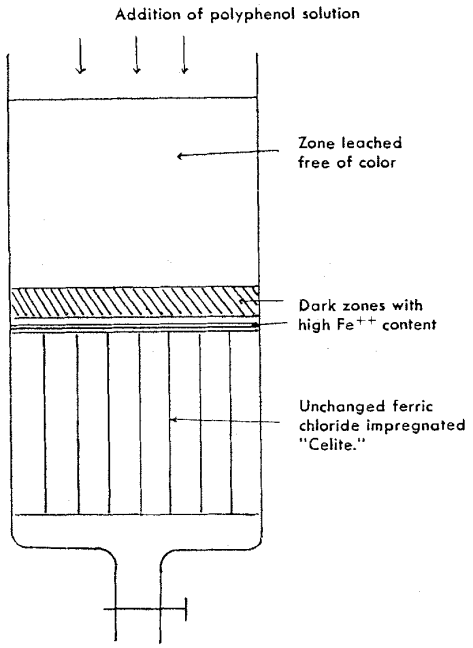


Figure 2. Model podzol praduced by polyphenols (*10*).

Two curious side-lights of the complexing properties should be mentioned here. In the course of searching for a suitable soil to mix with cement for road construction, Clare and Sherwood (*11*) of the Road Research Laboratories in England, found that small amounts (less than 0.5%) of humic acid prevented the concrete mixture from hardening. Addition of calcium chloride to the mixture of cement and soil restored the ability to harden. The investigators attributed the loss of hardening to the complexing action of the humic material on calcium liberated by the hydration process.

Another unusual aspect of the complexing property is illustrated by the following incident. Some years ago, this author received a letter from an old Arizona "placer" miner, who had written in response to an article on humic acid in a local journal. In the letter, the miner claimed that secondary enrichment of some sedimentary gold deposits could be due to the chemical solution of gold and the subsequent deposition of the

[1] Some investigators have claimed that free, uncomplexed humic acid is water and alcohol soluble. Experience in our laboratory has supported this claim, since humic acid with low ash contents are definitely more soluble in alcohol or water than high-ash sample.

metal downstream from the original source. The agent allegedly responsible for the solution was humic acid, which presumably complexed the metal under oxidative conditions. Subsequent reduction of the gold complex, as it was carried in a stream over reducing agents, would account for the enriched deposits.

The above process could be analogous to the solution of gold by the oxidative complexing of cyanide ion, as illustrated by the equations below.

$$Au + H^+ + \text{humic acid} + O_2 = Au(\text{humate})^{3+} + H_2O$$
$$Au(\text{humate})^{3+} + R = Au + \text{humic acid} + R^+$$
(R = reducing agent, such as Fe^{++} or organic compounds)

Literature references to this process are scattered (*12*) and conflicting, but indicate that such a role for humic acid has received serious attention.

In another recent communication to this author (*13*), it was reported that alkaline solutions of humic acid had dissolved native copper. Stevenson (*14*) and others have measured stability constants for various humic acid-metal complexes.

Attempts to characterize humic acid by infrared and ultraviolet absorption spectrophotometry have been unsuccessful. The infrared spectrum (Figure 3) contains a few sharp bands indicating hydroxyl and carboxyl functions, but the major part of the spectrum is featureless. Broad bands around 1600 cm^{-1} have been variously interpreted to represent carboxylate, quinoid, aromatic, and ethylenic moieties.

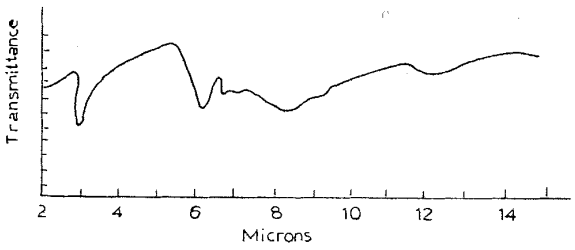


Figure 3. Infrared spectrum of humic acid (33).

The ultraviolet and visible spectra are monotonic, with no observable bands, and indicate only high absorption in the region below 400 mμ. Extinction coefficients have been used as a measure of degree of condensation by Kononova (*1*). Thus, she attributes high values of E^{465} to a polynuclear aromatic structure, and low values of E^{465} to structures with a high aliphatic content. This particular assignment has been consistent with another property of humic acid: its degree of dispersion as measured by coagulation experiments with electrolytes. The more highly aromatic structures have a higher degree of dispersion. It can readily be seen that this property of humic acid is of great interest to soil scientists, since degree of dispersion could affect many soil processes, including mobility of soil-mineral complexes, formation of soil structure, and translocation of humic substances in a soil profile.

In common with lignins, humic acids from different sources vary somewhat in their elemental composition. This is illustrated in Table 1.

Particular attention has been directed to the nitrogen content, which may vary from 0.5 to 5%, but which most commonly occurs in the range 3–5%. Bremner (*16*) and others have investigated the nature of the nitrogen compounds and have found that up to 75% of the nitrogen may be amino-acid (presumably from bound protein material) and glycosamines. On the other hand, some humic acids contain up to 75% unhydrolyzable nitrogen; in these acids, the element appears to have been irreversibly incorporated into the macromolecule and to exist in heterocyclic moieties (*17*). Interest in the nature of the fixed nitrogen is based on the possible role of humic acid as a denitrifying agent in the soil. *In vitro* experiments with natural and artificial humic acids (*18*) have shown that nitrite, ammonia, and amino acids can be irreversibly fixed under oxidative conditions. Since the amount of nitrogen reserve (fixed form) in the soil is a very important agricultural quantity, interest in the chemistry of N-containing humic acids has been intense.

Many investigators have used the C/H ratio of humic acids as a measure of the degree of condensation of aromatic rings, or as a measure of the state of oxidation; regular trends have been observed in certain types of acids, for example from podzol humic acids to chernozem humic acids. There have been some attempts to use this as an index for a rational classification of soil humic acids.

Chemical Studies

Should a young, ambitious PhD in organic chemistry examine this description of humic acid, the chances are that he would not be enticed to work with the compound. He would see a poorly-defined substance of varying elemental composition which was polymeric and non-crystalline. Furthermore, if he surveyed the literature carefully, he would find chemical references of countless papers dating back more than one hundred years. Very few of these references would contain any significant structural information about the compound. A formidable assignment would face the hardy or stubborn scientist who would willingly forego the opportunity of tackling a productive organic problem to work with humic acid.

Yet, there is a small number of chemists who are now actively investigating the structure of this substance. Most of those who have devoted their time and energy to probing the structure of this intractable material are associated with soil institutes in Russia, Germany, and Great Britain; relatively few Americans and Canadians are currently active in this field.

Chemical studies of humic acid fall into two broad categories: functional group analysis and degradative experiments. By the use of titration, acetylation,

Table 1. Elementary Composition of Humic Acids from Various Soils (*1*)

Soil	C	H	N	C/H
Podzolic	52.39	4.82	3.74	10.9
Rendzina	54.90	4.36	4.07	12.6
Degraded chernozem	56.34	3.54	3.58	15.9
Deep chernozem	57.47	3.38	3.78	17.0
Ordinary chernozem	58.37	3.26	3.70	17.9
Chestnut soil	58.56	3.40	4.09	17.2
Podzolic soil	56.67	4.79	5.14	11.8
Forest podzol B	52.77	3.62	0.51	14.6[a]

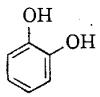
[a] See reference (*15*).

and methylation procedures chemists have shown the presence of carboxyl, phenolic, and aliphatic hydroxyl groups. Some evidence has been presented also for carbonyl and quinoid functions. An example of such a functional group analysis for a forest podzol-B soil humic acid is given in Table 2 below:

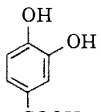
Table 2. Functional Groups in Humic Acid (19)

Functional group	Millieq. per gram of dry ash-free acid
Total acidity	11.5
Carboxyl	8.6
Total hydroxyl	5.1
Phenolic hydroxyl	2.9
Alcoholic hydroxyl (total-phenolic)	2.2
Carbonyl	5.5
Methoxyl	0.2

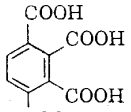
Both oxidative and reductive degradations have been carried out with humic acid, in both acid and basic media. Prior to the use of paper chromatography in the past ten years, relatively few degradation products were isolated and identified. These were mainly derived from catechol (I), protocatechuic acid (II), and benzene polycarboxylic acids (III). These compounds are typical breakdown products of lignin and are also found in coals.



I



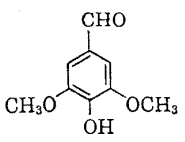
II



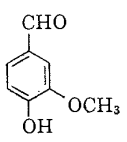
III

Most of the early chemical studies were carried out under drastic conditions, such as KOH fusion, oxidation with boiling nitric acid, and oxidation with alkaline permanganate. Under such conditions, extensive destruction of the substance occurred, and only 1–2% of the original compound could be recovered as recognizable monomers. Even many of the monomeric phenols and phenolic acids had suffered radical changes under some of the reaction conditions.

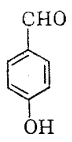
Recently, milder conditions have been employed in an attempt to recover molecular species which bear some resemblance to their original structure in the humic acid molecule. These newer techniques, coupled with paper chromatography, have enabled investigators to isolate a number of components in complex reaction mixtures. Thus, Morrison (*20*) in 1958, utilizing the alkaline nitrobenzene oxidation procedures (commonly employed in lignin chemistry), found syringaldehyde (IV), vanillin (V), and *p*-hydroxybenzaldehyde (VI) in low yields (less than 2%) in peat humic acids.



IV

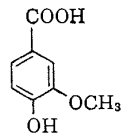


V

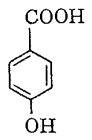


VI

Later, Steelink and Green (*21*), using the CuO-NaOH oxidation method, isolated the same compounds as Morrison had, plus the corresponding acid derivatives, vanillic acid (VII) and *p*-hydroxybenzoic acid (VIII). All of these compounds would be expected to

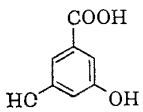


VII

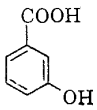


VIII

arise from the chemical breakdown of lignin. What was novel in the results of Steelink and Green was the detection of two additional phenolic acids, 3,5-dihydroxybenzoic acid (IX) and meta-hydroxybenzoic acid (X), in the reaction mixture containing compounds (IV) through (VIII).

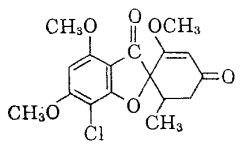


IX

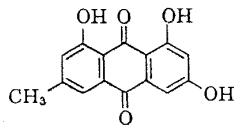


X

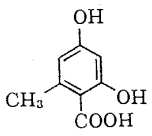
Clearly, compounds (IX) and (X) could not arise from lignin under the reaction conditions which were employed and must be assumed to be derived from resorcinol moieties in the humic acid molecule. This was the first chemical evidence for the existence of non-lignin components in the humic acid macromolecule, a fact which had been previously suspected by many investigators. Resorcinol-derived compounds could be incorporated into the humic acid molecule by the oxidative polymerization of a number of polyphenols (*5*) which occur in the soil environment. For example, numerous plant extractives such as pinosylvin and quercetin possess resorcinol hydroxylation patterns; many microorganisms also synthesize compounds based on resorcinol, such griseofulvin (XI), emodin (XII), and orsellinic acid (XIII). Since these compounds, or



XI



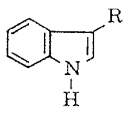
XII



XIII

their degradation products, could co-exist with lignin compounds in the soil, their copolymerization into the humic acid polymer is not unlikely.

Indole-derived substances (XIV) have also been detected by chromatographic techniques in nitrogen-containing humic acids by Flaig (*17*), indicating that part of the nitrogen is irreversibly fixed in a heterocyclic



XIV

system. Other workers (*22*) have analyzed the acid-hydrolyzable fraction of humic acid and have found 22 amino acids in addition to a variety of aminosugars.

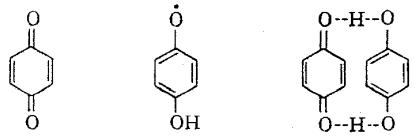
If one surveyed the present-day knowledge of the chemical structure of humic acid, he would conclude that it is a macromolecule in the molecular weight range of 20,000–50,000 which contains an easily hydrolyzable protein and carbohydrate fraction attached to a highly condensed "core." This "core" is partly aromatic in character, with structural elements derived from lignin phenols and resorcinol phenols. In addition, alcohol, carboxyl, carbonyl, and quinoid groups are present, together with heterocyclic nitrogen. The substance is invariably complexed with 1–20% inorganic matter.

Because of the difficulty inherent in direct chemical studies of this substance due to extensive destruction into uncharacterized fragments, other approaches have been attempted. Flaig in Germany (*23*) and Erdtman in Sweden (*24*) have studied the oxidative polymerization of simple phenols into brown, artificial "humic acids," in an attempt to simulate natural processes in the soil. A proposed model for the formation of humic acid, based on extensive studies with model systems, is in Figure 4.

While most investigators have accepted the lignin origin of humic acid, microbiologists have challenged this assumption. Kononova in Russia (*26*) has shown that *aspergillus* and *penicillium* molds produce humic-like substances when grown on glucose. R. J. Swaby in Australia (*27*) has proposed that humic substances are formed by the rapid copolymerization of simple phenols in dead plant cells, and not by the slow decomposition of lignin. Such a view has received support from the findings of Raudnitz (*4*) who showed evidence for the presence of humic acid-phosphate complexes in the leaves of rhododendrum species.

Although studies of the biogenesis of humic acid are important and necessary, they cannot be definitive until more direct information is available about the molecular structure. The primary task of the organic chemist still remains: to discover techniques which will provide this information without destroying a major portion of the molecule in the process. Two recent developments in the field show promise of meeting this requirement. One is the reductive pyrolysis of humic acid in the presence of steam, a process which tends to preserve the structural integrity of quinoid and other labile systems, which are normally fragmented or polymerized under strong oxidative conditions. Feldbeck, at the University of Delaware (*28*), has analyzed the volatile products of this pyrolysis reaction by means of vapor phase chromatography. He has been able to account for more than 30% of the original weight of humic acid as phenolic constituents. Hopefully, such studies should provide a much more complete picture of the variety of components in the macromolecule than is now available.

A second technique is based on the observation by Rex (*29*) that humic acid is a stable free organic radical. Using electron paramagnetic resonance spectrometry, Steelink and Tollin (*30*) at the University of Arizona have carried out a series of measurements to determine the nature of this free radical. Quite recently, the Arizona group (*31*) has been able to deduce certain structural elements in humic acid by observing the change in the EPR spectra as a function of chemical reduction. The spectra very clearly resemble that expected from a mixture of quinones (XV), semiquinones (XVI), and quinhydrones (XVII). If humic



XV XVI XVII

acid were derived in part (or totally) from lignin phenols, then these results would indicate that these phenolic elements have suffered bacterial or chemical oxidation. The same reasoning would apply to resorcinol-derived compounds, since these too are capable of being oxidized to stable free radicals. If Flaig's assumptions (Figure 4) are reasonable, then compounds (XV), (XVI), and (XVII) would be expected to arise from the oxidation of lignin.

The task that remains in this area is to determine the exact nature of these suspected quinone species and to determine their concentration in the macromolecule. Future EPR studies with controlled reduction and oxidation should provide a basis for the quantitative estimation of these moieties.

It is interesting to note that the occurrence of stable free radicals is quite consistent with the proposals of other workers regarding the biosynthesis of humic acid by the oxidative polymerization of phenolic substances. Two common natural polymers, lignins and melanins, also possess stable free radicals; the exact

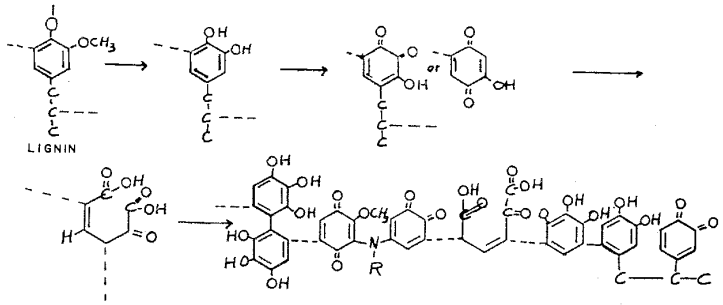


Figure 4. Possible formation of humic acid from lignin (25).

nature of the system which stablizes these free radicals is not known, but semiquinone structures which provide ient delocalization of the unpaired electron are most consistent with present evidence. All three of these substances have their origin in naturally-occurring polyphenols.

Conclusion

While the answer to the question "What is Humic Acid?" is still only fragmentary, newer chemical techniques for elucidating structural features are currently adding to the present knowledge of the subject. Information will also come from related disciplines as well as from the studies of related compounds. Microbiologists in Germany and Switzerland have recently carried out experiments with rotting wheat straw, which suggest that the bacterial decomposition of lignin leads directly to humic acid (*32*). Other investigators (*1*) have concentrated on the fulvic acid fraction of soil organic matter (that material extracted with dilute base, which is soluble in mineral acid) on the assumption that this substance is a precursor of humic acid and also has a simpler structure. These scientists hope to gain insights into the humic acid structure by studying the simpler fulvic acids, in much the same manner that lignin chemists have studied the structures of the simpler lignans.

No doubt, the vast majority of organic chemists will choose greener pastures to investigate in preference to ing with a substance which is so poorly defined and so poorly characterized as humic acid. The few chemists who persist in the field, however, can take heart at the progress which has been made in the last forty years with natural polymeric substances of equally frustrating properties—lignins, tannins, proteins, viruses—and continue to probe for information which will eventually provide a rational solution. Impetus to seek this solution also comes from the ever-increasing commercial uses of humic acid, such as drilling muds, boiler scale removal, pigments for printing inks, and fertilizers, as well as the increasing number of reports of its growth-hormone activity for plants and its role in transport of trace minerals.

Finally, out of sheer cussedness, there will always be a few hardy souls who will want to know the fundamental reason for Nature's wide distribution of this substance, and the method by which she synthesized it.

Literature Cited

(1) Kononova, M. M., "Soil Organic Matter," Pergamon Press, New York, 1961.
(2) Berthelot, M., and Andre, G., *Compt. Rend.*, **112**, 916 (1897).
(3) Maillard, L. C., *Ann. Chim. Phys.*, **9**, 258 (1917); Stevenson, F. J., *Soil Sci. Soc., Amer. Proc.*, **24**, 472 (1960); and Hodge, J. E., *Agric. and Food. Chem.*, **1**, 928 (1953).
(4) Raudnitz, H., *Chem. & Ind.*, **1957**, 1651.
(5) Davies, R. I., Coulson, C. B., and Lewis, D. A., *Sci. Proc. Roy. Dublin Soc.*, Series A, Vol. 1, 183 (1960).
(6) Forsythe, W. G. C., *Chem. & Ind.*, **1948**, 515.
(7) Piret, E. L., White, R. G., Walther, H. C., Jr., and Madden, A. J., Jr., *Sci. Proc. Roy. Dublin Soc.*, Series A, Vol. 1, 69 (1960).
(8) Mukerjee, H., *J. Ind. Chem. Soc.*, **33**, 744 (1956).
(9) Martin, A. E., and Reeve, R., *J. Soil Sci.*, **9**, 89 (1958).
(10) Bloomfield, C., *J. Sci. Food. Agric.*, **8**, 389 (1957).
(11) Clare, K. E., and Sherwood, P. T., *J. Applied Chem.*, **6**, 317 (1956).
(12) Freise, F. W., *Econ. Geology*, **26**, 421 (1931); Fetzer, W. G., *Econ. Geology*, **41**, 47 (1946).
(13) Tinsley, J., Department of Soil Science, University of Aberdeen, Scotland, Private communication.
(14) Stevenson, F. J., and Khanna, S. S., *Soil Sci.*, **93**, 298 (1962).
(15) Steelink, C., Berry, J. W., Ho, H., and Nordby, H., *Sci. Proc. Roy. Dublin Soc.*, Series A, Vol., 1, 59 (1960).
(16) Bremner, J. M., *J. Agric. Sci.*, **46**, 247 (1955).
(17) Flaig, W., and Breyhan, Th., *Z. Pflanzenernaehr., Dueng., Bodenk.*, **75**, 132 (1956).
(18) Bremner, J. M., *J. Agric. Sci.*, **48**, 352 (1956); Brusset, H., and Charpin, P. *Compt. Rend.* **240**, 2315 (1955).
(19) Schnitzler, M., and Wright, J. R., *Nature*, **184**, 1462 (1959).
(20) Morrison, R. I., *J. Soil. Sci.*, **9**, 130 (1958).
(21) Steelink, C., and Green, G., *J. Org. Chem.*, **27**, 170 (1962).
(22) Bremner, J. M., and Shaw, K., *J. Agric. Sci.*, **44**, 152 (1954); Bremner, J. M., *J. Sci. Food Agric.*, **9**, 528 (1958).
(23) Flaig, W., *Sci. Proc. Roy. Dublin Soc.*, Series A, Vol. 1, 149 (1960).
(24) Erdtman, H., and Stjernstrom, N. E., *Chem. & Ind.*, 1599 (1960).
(25) Flaig, W., *Suomen Kemistilehti* A., **33**, 229 (1960).
(26) Kononova, M. M., and Aleksandrova, I. V., *Soils & Fertilizers*, **22**, 77 (1959).
(27) Swaby, R. J., CSIRO, Division of Soils, Adelaide, Australia, private communication.
(28) Feldbeck, G. T., *Chem. Eng. News*, Dec. 11, 1961.
(29) Rex, R. W., *Nature*, **188**, 1186 (1960).
(30) Steelink, C., and Tollin, G., *Biochim. et Biophys. Acta*, **59**, 25 (1962).
(31) Steelink, C., Reid, T., and Tollin, G., *Chem. Eng. News*, Sept. 24, 1962.
(32) Flaig, W., Schobinger, U., and Deuel, H., *Chem. Ber.*, **92**, 1973 (1959).
(33) Elofson, E. M., *Can. J. Chem.*, **35**, 926 (1957).

Journal of
APPLIED
BIOMEDICINE

J. Appl. Biomed.
3: xxx–xxx, 2005
ISSN 1214-0287

REVIEW

Humic substances – compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine

Eladia M. Peña-Méndez[1], Josef Havel[2], Jiří Patočka[3]

[1] Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Chemistry, University of La Laguna, Spain
[2] Department of Analytical Chemistry, Faculty of Science, Masaryk University, Brno, Czech Republic
[3] Department of Toxicology, Military Medical Academy, Hradec Králové and Department of Radiology, Faculty of Health and Social Studies, University of South Bohemia, České Budějovice, Czech Republic

Received 20th September 2004.
Revised 20th October 2004.
Published online 22nd November 2004.

Summary
Humic substances as part of humus-soil organic matter – are compounds arising from the physical, chemical and microbiological transformation (humification) of biomolecules. They are important because they constitute the most ubiquitous source of non-living organic material that nature knows. Approximately 80% of the total carbon in terrestrial media and 60% of the carbon dissolved in aquatic media are made up of humic substances. Humic substances have important roles in soil fertility, and are considered to have primal relevance for the stabilization of soil aggregates. They can be divided into three components according to their solubility: humic acids, fulvic acids and humin. Humic acids are the most explored group of humic substances. Beyond their relevance for life these substances have industrial applications in the development of absorbents to be used at the sources of metal-poisoning. Being natural substances, their purification process is cheaper than the synthesis of any other sorbent and, moreover, due to their high operability, they absorb more than the absorbents used to date, such as active charcoals or clays. The specific properties of humic acid products enable their application in industry, agriculture, environmental and biomedicine.

Keywords: humification – humin – humic acids – fulvic acids

✉ Eladia M. Peña-Méndez, Department of Analytical Chemistry, Nutrition and Food Science, Faculty of Chemistry, University of La Laguna, 38071-La Laguna, Spain
empena@ull.es

INTRODUCTION

Humic substances (HS) are the most widely-spread natural complexing ligands occurring in nature. The presence of HS in soils have also been detected, even in the Antarctic continent where the humification process under Antarctic conditions is very specific and different from the other

continents (Gajdošová et al. 2001, Pacheco and Havel 2002, Gajdošová et al. 2003). They make up the bulk of organic matter, because they represent most of the organic materials of soil, peat, lignites, brown coals, sewage, natural waters and their sediments. Humic substances can be divided into three components: fulvic acids (FAs), humic acids (HAs) and humin. One of the most important parts of HS is HAs. Humic acids and FAs represent alkali-soluble humus fragments, humin represents the insoluble residue. Because of their molecular structure, they provide numerous benefits to crop production. They help break up clay and compacted soils, assist in transferring micronutrients from the soil to the plant, enhance water retention, increase seed germination rates and penetration, and stimulate the development of microflora populations in soils (Senesi et al. 1991).

The remarkable properties of humic acids have attracted the attention of many investigators. The results over the years have brought new knowledge on their structure and physicochemical properties and pointed to the use of these interesting natural compounds in many practical applications. The aim of this article is to review the current state of knowledge and to present current applications in agriculture, industry, the environment, and biomedicine (see, e.g. Ziechmann 1994).

HISTORY

The term "humus" originates from the Romans, when it was familiarly used to signify the entire soil. Later the term was used to denominate soil organic matter and compost or for different parts of this organic matter, as well as for complexes created by chemical agent treatments to a wide palette of organic substances. The principal definition of humus, as decomposed organic matter, originates from 1761 (Stevenson 1982).

The first relevant study of the origin and chemical nature of HS was worked out by Sprengel (1839). His comprehensive study on the acidic nature of HAs is thought to be his most important benefit to humus chemistry. Research on the chemical properties of HS was extended by the Swedish researcher Berzelius, whose main contribution was the isolation of two light-yellow-coloured HS from mineral water and slimy mud rich in iron oxides (Berzelius 1839).

Enormous advances have been made during the last decade thanks to modern physicochemical methods. Nevertheless, the structural chemistry of lignin and HS did not advance so fast as the chemistry of animal-originated biopolymers.

WHERE DO HUMIC SUBSTANCES COME FROM?

Although the formation process of HS has been studied hard and for a long time, their formation is still the subject of long-standing and continued research. Some theories have lasted for years; for example, the 'sugar-amine condensation' theory, the 'lignin' theory or the 'polyphenol' theory. A review of such theories can be found in a monograph of Davies and Ghabbour 1999. Nowadays, most investigators suppose that humic substances originated in lignin (Oglesby et al. 1967).

Polyphenols come mostly from lignin during its biodegradation, and probably play a key role in the formation process. Polyphenols are also regarded as the main agents in the formation of humic substances from some plants that do not contain much lignin and/or from non-lignin containing plants. Polyphenols can be considered as humic acid precursors. They themselves possess enough reactive sites to permit further transformations, for example some condensation reactions.

The humic substances system is created. by the association of various components present in the humification process, such as amino acids, lignins, pectins or carbohydrates, through intermolecular forces (donor-acceptor, ionic, hydrophilic, and hydrophobic) It is evident that the mechanisms of the formation of humic substances can be slightly different, depending on geographical, climatic, physical and biological circumstances, respectively. These compounds can be formed in several ways, and the role of lignin is important in the majority of these processes (Burdon 2001, Davies et al. 2001). Burdon (2001) proposed that humic organic matter consists mainly of a mixture of plant and microbial constituents plus the same constituents in various stages of decomposition (i.e. plant/microbial mixtures of carbohydrates, proteins, lipids and partially degraded lignins, tannins, melanins, etc.).

CHARACTERIZATION OF HUMIC SUBSTANCES

As already pointed out, all the humic substances can be divided into components according to their solubility in different media (Fig. 1). Humic acids and FAs represent alkali-soluble humus fragments; HAs are commonly extracted using diluted alkali and precipitated with an acid, and so are separated from the soluble FAs. Humin represents the insoluble residue (Thorn 1996).

From a geological point of view, humic substances are chemical intermediates between plants and fossils. The chemical nature of soils,

sludge and sediments can subsequently, continually and selectively vary via the conversion and degradation of organic matter (Ziechman 1993). Extinct vegetation represents the main source of organic matter transforming to humic substances in the environment. Humification is a continuous historical process, and soil humus is a dynamic system of both chemically active and passive components (Gonzalez et al. 2003). The amount of organic carbon in the Earth in the form of humic substances exceeds that which makes up living organisms (Engel and Macko 1993).

Humic acids, one of the most important components of HS, help break up clay and compacted soils, assist in transferring micronutrients from soil to plants, enhance water retention, increase seed germination rates, and stimulate the development of microflora populations in soils. Humic acids also slow down water evaporation from soils. This is especially important in soils where clay is present at low concentration or not at all, in arid areas, and in sandy soils without the capability to hold water. Humic acids provide also sites for microflora to colonize. Bacteria secrete enzymes which act as catalysts, liberating calcium and phosphorous from insoluble calcium phosphate, and iron and phosphorous from insoluble iron phosphate. The chemical structure of HAs is very complicated and depends on their source.

The elemental composition of different FAs and HAs shows that the major elements in their composition are C, H, O, N, and S. These major elements are always present regardless their origin and country or continent (Gajdošová et al. 2001, Tan 2003, Kurková et al. 2004). Moreover, besides elemental composition, group composition is used to characterize HS as it gives information about the chemistry and structural properties of HS (Purdue 1988, Tan et al. 2000). Fulvic acids contain more functional groups of an acidic nature, particulary –COOH. The total acidities of fulvic acids (900–1400mmol/100g) are considerably higher than for humic acids (400–870mmol/100g). Another important difference is that while the oxygen in fulvic acids is largely in known functional groups (–COOH, –OH, –C=O), with a high oxygen content, the acidity and degree of polymerisation all change systematically with increasing molecular weight. The proportion of oxygen in humic acids seems to occur as a structural component of the nucleus.

The formation of aggregates within solutions of humic acids was studied by capillary electrophoresis for the first time by Fetsch et al. (1998a, 1998b). Humic acids (HAs) aggregation has also been studied in aqueous solution by high performance size exclusion chromatography (Peuravuori and Pihlaja 1997), light scattering (Manning et al. 2000), vapor pressure osmometry (VPO) (Marinsky et al. 1990), ultrafiltration (Aiken and Malcom (1987), conductometry and spectrophotometry in combination with factor analysis (Peña-Méndez et al. 2004). HAs consist of a mixture of molecules with much lower molecular weight than proposed before in the literature. HAs components are low molecular weight compounds but they aggregate step by step to give higher molecular weight aggregates and supramolecules of higher molecular weight. Hosse and Wilkinson (2001), using fluorescence correlation spectroscopy observed that the aggregation of HAs is due to the formation of dimers and trimers at low pH.

The characterization of complex mixtures of FAs and HAs is considered to be one of the most important items in HS research nowadays. Thanks to the development of analytical techniques and computer technologies, great efforts have been made to elucidate the molecular structures of FAs and HAs. From the work of Stevenson (1982), Buffle et al. (1977) to more recent models of Shulten (2002, 2003), Kujawinski et al. (2002a, 2002b) and Stenson et al. (2002, 2003), several molecular structures describing the structure of humic acids have been proposed. For a long time, it has been suggested using different analytical techniques that HAs are high molecular weight compounds. However, five years ago, we proved by CE that HAs are low molecular weight compounds. This was announced for the first time at the 9th International Meeting of the IHSS, Adelaide Australia, September 20–25, 1998, IHSS, Atlanta (Havel et al. 2001) and in a subsequent publication. Mass spectra of HAs (Fig. 4) obtained by LDI-TOF MS shows the presence of low molecular weight molecules from low m/z values to higher. The analysis of the mass spectra obtained for different HAs showed that several m/z values are the same for all the HAs, suggesting that some compounds are the same and that they are present in HAs from very different origins and sources. The latest results from mass spectrometry (MS) combined with other analytical techniques have confirmed our previous results. As for fulvic acids, several thousand compounds have been identified, and are considered to be mostly derived from lignin (Kujawinski et al. 2002a, Stenson et al. 2003).

The chemical formulas of individual fulvic acids have been determined (Stenson et al. 2003). In the case of HAs, interesting results have been achieved recently applying Electrospray Ionization (ESI) (Kujawinski et al. 2002a, Brown and Rice 2002, Kujawinski et al. 2002b, Stenson et al. 2002, Stenson et al. 2003) coupled with Fourier Transform ICR mass spectrometry (FT-ICR) and Laser Desorption/Ionization time of flight (LDI-TOF) (Pokorná et al. 1999, Havel et al. 1999, Gajdošová et al. 2000, Gajdošová et al. 2003). Based on the latest experimental results obtained by LDI-TOF MS and also on the base of the

isotopic patterns observed for compounds present in HAs (Fig. 4), Pacheco and Havel (2004) have suggested the latest empirical formula for HAs; the results from their model are in a good agreement with those obtained by ESI (Kujawinski et al. 2002a, Brown and Rice 2002, Kujawinski et al. 2002b, Stenson et al. 2002, Stenson et al. 2003).

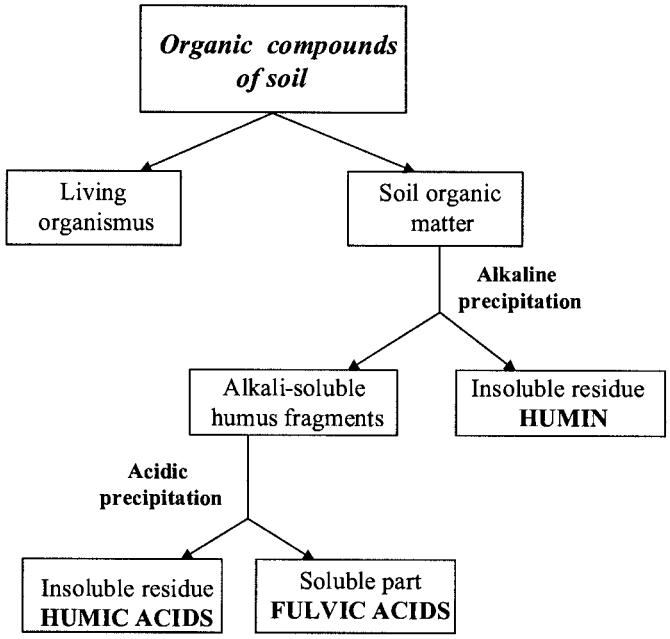


Fig. 1. **Scheme of division of humic substances in dependence of their solubility.**

APPLICATIONS OF HUMIC SUBSTANCES

Humus represents one of the greatest carbon reservoirs on Earth. So far, industrial applications of humus and humus-derived products are rare. On the contrary, the usage of coal was more abundant and essentially, it constituted the basis of the chemical industry in the second half of the 19th century and the first half of the 20th century. Petroleum was also an application and it was regarded as the main raw material for the chemical industry of the 20th century. Nowadays, applications of HS can be divided into four main categories: agriculture, industry, environment and biomedicine.

Agriculture applications

HS play an important role from the agronomical point of view. They influence significantly the quality and productivity of the soil. In addition to the improvement of the soil's physical properties and moisture conditions mentioned above, HS also show a high base exchange capacity, which is important for soil fertility (Lotosh 1991, Zhang and He 2004).

Currently, humic materials are used as additives in fertilizers (García et al. 1994, Madejon et al. 2001, Albiach et al. 2001, Kerek et al. 2003, Arancon et al 2004). Different salts of humic substances, such as calcium humate, were used to increase soil fertility (e.g. Buckau et al. 2000). The fertilizing effect of sodium humate on plant leaves has been described. Ammonium humate was also found to have a significant growth-stimulating effect (Lotosh 1991). The characteristics of and applications for humic acids extracted from different compost have also been studied (Ceppi et al. 1999, Madejon et al. 2001, Arancon et al 2004).

The growth-promoting effect of humic substances has been observed by many investigators and humates are often part of different preparations for growth-improvement of plants. Productivity of soil is increased by different ways in the presence of humic materials. The indirect effects of humic substances are very important as they integrate iron to the chelates and make it available to plants. Another role of humic substances lies in the enhancement of the quality of

soils when they are very poor in organic matter. Recent research shows that humic acid can be used as farm animal feed thanks to its growth-promoting effect (e.g. Kocabagli et al. 2002).

Industrial applications

Humus and humus-containing materials have been used in large-scale building, for instance, as additives to control the setting rate of concrete. Humic materials found use also in the preparation of leather. Initially, they were used as a leather dye, later on as an agent for tanning leather and, finally, as an ingredient of a solution to finish leather.

The woodworking industry is another field where HS have been applied. They were used to prepare a "natural indigo" to dye wood veneer. In addition to this use, humic materials appeared to be suitable agents as a component of water-soluble stains for wood furniture.

In the ceramic industry, humic substances were employed mainly as additives to enhance the mechanical strength of unprocessed ceramics, to improve the casting properties of ceramics (Waksman 1938), to color clay tiles and among many other uses they were also applied in the preparation of earthenware.

Furthermore, humic materials have found application in the production of plastics, especially as dyes for coloring Nylon 6 or PVC plastics, hardeners of polyurethane foams or as plasticizer ingredients for PVC (Majakova and Proskurjakov 1972).

Humic materials found numerous applications in the paper industry too. They are included in different manufacturing procedures, for example in the production of electricity conducting paper sheets or in the manufacture of high tensile strength paper, and also in the recycling of paper. Other industrial applications can be mentioned: as an ion exchanger, as a source of synthetic hydrocarbons and fuel oils (Duncan et al. 1981), in food-processing or to enhance the extraction of uranium from its ores (Schmeide et al. 2000). Humic substances have a large capacity to retain transition metals, forming metalorganic complexes, which cause these metals to be more or less available for plants which include then them into the food chain.

Interesting possibilities are the manufacture of humic substances from the waste processing industry. Results showed that the conversion of cellulose into humic acids is clearly underway in the production of recycled liquid packaging board (LPB) bales (Koivula and Hanninen 1999)

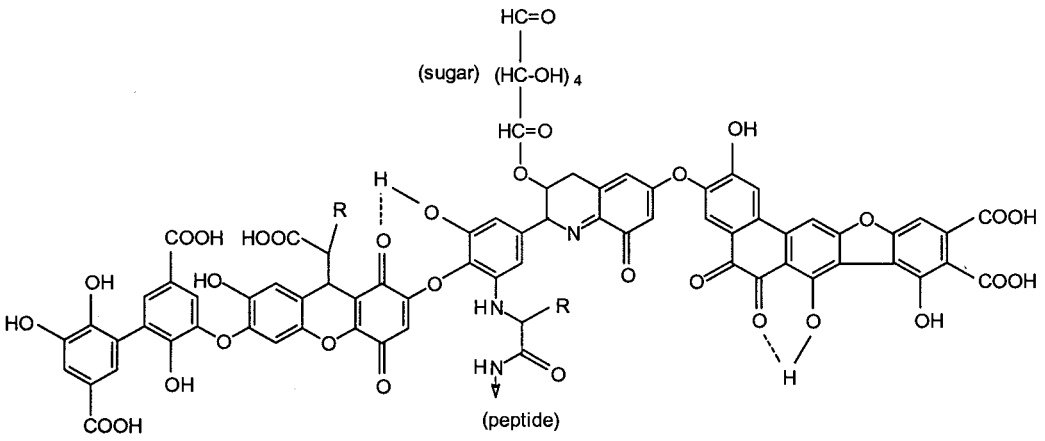


Fig. 2. **Model structure of humic acid according to Stevenson (1982);** R can be alkyl, aryl or aralkyl.

ENVIRONMENTAL APPLICATIONS

Natural organic colloids (humic and fulvic acids) are important because they form water-soluble complexes with many metals including radionuclides (Lubal et al. 1998, Lubal et al. 2000, Pacheco and Havel 2001, Ghabbour et al. 2001). These organics may therefore be important as radionuclide transport agents through the environment. It is known that the presence of humic substances in natural waters can influence the uptake of radionuclides by natural solids and thus their migration to surface and ground waters (Bondietti 1982, Samanidou et al. 1991). The main task of humic substances in environmental chemistry is to remove toxic metals, anthropogenic organic chemicals and other pollutants from water. Ion-exchange materials based on calcium humate were found suitable for the removal of such heavy metals as iron, nickel, mercury, cadmium and

copper from water and also to remove radioactive elements from water discharges from nuclear power plants. Their selective binding capabilities are also exploited for the destruction of munitions and chemical warfare agents (Ghabbour and Davies 1999). Humus-based filters have been developed for sewage purification, with many applications. The filters are useful to clean chromate smelter wastewater, to remove oil and dyes from wastewaters and aquatic systems (Versraete and Devliegher 1997), to filter urban and industrial

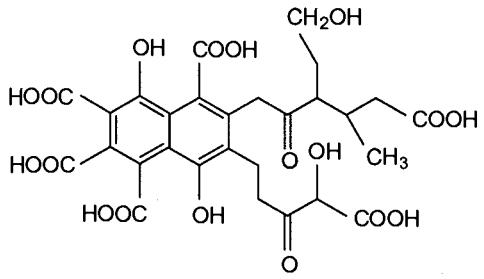


Fig. 3. **Model structure of fulvic acid** according to Buffle et al. (1977).

wastewaters, to remove pesticides from sewage and to remove phenol from water.

Humus-containing materials have been also utilized for sorbing gases, e.g. the removal of waste gases from an animal-carcass rendering plant. Slightly modified humates can be applied to remove hydrogen sulfide and mercaptans from municipal gas supplies, and sulfur dioxide from stack gases (Green and Manahan 1981).

Different groups of compounds such as herbicides, fungicides, insecticides, nematicides, dioxins and also some pharmaceutical products like estrogenic compounds were determined as possible environmental endocrine disruptors. Thanks to their ability to adsorb organic pollutants from the environment, humic substances were found to be useful to remove those contaminants from water, soil and sewage sludges (Shin et al. 1999, Lofredo et al. 2000). The complex nature of the interaction between HS and xenobiotics and their influence in the environmental quality (water, soil, and atmosphere) has been studied by different authors. The study of the acido-basic and complexation properties of HAs with several inorganic and organic compounds has attracted increased attention due to their influence on many aspects of soil and water quality, and industrial processes. Pacheco et al. (2003) found that some inorganic and/or organic pollutants were strongly complexed (bound) only with some of the HAs components. As a consequence of such interaction quite stable entities of the supramolecular kind were formed.

The interactions between humic materials and microorganisms have been intensively studied for the past 30 years. It was found that fermenting bacteria could reduce humic substances. This fact has significant implications for the autecology of anaerobic bacteria in soils and sediments. The cumulative production of acetate during this process seems to be energetically advantageous for fermenting bacteria (Benz et al. 1998). Utilization of humics as the energy supply for specific bacteria, fungi and higher microorganisms was studied by many investigators (Bhardwaj and Gaur 1971) but it was noted that they can not exploit humic materials as a food source.

BIOMEDICAL APPLICATIONS

Humic substances produced on a commercial scale are used in veterinary and human medicine. Several studies of the medicinal properties of humic materials have been reported (Mund-Hoym 1981, Brzozowski et al. 1994). It was found that humic acids administered prophylactically to rats decreased significantly the extension of gastric damage induced by ethanol. TPP administered to rats with experimental gastric and duodenal ulcers significantly accelerated the healing process (Brzozowski et al. 1994)

Pflug and Ziechman (1982) reported that humic acids are able to interact with the bacterium Micrococcus luteus. In this case humic materials protected the organism against cell-wall disruption by the enzyme lysozyme. Thiel et al. (1981), who found that preincubation of cell cultures with ammonium humate avoided infection by the herpes virus, noted the function of humic substances as protectors of the organism.

In the last decade there has been an increasing interest in the employment of humic materials in medicine and biology. The possibility of soil humus extract with amino acid complexes and vitamin B analogues being a candidate as a base of

cosmetic and pharmaceutical products has been studied. The main reason for the increasing attention devoted to humic acids can be explain by their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities (Yamada et al. 1998). The potential of humic substances to form chelate complexes with heavy metals (such as cadmium) enable them to be used for the elimination of heavy metals from living organisms (Klöcking 1992). Humic materials in aquatic systems and water sediments have been observed to be closely connected with efficacy of hydrotherapy and

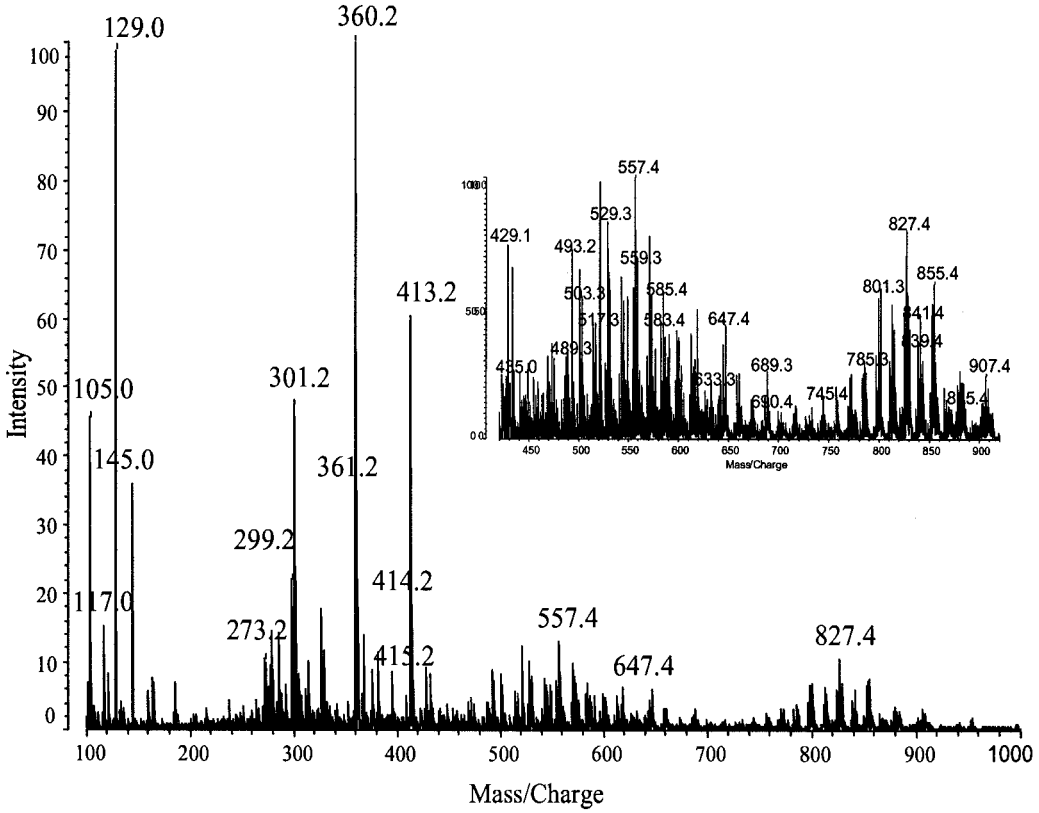


Fig. 4. **LDI TOF MS mass spectra of HAs soil (IHSS).**

balneotherapy (Gadzhieva et al. 1991, Hampl et al. 1994).

Antibacterial (Ansorg 1978, Skliar et al. 1998) and antiviral (Klöcking and Sprossig 1972, Thiel et al. 1977 1981, Schiller et al. 1979, Klicking 1991, Klöcking et al. 2002) properties of humic substances represent new possibilities for their medical application.

Of great interest is that hospital studies show that difficult viral respiratory illnesses common in children are readily resolved with fulvic acid dietary supplementation. Fulvic acid is a humic extract common to rich organic humus soil and also certain ancient plant deposits. Many medical studies show that humic substances, especially fulvic acids, have the power to protect against cancer and related cancer-causing viruses. Studies often show reversal of deadly cancers and tumors using special humic substance therapies (Schneider et al. 1996, van Rensburg et al. 2002, Joone et al. 2003).

On the other hand, humic acid has been shown to be a toxic factor for many mammalian cells, but the specific mechanism of its cytotoxicity remains unclear. Its redox properties make humic acid capable of reducing iron(III) to iron(II) in aqueous conditions over a broad range of pH values (from 4.0 to 9.0) and of reducing and releasing iron from ferritin, but this process is partially inhibited by superoxide scavengers. Subsequently, the iron released from ferritin has been shown to accelerate the humic acid-induced lipid peroxidation. Humic acids have the ability to reduce and release iron from ferritin storage as well as to promote lipid peroxidation. Therefore, HAs coupled with released iron can disturb the redox balance and elicit oxidative stress within a biological system. This may be one of the most important mechanisms for HA-induced cytotoxicity (Ho et al. 2003).

Humus represents one of the greatest carbon reservoirs on the Earth. Although industrial applications of humus and humus-derived products

were a relatively low scale, usage of coal was more abundant and was the basis of the chemical industry in the second half of the 19th century and in the first half of the 20th century. Petroleum was also applied and it is considered as the main raw material for the chemical industry of the 20th century (Ghabbour and Davies 2001).

Now it is time for new applications of humic substances in less traditional arenas, mainly in biomedicine (Laub 1999, Laub 2003a, Laub b, Ghosal, 2003).

CONCLUSIONS

Humic substances are group of organic compounds formed by the association of high-molecular-mass substances from microbiological, vegetative and animal origin. They are organic macromolecules with multiple properties and high structural complexity. They exist abundantly in soil, natural water and various terrestrial and aquatic environments. The humic substances group can be divided into three components based on their solubility: fulvic acids, humic acids, and humin. Fulvic acids and humic acids represent alkali-soluble humus fragments and humin represents the insoluble residue. As for the structure, it is still not known, although there has been a breakthrough by Havel's research group using CE and MALDI-TOF MS indicating that HAs should not be considered as high molecular weight compounds Regardless of the still unknown structure of humic substances and the great efforts to elucidate, it is known that their major functional groups include carboxylic, phenolic, carbonyl, hydroxyl, amine, amide and aliphatic moieties, among others. Due to this polyfunctionality, humic substances are one of the most powerful chelating agents among natural organic substances. The zwitterionic character of humic substances allows the interaction of anions with positively charged groups of humics and cations with negative charged groups of these substances. The unique chemical properties of humic substance products enable their application in industry, agriculture, environmental and biomedicine. In HAs there are compounds with potentially important pharmaceutical or medicinal properties and the search for or and investigation of these compounds is of great importance for pharmaceutical and biomedical applications.

ACKNOWLEDGEMENT

This work was supported by Ministry of Education, Youth and Sport, MSM 143100007 and MSM 143100011, Czech Republic. E.M. Peña-Méndez would like to thank the Canarian Government (Spain) for financial support.

REFERENCES

Aiken G.R. and Malcom R.L.: Molecular weight of aquatic fulvic acids by vapor pressure osmometry. Geochim. Cosmochim. Acta 51:2177–2184, 1987.

Albiach R., Canet R., Pomares F., Ingelmo F.: Organic matter components, aggregate stability and biological activity in a horticultural soil fertilized with different rates of two sewage sludges during ten years. Biores. Technol. 77:109–114, 2001.

Ansorg R. and Rochus W.: Studies on the antimicrobial effect of natural and synthetic humic acids. Arzeimittelforschung 28:2195–2198, 1978.

Arancon N.Q., Edwards C.A., Bierman P., Welch C., Metzger J.D.: Influences of vermicomposts on field strawberries: 1. Effects on growth and yields. Bioresource Techn. 93:145–153, 2004.

Benz M., Schink B., Brune A.: Humic acid reduction by propionibacterium freudenreichii and other fermenting bacteria. Appl. Environ. Microbiol. 64:4507–4512, 1998.

Berzelius J.J.: Lehrbuch der Chemie. Wöhler, Dresden and Leipzig 1839.

Bhardwaj K.K. andGaur A.C.: Studies on the growth stimulating action of humic acid on bacteria. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. 126:694–699, 1971.

Buffle J., Greter F.L., Haerdi W.: Measurement of complexation properties of humic and fulvic acids in natural waters with lead and copper ion-selective electrodes. Anal. Chem. 49:216–222, 1977.

Buckau G., Hooker P., Moulin V., et al.: Versatile components of plants, soils and water In: Ghabbour E.A. and Davies G. (eds): Humic Substances, RSC, Cambridge 2000.

Burdon J.: Are the traditional concepts of the structures of humic substances realistic? Soil Sci. 166:752–769, 2001.

Bondietti E.: Environmental migration of long-lived radionuclides. IAEA, Vienna 1982.

Brzozowski T., Dembinski A., Konturek S.: Influence of Tolpa Peat Preparation on gastroprotection and on gastric and duodenal ulcers. Acta Pol. Pharm. 51:103–107, 1994.

Brown T. L. and Rice J.A.: Effect of experimental parameters on the ESI FT-ICR mass spectrum of fulvic acid. Anal. Chem. 72:384–390, 2000.

Davies G. and Ghabbour E.A. (eds.): Humic substances, structures, properties and uses. The RSC, Cambridge 1999.

Davies G., Ghabbour E.A., Steelink C.: Humic acids: Marvelous products of soil chemistry. J. Chem. Educ. 78:1609–1614, 2001.

Duncan D.A., Bodle W.W., Banejerd D.P.: Energy from biomass and waste. 5th Symposium,

Papers: Institute of Gas Technology, Chicago, 1981, pp. 917.

Engel M.H. and Macko S.A. (eds.): Organic Geochemistry, Plenum Press, New York and London 1993.

Fetsch D., Albrecht-Gary A.M., Peña-Méndez E.M., Havel J.: Capillary zone electrophoresis of humic acids. Scripta Fac. Sci. 1997, pp. 27–37.

Fetsch D., Hradilová M., Peña-Méndez E.M., Havel J.: Capillary zone electrophoresis study of humic substances aggregation. J. Chromatogr. A. 817:313–323, 1998a.

Fetsch D., Fetsch M., Peña-Méndez E.M., Havel J.: Humic acids capillary zone electrophoresis adsorption on capillary walls, separation in metal ion supplemented buffer and the fingerprints. Electrophoresis 19:2465–2473, 1998b.

Fetsch D. and Havel J.: Capillary zone electrophoresis for the separation and characterization of humic acids. J. Chromatogr. A. 802:189–202, 1998c.

Gadzhieva N.Z., Tsoi E.P., Turovskaia S.I., Ammosova I.M.: The antibacterial activity of a humic preparation made from the therapeutic peat mud of the Dzalal Abad deposit in Kirgizia. Naucn. Dokl. Vyss. Skoly Biol. Nauki 10:109–113, 1991.

Gajdošová D., Pokorná L., Kotz A., Havel J.: Mass spectrometry and capillary electrophoresis analysis of coal-derived humic acids produced from oxyhumolite. A comparative study. In: Humic substances. Versatile components of plants, soils and water. Ghabbour E.A. and G. Davies (eds.):RSC, Cambridge 2000, pp. 289–298.

Gajdošová D., Pokorná L., Láska S., Prošek P., Havel J.: Are there humic acids in Antarctica? In Ghabbour E.A. and Davies G. (eds): Humic Substances. Structures, Models and Functions. RSC, Cambridge 2001, pp. 121–131.

Gajdošová D., Novotná K., Prosek P., Havel J.: Separation and characterization of humic acids from Antarctica by capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry inclusion complexes of humic acids with cyclodextrins. J. Chromatogr. A. 1014:117–127, 2003.

Garcia D., Cegarra J., Roig A., Abad M.: Effects of the extraction temperature on the characteristics of a humic fertilizer obtained from lignite. Biores. Technol. 47:103–106, 1994.

Ghabbour E.A., Davies G., Ghali N.K., Mulligan M.D.: The effect of temperature on tight metal binding by peat and soil derived solid humic acids. Canad. J. Soil. Sci. 81:331–336, 2001.

Ghabbour E.A. and Davies G. (eds.): Humic substances: structures, models and functions. Based on proceedings, RSC, Cambridge 2001, p. 401.

Gonzalez M.G., M.E. Conti, R.M. Palma, N.M. Arrigo: Dynamics of humic components and microbial activity under no-tillage or reduced tillage, as compared with native pasture (Pampa Argentina). Biol. Fert. Soils 39: 135–138, 2003.

Green J.B. and S.E. Manahan: Absorption of sulphur dioxide by sodium humates. Fuel 60: 488–494, 1981.

Havel J. and D. Fetsch: Humic substances. Capillary zone electrophoresis. In Wilson I. (ed.): Encyclopedia of Separation Science. Academic Press Ltd., London 2000.

Havel J., D. Fetsch, E.M. Peña-Méndez, P. Lubal, J. Havliš: Recent developments in humic acid characterization. Acidobasic and complexation properties, separation and reliable fingerprints by capillary electrophoresis and MALDI-TOF mass spectrometry. Swift R.S. and K.M. Spark (eds.). Understanding and Managing Organic Matter in Soils, Sediments, and Waters. 9th International Meeting of the IHSS, Adelaide 1998, pp. 20–25, IHSS, Atlanta 2001, pp. 77–82.

Hosse M. and K.J. Wilkinson: Determination of electrophoretic mobilities and hydrodynamic radii of three humic substances as a function of pH and ionic strength. Environ. Sci. Technol. 35: 4301–4306, 2001.

Ho K.J., T.K. Liu, T.S. Huang, F.J. Lu:.Humic acid mediates iron release from ferritin and promotes lipid peroxidation in vitro: a possible mechanism for humic acid-induced cytotoxicity. Arch. Toxicol. 77: 100–109, 2003.

Joone G.K., J. Dekker, C.E. van Rensburg: Investigation of the immunostimulatory properties of oxihumate. Z. Naturforsch. 58: 263–267, 2003.

Kiss G., E. Tombácz, B. Varga, R. Alsberg, L. Persson: Estimation of the average molecular weight of humic-like substances isolated from fine atmospheric aerosol. Chemosphere 37: 3783–3794, 2003.

Klöcking R. and M. Sprossig: Antiviral properties of humic acids. Experientia 28: 607–608, 1972.

Klöcking R.: Interaction of humic acids and humic-acid-like polymers with herpes simplex virus type 1. Humanic Substances in the Aquatic and Terrestrial Environment, Berlin 1991, pp. 408–412.

Klöcking R.: Humic substances in the global environment and implications in human health. Monopoli 1992, p. 129.

Klöcking R., B. Helbig, G. Schotz, M. Schacke, P. Wutzler: Anti-HSV-1 activity of synthetic humic acid-like polymers derived from p-diphenolic starting compounds. Antivir. Chem. Chemother. 13: 241–249, 2002.

Kocabagli N., M. Alp, N. Acar, R. Kahraman: The effects of dietary humate supplementation on broiler growth and carcass yield. Poult. Sci. 81: 227–230, 2002.

Koivula N. and K. Hanninen: Biodeterioration of cardboard-based liquid containers collected for fibre reuse. Chemosphere 38: 1873–1887, 1999.

Kujawinski E.B., P.G. Hatcher, M.A. Freitas: High-resolution fourier transform ion cyclotron resonance mass spectrometry of humic and fulvic acids: improvements and comparisons. Anal. Chem. 74: 413–419, 2002a.

Kujawinski E.B., M.A. Freitas, X. Zang et al.: The application of electrospray ionization mass spectrometry to the structural characterization of natural organic matter. Org. Geochem. 33: 171–180, 2002b.

Kurková M., Z. Klika, Ch. Kliková, J. Havel: Humic acids from oxidised coal I. Elemental composition, titration curves, heavy metals in HA symplex, nuclear magnetic resonance spectra of HAs and infrared spectroscopy. Chemosphere 54: 1237–1245, 2004.

Laub R.J.: Process for preparing synthetic soil-extract materials and medicaments based thereon. US Patent 5,945,446, 1999.

Laub R.J.: Synthetic soil-extract materials and medicaments for herpes viruses based thereon. US Patent 6,524,566, 2003a.

Laub R.J.: Synthetic soil-extract materials and medicaments for human immunodeficiency viruses based thereon. US Patent 6,534,049, 2003b.

Ghosal S.: Delivery system for pharmaceutical, nutritional and cosmetic ingredients. US Patent 6,558,712, 2003.

Loffredo E., M. Pezzuto, N. Senesi: Humic Substances: Versatile Components of Plants, Soils and Water. E.A. Ghabbour and G. Davies (eds.): RSC, Cambridge 2000.

Lotosh T.D.: Experimental bases and prospects for the use of humic acid preparations from peat in medicine and agricultural production. (In Russian) Naucn. Dokl. Vyss. Skoly. Biol. Nauki 10: 99–103, 1991.

Lubal P., D. Široký, D. Fetsch, J. Havel: The acidobasic and complexation properties of humic acids. Study of complexation of Czech humic acids with metal ions. Talanta 47: 401–412, 1998.

Lubal P., D. Fetsch, D. Široký et al.: Potentiometric and spectroscopic study of uranyl complexation with humic acids. Talanta 51: 977–991, 2000.

Madejón E., R. López, J.M. Murillo, F. Cabrera: Agricultural use of three (sugar-beet) vinasse composts: effect on crops and chemical properties of a Cambisol soil in the Guadalquivir river valley (SW Spain). Agric. Ecosyst. Environm. 84: 55–65, 2001.

Majakova E.F. and V.A. Proskurjakov: Proc. 4th International Peat Congress, Ontaniemi 1972 p. 235.

Manning T.J., T. Bennett, D. Milton: Aggregation studies of humic acid using multiangle laser light scattering. Sci. Total Environ. 257: 171–176, 2000.

Marinsky J.A. and M.M. Reddy: Vapor-pressure osmometric study of the molecular weight and aggregation tendency of a reference-soil fulvic acid. Anal. Chim. Acta 232: 123–130, 1990.

Mine K., R.A. Drijber, R.E. Gaussoin: Labile soil organic matter as a potential nitrogen source in golf greens. Soil Biol. Biochem. 35: 1643–1649, 2003.

Mund-Hoym W.D.: The effect of salhumin baths on rheumatic and traumatic joint diseases. Med. Welt. 32: 1378–1381, 1981.

Oglesby R.T., R.F. Christman, C.H. Driver: The biotransformation of lignin to humus facts and postulates. Adv. Appl. Microbiol. 9: 171–84, 1967.

Pacheco M.L. and J. Havel: Capillary zone electrophoretic study of uranium(VI) complexation with humic acids. J. Radioanal. Nucl. Chem. 248: 565–570, 2001.

Pacheco M.L. and J. Havel: Capillary zone electrophoresis of humic acids from American continent. Electrophoresis 23: 268–277, 2002.

Pacheco M.L., E.M. Peña-Méndez, J. Havel: Supramolecular interactions of humic acids with organic and inorganic xenobiotics studied by capillary electrophoresis. Chemosphere 51: 95–108, 2003.

Pacheco M.L. and J. Havel: New results from capillary electrophoresis and MALDI TOF MS studies of humic acids interactions with various compounds and xenobiotics. In Ghabbour E.A. and G. Davies (eds): Humic Substances: Molecular Details and Applications in Land and Water Conservation. Taylor and Francis Inc., New York 2005, in print.

Peña-Méndez E.M., D. Fetsch, J. Havel: Aggregation of humic acids in aqueous solution vapor pressure osmometric, conductivity, spectrophotometric study. Anal. Chim. Acta 2004, in print.

Peuravuori J. and K. Pihlaja: Molecular size distribution and spectroscopic properties of aquatic humic substances. Anal. Chim. Acta 337: 133–149, 1997.

Pflug W. and W. Ziechman: Humic acids and the disruption of bacterial cell walls by lysozyme. Soil Bio. Biochem. 14: 165–166, 1982.

Pokorná L., D. Gajdošová, J. Havel: Characterization of humic acids by capillary zone electrophoresis and matrix assisted laser desorption ionisation time of flight mass spectropmetry. In Ghabbour E.A. and G. Davies (eds.): Understanding Humic Substances:

Advanced Methods, Properties and Applications. RSC, Cambridge 1999.

Pokorná L., M.L. Pacheco, J. Havel: Highly reproducible capillary zone electrophoresis of humic acids in cyclodextrines or oligosaccharides modified background electrolytes. J. Chromatogr. A. 895: 345–350, 2000.

Pokorná L., D. Gajdošová, S. Mikeska, J. Havel: Analysis and characterization of a "standard" coal derived humic acid, humic substances. In: Versatile components of plants, soils and water. Ghabbour E.A. and G. Davies (eds.): RSC, Cambridge 2000, pp. 299–329.

Pokorná L., D. Gajdošová, S. Mikeska, P. Homoláč, J. Havel: The stability of humic acids in alkaline media. In: Humic Substances. Structures, Models and Functions. Ghabbour E.A. and G. Davies (eds.): RSC, Cambridge 2001, pp 133–149.

Purdue E.M.: Measurements of binding site concentrations in humic substances. In: Metal Speciation – Theory, Analysis, and Applications. Kramer J.R. and H.E. Allen (eds): Lewis Publ., Chelsea 1998, pp. 135–154.

Samanidou V., I. Papadoyannis, G. Vasilikiotis: Mobilization of heavy-metals from river sediments of Northern Greece, by humic substances. J. Environm. Sci. Health A26: 1055–1068, 1991.

Schmeide K., S. Pompe, M. Bubner, K.H. Heise, G. Bernhard, H. Nitsche: Uranium(VI) sorption onto phyllite and selected minerals in the presence of humic acid. Radiochim. Acta 88: 723–728, 2000.

Schulten H.-R.: Models of humic structures: Association of humic acids and organic matter in soils and water. In Clapp C.E, M.H.B. Hayes, N. Senesi, P.R. Bloom, P.M. Jardine (eds.): Humic Substances and Chemical Contaminants: Proc. Workshop and Symp. Int. Humic Substances Soc., Soil Sci. Soc. Am., Am. Soc. Agronomy, Anaheim 1997, pp. 26–27. Soil Sci. Soc. Am., Inc., Madison 2001, pp. 73–87.

Schulten H.-R.: New approaches to the molecular structure and properties of soil organic matter: Humic-, xenobiotic-, biological-, and mineral-bounds:. In Violante A., P.M. Huang, J.-M. Bolag, L. Gianfreda (eds.): Soil Mineral-Organic Matter-Microorganisms Interactions and Ecosystem Health. Dynamics, Mobility and Trasformations of Pollutans and Nutrients. Development in Soil Science 28A, Elsevier, Amsterdam 2000, pp. 351–381.

Senesi N., T.M. Miano, M.R. Provenzano, G. Brunetti: Characterization, differentiation, and classification of humic substances by fluorescence spectroscopy. Soil Sci. 152: 259–271, 1991.

Šenkýř J., D. Ročáková, D. Fetsch, J. Havel: The acidobasic and complexation properties of humic acids. Study of humic acids complexation with cadmium and lead (II) by pulse anodic strippping voltametry. Tox. Environ. Chem. 33: 1–15, 1998.

Schiller F., R. Klöcking, P. Wutzler, I. Farber: Results of an oriented clinical trial of ammonium humate for the local treatment of herpesvirus hominis infections. Dermatol. Monatsschr. 165: 505–509, 1979.

Schneider J., R. Weis, C. Manner et al.: Inhibition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: mechanism of inhibition. Virology 218: 389–395, 1996.

Shin D., Y. Chung, Y. Choi et al.: Assessment of disinfection by-products in drinking water in Korea. J. Expo. Anal. Environ. Epidemiol. 9: 192–199, 1999.

Skliar T.V., A.V. Krysenko, V.G. Gavriliuk, A.I. Vinnikov: A comparison of the developmental characteristics of Neisseria gonorrhoeae and staphylococcus aureus cultures on nutrient media of different compositions. Mikrobiol. Z. 60: 25–30, 1998.

Sprengel K.: Die Bodenkunde oder die Lehre vom Boden. Műller, Leipzig 1839.

Stenson A.C., W.M. Landing, A.G. Marshall, W.T. Copper: Ionization and fragmentation of humic substances in electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry. Anal. Chem. 74: 4397–4409, 2002.

Stenson A.C., A.G. Marshall, W.T. Copper: Exact masses and chemical formulas of individual suwannee river fulvic acids from ultrahigh resolution ESI FT-ICR mass spectra. Anal. Chem. 75: 1275–1284, 2003.

Stevenson F.J.: Humus chemistry genesis, composition, reactions. Willey Interscience, New York 1982.

Tan K.H., R.A. Leonard, L.E. Asmussen, J.C. Lobartine, A.R. Gingle: Composition of humic acids extracted under air and nitrogen atmosphere. Comm. Soil Sci. Plant. Anal. 21: 1999–2016, 2002.

Tan K.H.: Chemical composition of humic matter. In: Humic Matter in Soil and the Environment. Principles and Controversies. Marcel and Dekker, New York 2003.

Thiel K.D., R. Klöcking, H. Schweizer, M. Sprossig: In vitro studies of the antiviral activity of ammonium humate against herpes simplex virus type 1 and type 2. German Zentralbl. Bakteriol. 239: 304–321, 1977.

Thiel K.D., B. Helbig, R. Klöcking, P. Wutzler, M. Sprossig, H. Schweizer: Comparison of the in vitro activities of ammonium humate and of enzymically oxidized chlorogenic and caffeic

acids against type 1 and type 2 human herpes virus. Pharmazie 36: 50–53, 1981.

Thorn K.A., W.S. Goldenberg, S.J. Youngerand, E.J. Weber: Humic and fulvic acids: isolation, structure, and environmental role. In Gaffney J.S., N. A. Marley, S. B. Clark (eds.): ACS Symposium Series 651, 1996, p. 299.

Van Rensburg C.E., J. Dekker, R. Weis, T.L. Smith, J. van Rensburg, J. Schneider: Investigation of the anti-HIV properties of oxihumate. Chemotherapy 48: 138–143, 2002.

Verstraete W. and W. Devliegher: Formation of non-bioavailable organic residues in soil: perspectives for site remediation. Biodegradation 7: 471–485, 1997.

Waksman S.A.: Humus: origin, chemical composition, and importance in nature. Williams and Wilkins Co., Baltimore 1938.

Yamada E., T. Ozaki, M. Kimura: Determination and behavior of humic substances as precursors of trihalomethane in environmental water. Anal. Sci. 14: 327–332, 1998.

Zhang M. and Z. He: Long-term changes in organic carbon and nutrients of an Ultisol under rice cropping in southeast China. Geoderma 118: 167–179, 2004.

Ziechmann W.: Humic Substances. George August Universität Göttingen, Bibliographischer Institut. Wissenschaftsverlag, Mannheim 1994.

DRUG DEVELOPMENT RESEARCH 57:140–159 (2002)

DDR

Research Overview

Medical Drugs From Humus Matter: Focus on Mumie

Igor Schepetkin,[1] Andrei Khlebnikov,[2] and Byoung Se Kwon[1,*]

[1]*Immunomodulation Research Center and Department of Biology Science, University of Ulsan, Ulsan, Korea*

[2]*Altai State Technical University, Barnaul, Russia*

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT In this review, we focus on the medicinal drugs from humus matter such as peat, sapropel, and mumie. The most clinically available medicines, containing peat and sapropel extracts, are Torfot, Tolpa Peat Preparation (TPP), Peloidodistillate, Humisol, Peloidin, FiBS, and Eplir. Much attention in the review is concentrated on mumie composition, its pharmacological properties, and new pharmacological drugs with mumie (Shilagen, Abana, Cystone, Diabecon 400, EveCare, Geriforte, Lukol, Pilex, Rumalava, Tentex forte, Nefrotec, Adrenotone, Siotone, La-Tone Gold, Andro-Surge, Solanova Libidoplex). It was concluded that therapeutic properties of crude extracts from peat, sapropel, and mumie have similarity to the ones of fulvic and humic acids. They are antibacterial, antitoxic, antiradical, antiulcerogenic, antiarthritic, immunomodulatory, and antiinflammatory properties. Possible directions for better development of new drugs from humus matter are discussed. Drug Dev. Res. 57 140–159 2002.

Key words: plant humification; peat; sapropel; mumie; shilajit

PEAT, SAPROPEL, AND MUMIE AS HUMUS MATTER

In the last three decades, focus on research of humus matters has increased all over the world, and a large body of evidence has collected to show immense potential of peat, sapropel, and mumie used for developing new medicinal drugs.

Humus matter consists of organic residues that have lost their original structure after decomposition in the environment [Stevenson, 1994]. In contrast to the living cell, where the synthesis of biopolymers is achieved in accordance with the genetic code, in the process of humification there is no established program of any kind; therefore, any substances can appear, both simpler and more complex than initial biomolecules. The resultant products again undergo the synthesis reactions or decomposition, and this process continues practically continuously. As a result of numerous reactions in the humus matter, only the most stable compounds are accumulated, which exist longer than more labile substances [Orlov, 1997]. Examples of biogenic sediments formed mainly of plants include peat, sapropel, and mumie.

Peat is organic soil formed as a result of incomplete disintegration and humification of died marsh plants in conditions of high humidity. The organic matter of peat in 90% consists of humin, humic and fulvic acids (up to 40%), lignin, polysaccharides, lipids, pectines, hemicellulose, and cellulose [Orlov, 1995; Mathur et al., 1993].

Contract grant sponsor: SRC Fund to IRC at UOU from the KOSEF and the Korean Ministry of Sciences and Technology.

*Correspondence to: Byoung S. Kwon, Immunomodulation Research Center, University of Ulsan, P.O. Box 18, Moo-Keo Dong, Nam-Ku, Ulsan, 680-749, Korea.
E-mail: bskwon@uou.ulsan.ac.kr

Received 6 April 2002; Accepted 10 June 2002

Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/ddr.10058

Sapropels are silted deposits of water reservoirs (lakes, peat marshes, sea estuaries) and contain a large quantity (>50%) of organic matter (lignin–humus complex, carbohydrates, bitumen, etc.) in colloidal state. The organic components of "mature" sapropel are produced by the slow decomposition and humification of plants and phytozooplankton in anaerobic conditions [Rohling, 1994]. This process is accompanied by the condensation of phenolcarboxylic acids with formation of new high-molecular organic compounds such as humic and fulvic acids. Peat and sapropel are used in pelotherapy as external remedies. The internal application of extracts from peat and sapropel became usual in Russia and Poland in connection with pioneer works of academician V. P. Filatov, who developed the theory of biogenic stimulators about 70 years ago [Filatov, 1961].

Mumie (common names: shilajit, mummiyo, asphaltum, vegetable asphalt, mineral pitch) is a semihard, brownish black to dark, greasy, black resin that has a distinctive coniferous smell and bitter taste. Mumie is found in mountain regions of Afghanistan, Bhutan, China, Nepal, Pakistan, Tibet, and some regions of the former USSR (Ural, Baykal, Sayan, Caucasus, Altai mountain regions, Kirgysia, Tajikistan, Uzbekistan, and Kazakhstan), where it is gathered in small quantities from steep rock faces at altitudes between 1 and 5 km [Khakimov, 1974; Ghosal et al., 1991b]. On the basis of special features of origin, mumie is divided into three types: petroleum mumie, plant mumie (mumie-asil), and mumie-kiem [Khakimov, 1974]. It is assumed that petroleum mumie is a result of transformation of deep petroleum products of mountains. Mumie-kiem is formed as a result of the long-term humification of guano (feces) of alpine rodents, in particular rock vole *Alticola strelzowi*. Mumie-asil is formed due to the long-term humification of *Euphorbia* and *Trifolium* (clover) plants and lichen [Ghosal et al., 1976, 1988; Korago, 1992]. In fact, radioisotope analysis of mumie was shown that samples from Altai have an age between 500 and 1,500 years, and the age of Central-Asian samples is up to 15,000 years. Mumie-asil has the highest therapeutic quality, and it is this type of mumie and its aqueous extract that are under discussion in the review.

MEDICAL DRUGS FROM PEAT

The humification process leads to change in pharmacological properties of peat extracts, in particular, to enhance in antiulcerogenic and antiradical activity of peat extracts [Yudina et al., 1998a, 1998b]. For some peat humates, the antitoxic properties are also characteristic [Lotosh, 1991]. Pharmacological properties of peat extracts are studied mainly on such patented drugs as Torfot (Russia) and Tolpa Peat Preparation (TPP) (Poland). The chemical composition of these preparations is standardized, which makes it possible to conduct systematic studies on the influence of peat extracts on biological systems.

Torfot

Torfot is a product of distillation of specific peat layers. As a medicinal drug, it is a sterile liquid with the characteristic smell of peat. Torfot is administered in the form of hypodermic or subconjunctival injections in ophthalmology for treatment of patients with keratitis, chorioretinitis, and vascular and degenerative processes in the retina [Bushmich and Golatska, 1972; Gorgiladze et al., 1984; Shpak et al., 1990]. Torfot possesses antibacterial and antiinflammatory action, and improves blood circulation and tissue regeneration. These properties of the drug are the reason for its application in stomatology [Dunaev et al., 1996]. It was shown that Torfot is also applicable for complex treatment for other chronic inflammatory diseases and pulmonary tuberculosis [Strelis et al., 1991].

Tolpa Peat Preparation

TPP was first produced in the laboratory of Polish professor Stanislaw Tolpa (1901–1996), and now it is manufactured by Torf Corporation (Wroclaw, Poland) on the base of peat extract obtained from selected peat deposits in ecologically clean and unpolluted areas in Poland. TPP contains organic substances, primarily bound sugars, amino acids, uronic and humic acids, and mineral salts. TPP (as tablets and gels) is a natural drug registered in Poland for medicinal use. No embryotoxic or teratogenic effects were observed in hamsters or rats after the administration of TPP in daily doses from 5 to 50 mg/kg [Juszkiewicz, 1993]. TPP was found to be neither mutagenic nor genotoxic in selected short-term tests [Koziorowska et al., 1993], and was unable to induce or enhance an allergic sensitization in mice and guinea pigs [Maslinski et al., 1993]. The cytotoxicity (CD50) of TPP for human peripheral blood leukocytes is 1–9 mg/ml (in vitro test) [Inglot et al., 1993].

TPP is an interferon-α and -γ and tumor necrosis factor-α (TNF-α) inducer in cultures of human peripheral blood leukocytes. The optimal concentration of TPP for cytokine response in leukocyte culture was 10–100 µg/ml [Inglot et al., 1993]. TPP (10 and 100 µg/ml) also enhanced the interferon-β and TNF-α production by mouse peritoneal macrophages [Blach-Olszewska et al., 1993]. The promoting effect of TPP (one intraperitoneal injection a day for 4 days) on mouse humoral response occurred for doses of 0.5–10 mg/kg, whereas the doses of 100 and 250 mg/kg had a suppressive effect. The impact of TPP on the

percentage of splenocytes forming E-rosettes was also dose dependent, but in this case stimulating activity was observed for the doses of 2.5–25 mg/kg [Obminska-Domoradzka et al., 1993b]. The intravenous administration of TPP to rabbits at a dose of 5 mg/kg increases the percentage of phagocytizing cells and phagocytic activity of neutrophils. A single administration of TPP (50 mg/kg) to rabbits with lipopolysaccharide (LPS)-induced fever leads to total inhibition of endotoxic shock syndrome [Obminska-Domoradzka et al., 1993a]. TPP increased the ability of human mononuclear leukocytes from patients with coronary artery disease to induce neovascularization in the local graft-versus-host reaction, and decreased the high activity of lymphocytes from rheumatoid arthritic patients [Skopinska-Rozewska et al., 1993]. The interleukin-1 release in cultures of mononuclear leukocytes from patients with rheumatoid arthritis was inhibited by TPP in concentration of 100 pg/ml [Skopinska-Rozewska, 1991]. Both TPP and its fractions suppress the lipid peroxidation in the mitochondria from human placenta [Piotrowska et al., 2000].

MEDICAL DRUGS FROM SAPROPELES

Sapropeles of different kinds are varied in their ability to correct hepatic function in rats with toxic hepatitis and have positive effect during experimental therapy of pancreatitis [Ioshchenko and Zyrianova, 1991; Kuzmenko et al., 1998]. The restoring influence of sapropeles appears to occur due to both their adaptogenic and antioxidant effects [Krylov et al., 1990; Nizkodubova et al., 1991; Yudina et al., 1998a]. At present, the medicinal remedies Humisol, Peloidin, and FiBS were produced from the different kinds of sea mud.

Peloidodistillate

Peloidodistillate is produced by distillation of sapropel from Tambukan lake (Caucasian region, Russia). The variety of this drug is "Vitapeloid" (1% solution of pyridoxine hydrochloride in Peloidodistillate). Therapeutic effect of the drug is caused by the presence of phenolcarboxylic acids, amines, vitamins, and microelements in its composition. The preparations stimulate metabolic processes in organism, accelerate regeneration ability, increase the organism resistance to unfavorable factors, and activate immunity. The drug does not possess allergic, teratogenic, and carcinogenic properties. It is applied in ophthalmology for treating patients for degenerate processes of cornea and retina, and initial forms of optical nerve atrophy [Degtiarenko et al., 1989]. The drug is also recommended against radiculites and neuralgias. In gynecology it is used against chronic inflammatory processes.

Humisol

Humisol (produced in Tallinn, Estonia) is 0.01% solution of humic acid fractions from Haapsalu (Baltic Sea) estuarine mud in 0.9% NaCl solution. Humisol is used (intramuscularly or via electrophoresis) in the cases of chronic radiculites, plexitises, neuralgia, rheumatoid arthritis, arthroses, chronic diseases of tympanum, paranasal sinuses, rhinitis, and other diseases for stimulation of immunity. For the patients treated with Humisol as adjuvant drug, the salmonellosis course was more favorable, the period required for recovery was shorter, and immunity indices earlier became normal [Vereshchagin and Golosnoi, 1994]. The antimutagenic effect of Humisol in cultures of blood T-lymphocytes exposed to cyclophosphane was discovered [Sevostianova, 1998].

Peloidin

Peloidin is a filtrate of specific kind of mud solution from Odessa (Black Sea) estuarine sapropel. Oral administration and electrophoresis of Peloidin are beneficial in lesions of gastric and duodenal mucosa [Komarova et al., 1991] and diseases of the gallbladder and biliary tract [Kuberger and Kalmanovskaia, 1966]. Peloidin phonophoresis proved to be valuable in the treatment for pathogenetic condition at all stages of lumbar osteochondrosis [Shmakova et al., 1990]. The drug is also applicable for treating patients with inflammatory processes of the genital system [Riabtseva et al., 1975].

Peloidin (with phonophoresis) and Humisol (as intramuscular injections) are demonstrated to cause a distinct increase of cellular immunity indices, and a positive trend in biochemical parameters and cardiovascular function in patients with pulmonary tuberculosis [Strelis et al., 1989, 1991; Strelis and Zhivotiagina, 1991].

FiBS

FiBS (abbreviation of author names: *Fi*latov VP, *B*iver VA, *S*korodinskaya VV) is a product obtained by distillation of specific kind of sea mud. It contains cinnamic acid and coumarins. FiBS possesses immunomodulating action on primary humoral immune response and does not increase a delayed-type hypersensitivity reaction [Degtiarenko et al., 1989, Degtiarenko, 1990].

Eplir

Eplir is a 1% oil solution of lipid fraction from specific sulfide mud. Eplir administration to rats

with CCl_4-induced hepatitis protects the liver parenchyma against dystrophy, necrosis, and inflammation [Vengerovskii et al., 2001]. The mechanism of Eplir biological action is determined by its antioxidant properties [Saratikov et al., 1990].

PHARMACOLOGICAL PROPERTIES OF MUMIE

Mumie is a complex natural mixture of organic (60–80%) and inorganic (20–40%) compounds and trace elements [Korago, 1992; Peerzada et al., 1999]. Mumie samples from different regions of the Earth have similar physical properties and qualitative chemical composition, but they differ in percent ratio of components. The levels of biologically active ingredients vary with geographic region, so some mumie samples may have high levels of the active ingredients. On dissolving in water, nearly 30–50% of the weight of mumie passes into the supernatant liquid, and the sediment includes mineral and plant residues in quantities depending on purity of the samples used.

Although mumie has been used in the folk medicine of different countries for almost 3,000 years, there are still many legends involving its mysterious origin. In Arabic countries mumie is called "mountain sweat"; in Burma, "mountain blood"; in Tibet and Mongolia, "rock juice"; and in Altai, "mountain oil." "Shilajit" in Sanskrit means "conqueror of mountains and destroyer of weakness." The name "mumie" was devised by the Arabs. Initially this medicine was called "Mum" in Persian. In ancient Egypt, this wonderful resin was used for embalming mummies.

Mumie was traditionally used in Asian herbal medicine both inwardly and outwardly against injuries, bone fractures, dislocations, diseases of skin, diseases of peripheral nervous system (neuralgia, radiculitis), and also as a soothing and antiinflammatory agent. Greek physicians used this medicine as an antidote to poisons and in the treatment of various problems including arthritis and inflammation. Avicenna in *Canon Medicinae* wrote that mumie possessed the ability to resorb tumors and pimples. "Mountain wax" in the form of drinking and rubbing is an excellent remedy for pains with dislocations and fractures, injuries, wounds, and paralysis of facial nerve. Mumie is useful in cases of migraine, vertigo, diseases of the ear, angina, hemorrhages, diseases of the gastrointestinal tract and urinary organs, and bites from snakes and scorpions [Rasulov, 1964]. Mumie was used as a rejuvenator in traditional Russian and Ayurveda medicine [Tiwari and Tiwari, 1973; Khakimov, 1974].

Mumie is prescribed for genitourinary diseases, diabetes, jaundice, adiposity, enlarged spleen, digestive disorders, epilepsy, nervous diseases, elephantiasis, tuberculosis, chronic bronchitis, asthma, anemia, amenorrhea, dysmenorrhea, menorrhagia, eczema, leprosy, anorexia, fracture of bones, and osteoporosis [Anisimov and Shakirzyanova, 1982; Nigam et al., 1984; Acharya et al., 1988; Bhattacharya, 1995]. Mumie is useful as an aphrodisiac, rejuvenator, alternative tonic, internal antiseptic, diuretic, lithontriptic [Rasulov, 1964; Kozlovskaya, 1968; Acharya et al., 1988]. Mumie can be applied as a remedy for the above-mentioned and some other disorders both as an independent preparation and as a component of herbomineral formulations (drugs and manufacturers, including location of manufacturers, are listed in Table 1).

Different companies manufacturing crude extract of mumie give their recommendations regarding the application of this preparation for humans. Best Nutrition Products (United States) recommended the following regimen for adults: 0.2 g of mumie extract, per os, two to three times a day for 25–30 days during intense training or stress; repeat course after 1–2 weeks. Russian doctors recommend administration of mumie for treating children depending on their age: from 3 months to 1 year: 0.01–0.02 g/day, from 1 to 5 years: 0.03–0.04 g/d, from 5 to 9 years: 0.05 g/d, from 9 to 14 years: 0.1 g/d.

Successful application of mumie in folk medicine as a remedy for many diseases focused the attention of researchers on studying the therapeutic properties and chemical composition of this multi-component natural substance.

Influence of Mumie on Enzyme Activity, Ion Transport, and Free Radical Processes

Mumie (0.2–0.8 mg/ml) activates mitochondrial respiration but suppresses activity of succinate-oxidase and NADH-oxidase in mitochondrion [Almatov and Akhmerov, 1977]. It is assumed that the stimulating action of Mumie is caused by activation of Ca^{2+} transport. Mumie induces a dose-related increase in superoxide dismutase, catalase, and glutathione peroxidase activities in frontal cortex and striatum of rats [Ghosal et al., 1993; Bhattacharya et al., 1995; Ghosal and Bhattacharya, 1996].

Processed extract of mumie efficiently traps hydroxyl radicals, NO· and SO· radicals and also has the ability to regenerate ascorbic acid [Ghosal, 1995]. Mumie is an inhibitor of lipid peroxidation induced by cumene hydroperoxide and ADP/Fe^{2+} complex in a dose-dependent manner. It decreases the rate of oxidation of reduced glutathione and inhibits ongoing lipid peroxidation, induced by these agents, immediately after its addition to the incubation system [Tripathi et al., 1996]. Antiradical properties of mumie extract can be attributed to the presence of dibenzo-α-pyrones and fulvic acid [Wang et al., 1996]. It is

assumed that the therapeutic properties of some multicomponent preparations, containing mumie in their composition, are caused by antiradical properties of this humus matter [Mitra et al., 1996a; Bhattacharya et al., 1997].

Antibacterial activity

Mumie possesses the antimicrobial activity with respect to different strains of the widespread pyogenic microbes (staphylococci, streptococci, coliform bacteria, enterococci, Proteus) [Shakirov, 1967, 1969] and is applied for treatment of suppurative wounds [Muratova and Shakirov, 1968]. The bactericidal activity of mumie extract may be related to some of its components, such as benzoic and fulvic acids [Van Rensburg et al., 2000]. The clinical studies carried out in Kazakhstan Tuberculosis Institute (Almaty) showed that the application of mumie extract (patented name "Olepet") for complex treatment of 300 patients with pulmonary tuberculosis shortened the treatment period by three to four times as compared with traditional chemotherapy.

Mumie as Anabolic Agent

To reach the anabolic effect of mumie extract, the short-term cycles were used (0.3–0.6 g/d, for 10–12

TABLE 1. Pharmaceutical Companies Producing Medical Drugs From Mumie, Peat, and Sapropeles

Medical Drug	Company	Company Location (City/State/Country)
Abana (HeartCare)	Hymalaya Drug Company	Banglore, India
Adrenotone	Rockwell Nutrition Company	Miami, FL
	Gaines Nutrition	Redlands, CA
Andro-Surge	Mineral Connection	Taylor, TX
Cystone (UriCare)	Ayurvedic Concepts	Segamat, India
	Hymalaya Drug Company	Banglore, India
	Stichting EISRA	Den Haag, The Netherlands
Diabecon D-400	Hymalaya Drug Company	Banglore, India
EveCare	Hymalaya Drug Company	Banglore, India
GeriCare	Hymalaya Drug Company	Banglore, India
Geriforte	Hymalaya Drug Company	Banglore, India
Kidney Formula	Banyan Botanicals	Albuquerque, NM
La-Tone Gold	LA-Medica, Pvt. Ltd.	Calcutta, India
Lukol	Indousplaza	New York, NY
	Hymalaya Drug Company	Banglore, India
Mumie	Ehinops	Sevastopol, Ukraine
	Evalar	Biysk, Russia
Mumie-Vitamustim	Avicenna	Moscow, Russia
Nefrotec	Hymalaya Drug Company	Banglore, India
Pilex (VeinCare)	Hymalaya Drug Company	Banglore, India
Rumalaya (JointCare)	Hymalaya Drug Company	Banglore, India
Shilagen	Sandusky's Health Alternatives	Sylvan Lake, Alberta, Canada
	Crucible Catalog	Sacramento, CA
Shilajit	Dabur India, Ltd.	New Delhi, India
	FabriChem	Fairfield, CT
	Aarogya Herbals (P), Ltd.	New Delhi, India
	SDR Shilajit	Amritsar, Punjab, India
Siotone	Albert David, Ltd.	Calcutta, India
	Resources International	Lexington, KY
Solanova Libidoplex	Wellness Tools	Colorado Springs, CO
	Solanova	Novato, CA
	Metafoods	Cottonwood, AZ
	Eckhart Corp.	Novato, CA
Somatomed (VesPro GHS)	VesPro Life Sciences	Overland Park, KS
StressCare	Hymalaya Drug Company	Banglore, India
Tentexforte	Hymalaya Drug Company	Banglore, India
Eplir	Biolit	Tomsk, Russia
FiBS	Biostimulator	Odessa, Ukraine
	Farmak	Kiev, Ukraine
Humisol	Tallinna Farmaatsiatehas	Tallinn, Estonia
Peloidodistillate	Biostimulator	Odessa, Ukraine
Tolpa Peat Preparation (TPP)	Torf Corp.	Wroclaw, Poland

days) in sportsmen. These cycles are often repeated three to four times after a 15–20-day break. The effect includes activation of anabolic processes on cell and molecular levels in different organs and tissues [Gupta et al., 1966]. Experimental investigations showed that mumie extract (0.5 g/kg, per os, daily, for 10 days) accelerated processes of protein and nucleic acid synthesis, stimulated the energy-providing reactions in liver, and promoted transportation of minerals, especially calcium, phosphorus, and magnesium, into muscle and bone tissues [Shvetskii and Vorobeva, 1978]. Studies of anabolic properties made it possible to use mumie extract in elite Russian military and sports establishments for nearly 4 decades for increasing strength and muscle mass as well as for its recuperative powers.

Mumie extract is a constituent part of several multi-component anabolic preparations, such as Ves-Pro GHS (Somatomed), Andro-Surge and Geriforte [Dubey et al., 1980].

Mumie as Adaptogen

Mumie extract is an adaptogenic agent that protects the human physiological system against diverse stressors and improve restoration (recovery) after exercises. However, it did not significantly change floating time on the swimming test in mice [Bose and Gupta, 1999]. Mumie extract is included in composition of several multicomponent drugs with adaptogenic activity, such as Adrenotone, Siotone, StressCare, and Geriforte [Dubey et al., 1984a; Bhattacharya et al., 2000].

Effect of Mumie on Endocrine System

Mumie contains steroid-like compounds; among them are sterols and dibenzo-α-pyrones. Plant sterols could be incorporated into mumie in humin bound form [Lichtfouse, 1999]. Mumie extract may act as an antidiabetic agent and can enhance the level of growth hormone in diabetic patients. It is a component of complex drugs VesPro GHS (Somatomed) and Andro-Surge that stimulate the human organism to produce more of its own growth hormone and testosterone.

Application of Mumie for Stimulation of Regeneration Processes

Mumie is highly effective in the treatment of thermal burns [Foigelman, 1972] and stimulation of hepatic regeneration after CCl_4 administration [Vaishwanar et al., 1976]. After burn injury, the application of mumie extract causes a decrease in pain, disappearance of inflammation, shortening of the periods of scab rejection and wound purification from the necrotic tissues, appearance of granulations, and early epithelization [Anisimov and Shakirzyanova, 1982]. The use of mumie (0.6 g/d, per os, for two 10-day cycles with 1-week break) for treating for postoperative cavities in 50 patients with chronic suppurative otitis contributed to the more rapid healing of trepanation cavity of the tympanum [Psakhis and Aizenberg, 1976]. It is possible that the positive role of mumie extract is caused by its stimulating action on bone regeneration. Ukrainian doctors used mumie for rehabilitation of patients after operation for vertebral–cerebrospinal injury [Perederko et al., 1998]. A significant positive action of mumie (irrigation by 0.5% water solution of mumie, daily, for 1 week) was noticed during the rehabilitative treatment after tonsillectomy [Gordievskii and Barulina, 1974].

Mumie extract (0.1 g/kg, daily) caused acceleration by 20–25% of primary callus formation on the focus of bone fracture of long bone in rabbits [Ismailova, 1965; Shakirov, 1965a; Kelginbaev et al., 1973]. Mumie in the same doses led to an increase of [^{32}P] uptake by 3–3.5 times on the focus of fracture [Shakirov, 1965b]. According to data of Tkachenko et al. [1979], the effect of mumie extract on bone regeneration in guinea pigs after fracture can be different depending on the dose of the preparation and time elapsed after operation. The maximum intensification of regeneration was observed when mumie was applied daily on the early periods after operation (1–7 days) at the dose of 0.3 g/kg. Under these conditions, mumie caused twofold intensification of osteoid formation and bone mineralization. Administration of mumie in these doses 2–3 weeks after operation was accompanied with reducing of osteoid mineralization by seven times [Tkachenko et al., 1979]. It was reported that mumie extract acted favorably on bone regeneration after fractures in children [Kelginbaev et al., 1973] and in patients subjected to surgery for osteoarticular tuberculosis [Suleimanov, 1972].

Antiinflammation Properties

Mumie extract is highly effective during treatment for paradontosis in humans [Habilov, 1971] and has significant antiinflammatory effect on osteoarthrosis, rheumatoid arthritis, ankylosing spondylitis, and cervical spondylosis [Soliev, 1983]. In folk medicine and clinics, mumie is used to treat patients for peptic ulcer (0.3 g/d, for 20–25 days). It is suggested that antiulcerogenic activity of mumie extract is determined by its antihistamine and antiserotonin effects. Antiulcerogenic activity of mumie extract was confirmed by experiments [Kozlovskaya, 1971]. Crude extract of mumie at the dose of 0.05 g/kg suppressed carrageenan-induced acute pedal edema (to 77%), granuloma pouch and adjuvant-induced arthritis in rats [Goel

et al., 1990]. It is also possible that antiinflammatory and antiulcerogenic properties of mumie can be explained by the presence of benzoic acid (up to 7–8%), fulvic acids, 4′-methoxy-6-carbomethoxybiphenyl, and tirucallane-type triterpenoids in its composition. These compounds are known to possess strong antiinflammatory properties [Ghosal et al., 1988, 1991b; Alam and Gomes, 1998; Rajic et al., 2001]. Due to bacteriostatic and antiinflammatory action, mumie extract facilitates the process of wound cleaning from necrotic tissues, granulation, and epithelization, and decreases the period of wound healing [Tazhimametov et al., 1987].

Immunomodulation and Antiallergic Properties

Application of mumie causes proliferation of lymphocytes of cortical thymus layer, and their intensive migration into thymus-dependent zones of lymph nodes and spleen [Agzamov et al., 1988]. Mumie extract activates macrophage cell migration in epithelioid granulomas in struck pulmonary tissue with experimental tuberculosis [Agzamov et al., 1988]. The specific epitheliocellular granulomas were transformed to macrophageal ones after mumie application. It was shown that the remedy promoted renewal of capillaries in inflammation focus and periphery, increased resorption of necrotic lesions and infiltrates, and improved transport of antibacterial drugs to the inflammation foci [Agzamov et al., 1988]. It is important that the preparation activates phagocytosis and releases cytokines in mouse peritoneal macrophages [Ghosal, 1990; Bhaumik et al., 1993]. The administration of mumie extract in the dose of 0.5 g/kg from the 1st to the 20th day after γ-irradiation (180–220 r/min, dose 600 r) stimulates lymphopoietic erythropoiesis in acute radiation disease. This is manifested by a more rapid restoration of the number of lymphocytes in peripheral blood, bone marrow, and spleen [Rogozkin and Tukhtaev, 1968]. Interestingly, for stimulation of immunity, in the Leningrad Zoo (St. Petersburg, Russia) chinchilla puppies were bottle-nursed with addition of mumie solution [Volkova, 2000].

Because mumie contains biphenyl and benzocoumarin compounds, which have significant antiallergic activity, mast cells are stabilized by mumie extract and have significantly less degranulation [Ghosal et al., 1988; Bhattacharya et al., 1989]. Positive results were obtained during the treatment for eczema and psoriasis (0.2 g/d, per os, two 10-day cycles with 5-day break) [Anisimov and Shakirzyanova, 1982].

Influence of mumie on central and peripheral nervous system

Mumie was used in Indian medicine to attenuate cerebral functional deficits, including amnesia, in geriatric patients. It was shown that this remedy promoted learning and memory [Bhattacharya and Ghosal, 1992; Jaiswal and Bhattacharya, 1992; Ghosal et al., 1993]. Administration of mumie extract (in doses of 0.04 g/kg for 7 days) decreased acetylcholinesterase activity restricted to the basal forebrain nuclei including medial septum and vertical limb of diagonal band in rat brain [Schliebs et al., 1997]. Mumie extract was considered as a prospective inhibitor of analgesic tolerance to morphine [Tiwari et al., 2001]. Treatment with mumie affected neither γ-aminobutyric acid (GABA) and benzodiazepine receptor binding nor NMDA and AMPA glutamate receptor subtypes in any of the cortical or subcortical regions, but increased muscarinic acetylcholine M_2 receptor binding [Schliebs et al., 1997]. Transina, an Ayurvedic herbal formulation comprising *Withania somnifera, Tinospora cordifolia, Eclipta alba, Ocimum sanctum, Picrorrhiza kurroa*, and mumie, exerts significant nootropic effect after subchronic treatment of rats (0.2 or 0.5 g/kg, per os, daily, for 21 days) that may be due to reversal of perturbed cholinergic function [Bhattacharya and Kumar, 1997]. Nevertheless, in the literature several cases were described when mumie application in treating neurologic diseases (multiple sclerosis) led to deterioration in patient state [Magidzon and Khmelevskii, 1982].

Mumie extract was recommended as an effective drug in treatment for radiculitis, plexitises, and neuralgias of different etiology [Kozlovskaya, 1968; Mamadjanov, 1975; Akhmedov and Aminov, 1979; Anisimov and Shakirzyanova, 1982]. In treating trigeminal nerve neuralgia, a combined procedure was used with the application of electrophoresis with 2% lidocaine and 4% mumie solution (in water) (10–12 times). Most of the results were remarkably positive, which was especially noticeable in the case of the neuritic stage of neuralgia with the central genesis and, in case of neuralgia, with predominantly peripheral genesis [Grechko et al., 1985].

Other Therapeutic Properties of Mumie

The mumie extract exhibited significant inhibition in the proliferation of the Ehrlich ascites tumor cells [Ghosal, 1990]. The preparation reduces the increased level of cholesterol in the blood and increases the removal of cholesterol with the bile. Mumie application with acute thrombophlebitis improves the general state of patients, decreases edema and pain, and leads to improvement in vessel pulsation and normalization of blood coagulability [Anisimov and Shakirzyanova, 1982]. Application of mumie under the conditions of intoxicating the animals by lead salts contributed to removal of this poison from the liver. The intravenous application of Caucasian mumie (2.5% solution in

water, 50 mg/ml/d, for 10 days) abolished the hematotoxic effect of thiotepa, a cytostatic agent that has been used in the treatment of malignant lymphomas and solid tumors [Kozlovskaya, 1972].

Mumie Toxicity

Mumie extract does not cause any mortality in mice up to the dose of 1 g/kg (intraperitoneal injection) [Acharya et al., 1988]. For toxicological study, the experimental animals received the preparation daily in the form of 1–10% aqueous solution (orally) for 1 month. The daily doses of mumie extract for rabbits and mice were 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 g/kg. On its application both once (0.5 g/kg) and on a multitime basis (total dose was from 1.5 to 15 g/kg) the investigators did not observe any morphological or histological changes in the internal organs of animals in comparison with the control group [Kelginbaev et al., 1973].

In the Ukrainian Gerontology Institute (Kiev), the study of toxicological properties of mumie picked from alpine regions of Central Asia was carried out. It was found that application of the remedy at the doses of 0.2 and 1 g/kg for 3 months did not lead to negative influence on the function of heart, liver, kidneys, blood cells, or nervous and endocrine systems. The study of specific teratogenic action showed that treatment of pregnant rats with mumie did not render embryotoxic or teratogenic actions. The postnatal development of young rats, whose parents received the preparation, was also normal.

Most of the investigators noted absence of side effects with mumie application at daily dose of 0.1–0.3 g inwardly. Some patients with bone fractures felt burning in the region of fracture. Patients with chronic colitis felt heat, burning, weakness, and sweating during 40–60 min after application of mumie extract. At higher doses (0.9-1.5 g/d) it can lead to increase in body temperature to 37.5°C, sweating, and headache. The duration of this reaction was from 20 min to 2–3 h [Anisimov and Shakirzyanova, 1982].

The use of standardized mumie extract provides the best opportunity of getting a positive result. Shilagen contains mumie that is standardized to contain at least 20% of fulvic acids is the new leader in high potency natural fulvic acid supplements. Raw mumie contains 0.01% wt/wt of dibenzo-α-pyrones. Their content enhances to 1% (wt/wt) in processed mumie extract. Standardization of mumie extract to contain 20% of fulvic acids and 1% of dibenzo-α-pyrones would ensure consistent nootropic activity, high immunomodulation, and very effective antioxidant activity [Ghosal and Bhattacharya, 1996]. The processing needs to remove free radicals, toxins, mycotoxins, and inactive ingredients.

Medical Drugs of Mumie

The pharmaceutical companies in India (Dabur, Aarogya Herbals (P) Ltd.), the United States (Fabri-Chem, Triple Crown), Ukraine, Kazakhstan, and Russia produce purified mumie extract without other biologically active additions (drugs: Shilajit, Mumie-Vitas, Olepet, Mumie), or mumie extract included in compositions of complex herbal and mineral formulas. Some of these preparations resulted from the development of Ayurvedic conceptions, the others were devised by contemporary research and patented. For example, Rowland [1999] suggested the mixture of vitamins, minerals, and mumie. Mumie is used in composition formulas of Shilagen, Abana (HeartCare), Cystone (UriCare), Diabecon 400, EveCare (MenstriCare), Geriforte (GeriCare/StressCare), Lukol, Pilex (VeinCare), Rumalava (JointCare), Tentex forte (VigorCare-Men), Nefrotec, Adrenotone, Siotone, La-Tone Gold, Andro-Surge, Solanova Libidoplex, and Renone cream [Jadhav and Bahga, 1971; Mardicar, 1975; Bhatta, 1982]. The content of mumie in some medical formulations is shown in Table 2.

Shilagen includes proven healing substances such as ashwagandha, ginkgo biloba, bacopin, and trace minerals to aid in the absorption and synergy of the primary mumie ingredients. The producers of Shilagen use a patented oxygen/nitrogen displacement extraction process that ensures the proper pH and increases the effectiveness of active ingredients of mumie by approximately 800%. They also use a standardized extract, so equal high levels of active ingredients are in each bottle. Shilagen has been recommend to treatment the same disorders for which mumie extract is applicable.

Mumie is incorporated in the herbal formula of *Adrenotone* that includes Chinese herbs used for centuries. Adrenotone has been recommend as a remedy to naturally support the adrenal function, high energy levels, and overall wellness during times of stress or immune weakness [Ziauddin et al., 1996]. Adrenotone is produced and distributed by Rockwell Nutrition Company and Gaines Nutrition.

Siotone is an herbal formulation in Ayurvedic medicine. It consists of components all of which, including mumie, are known to promote physical and mental health, and improve immunity. A study was undertaken to investigate the adaptogenic activity of Siotone against chronic unpredictable, but mild, foot-shock stress-induced disturbances in behavior in rats. Siotone (200 mg/kg, in mice) revealed significant decrease of chronic fatigue induced by Porsolt's forced

TABLE 2. Amount of Mumie in the Various Medical Formulations

Medical formulation	Weight of mumie (mg/capsule)	Percent of mumie (weight %; mumie per total active ingredients in the capsule)	References
Adrenotone	25	3.8	Ziauddin et al., 1996
Andro-Surge	50	22.6	The Mineral Connection Co.
Cystone	13	5.8	Singh et al., 1985
D-400	30	7	Sundaram et al., 1996
La-Tone Gold	30	4.6	LA-Medica Pvt Ltd.
Lukol	9	3	Gupta and Bhanot, 1973
Pilex	16	6	Misger et al., 1977
Rumalaya	16	4.2	Singh et al., 1984
Siotone	250	16.1	Bhattacharya et al., 2000
Tentex Forte	32	9.7	Bhatta, 1982

swimming test [Kaur and Kulkarni, 2000]. Siotone in granules (100 and 200 mg/kg) proved to be a good protector against convulsions induced by pentylenetetrazol, maximal electroshock, and strychnine. In hypoxic stress-induced convulsions, only 200 mg/kg were effective. The anticonvulsant action of Siotone granules was blocked by flumazenil (4 mg/kg), suggesting the involvement of a GABA-ergic mechanism [Kulkarni and Joseph, 1998a]. An extensive study of Siotone granules demonstrated its central nervous system depressant effect as well as its beneficial effect in anxiety and cognition in animals [Kulkarni and Joseph, 1998b]. Chronic stress-induced increase of tribulin activity in rat brain was also reversed by these doses of Siotone [Bhattacharya et al., 2000]. The drug is produced by Resources International.

La-Tone Gold is useful in treatment of sexual impotence. La-Tone Gold provides a rapid arousal by exerting generally stimulating and enhancing influence on the function related to the reproductive system that promotes enhanced genital blood circulation and engorgement, euphoria, and a satisfying tumescence besides improving stamina in males. La-Tone Gold is produced by LA-Medica Pvt Ltd..

Andro-Surge is an herbal formulation designed for optimal regulation of anabolic hormones and testosterone levels. This formula is recommended for male athletes, being especially effective for those over 38 years of age, or for adults with low levels of dehydroepiandrosterone. Andro-Surge is produced by Mineral Connection.

Solanova Libidoplex is a synergistic blend of specific herbal extracts and vitamins. It is a very powerful virility-enhancing formula designed to heighten energy and stamina. It can also be used as an overall body tonic. Benefits both men and women and is especially safe for use by women. Solanova Libidoplex is produced and distributed by Eckhart Corp., Solanova, and Metafoods.

Evecare (U-3107) is herb-mineral uterine tonic formulated by Himalaya Drug Company. The herbs used in this tonic are effective in various menstrual disorders. These components acting alone and in combination are responsible for the efficacy of the drug in dysmenorrhea, menorrhagia, and other uterine disorders [Mitra et al., 1998].

Mumie is one of the components of *StressCare* and *GeriCare*. StressCare is useful in stress-related conditions such as premature aging, fatigue, insomnia, or emotional imbalance. GeriCare is the ultimate general fitness product that promotes health and helps everyone to age gracefully without any adverse effects.

Geriforte is a completely natural product that regulates and balances all the body organs and systems for comprehensive health care maintenance. Geriforte is used as a restorative tonic to solve the problems of old age in India. Geriforte administration stimulates the antioxidant defense system [Pathania et al., 1998]. Antistress and anabolic properties of this drug were denoted in many reports [Singh et al., 1978; Dubey et al., 1980, 1984a]. Geriforte (feeding for 4 weeks) significantly increased catalase, superoxide dismutase, and glutathione peroxidase activities in liver of mice. In rats, in addition to these enzymes, the levels of reduced glutathione were also significantly enhanced [Pathania et al., 1998]. The contents of superoxide dismutase and catalase in brain of Geriforte-administered animals were increased by 24% and 30%, respectively [Singh et al., 1994].

Mumie is used for urinary disorders or stones in combination with *Kidney Formula*.

Renone cream is an external application ointment formulated for greater relief from muscular pains, joint pains, swelling, and inflammation. It is an exclusive

combination of many reputed and time-tested pain-relieving herbs and ingredients.

Lukol is an indigenous preparation, which is administered orally in the form of tablets for the treatment of leukorrhea. It is claimed to be completely free from toxic effects. The therapeutic value of Lukol in the oral therapy for nonspecific leukorrhea, menorrhagia, and other associated symptoms was established by several clinical trials in India [Bhagwat, 1962; Dabak et al., 1984].

Diabecon D-400 is a herbomineral formulation, main components of which among others are mumie, *Gymnema sylvestre, Pterocarpus mascupium, Casearia esculanta, Eugenia jambolana, Ocimum sanctum*, and *Momordica charantia* [Mitra et al., 1995a, 1995b, 1996a, 1996b]. Diabecon significantly potentiates the hypoglycemic action of insulin in alloxan-induced diabetic rats [Anturlikar et al., 1995]. Diabecon therapy caused a significant increase in islet number and beta cell count and appeared to bring about blood sugar homeostasis by increasing insulin secretion and regeneration of endocrine pancreas [Mitra et al., 1996c]. Clinical application of Diabecon showed that it can be used alone or as adjuvant in non-insulin-dependent diabetes mellitus patients, because it significantly reversed the changes in early diabetic retinopathy [Mitra et al., 1995b; Sundaram et al., 1996]. Experimental trials testify that Diabecon has a nephroprotective action against alloxan-induced renal damage in rabbits [Dubey et al., 1994]. Streptozotocin-induced histopathology changes in pancreas and liver of rats, as well as a decrease in pancreatic islet cell superoxide dismutase, were partially reversed by Diabecon. It was suggested that Diabecon helps in improving the glycogen stores in the liver and prevents the streptozotocin-induced damage through free radical scavenging activity of mumie extract [Mitra et al., 1996c].

Cystone, a patented herbal drug formulation, is claimed to maintain crystalloid–colloid balance and to dissolve the stone matrix, thereby disintegrating renal, bladder, and gall stones [Singh et al., 1985]. Cystone has been clinically used extensively for complex treating for many urinary tract complications such as urolithiasis [Rai, 1960; Khan, 1983], burning micturition [Garg and Singh, 1985], neuroureterolithiasis [Misger, 1982], urinary tract complications in pregnancy [Sengupta, 1987], urinary tract infections [Srivastava et al., 1991], and other urinary tract disorders [Chatterjee, 1982; Sharma et al., 1983]. Cystone has been shown to provide partial but significant protection against renal toxicity induced by the antitumor agent cisplatin in rats and mice [Rao and Rao, 1998; Rao et al., 1999]. The protection may be mediated through its ability to inhibit lipid peroxidation. In fact, Cystone inhibited the lipid peroxidation in renal cortical slices induced by cisplatin [Rao and Rao, 1998]. Cystone is manufactured by Ayurvedic Concepts (Bangalore, India) and Himalaya Drug Company.

Abana is an herbomineral medicinal preparation with the property of downregulation of β-adrenergic receptors. The therapy with Abana proved to be highly effective in hypertensive patients [Dadkar et al., 1990]. Administration of Abana for 3 days increased the basal amplitude and reduced the responses of atria to isoprenaline and norepinephrine in rabbits [Pasnani et al., 1988]. Abana pretreatment potentiated the inotropic responses of histamine and $CaCl_2$. These effects may be due to a specific depressant effect of Abana on the adrenergic receptors and to direct sensitization of the atrium manifested by the increased response to $CaCl_2$ [Pasnani et al., 1988]. Abana seems to reduce preload and afterload and improve diastolic and pump functions of heart that may be responsible for beneficial effect of Abana in patients with ischemic heart disease [Antani et al., 1990]. Histological pictures showed pronounced reduction in the atherosclerotic involvement of the coronary artery in rabbits with an atherogenic diet following treatment with Abana [Tiwari et al., 1993]. Abana protects mice against radiation-induced micronucleus formation and radiation-induced decline in cell proliferation [Jagetia and Aruna, 1997].

Pilex tablets are used for relief of hepatic congestion. The drug reduces venous engorgement and turgidity, tones up the venous walls, reduces portal pressure, prevents bleeding and inflammation, and is a mild laxative [Rangnekar and Arora, 1975; Misger et al., 1977].

Rumalaya is an indigenous formulation whose components, including mumie, have antiinflammatory and antiarthritic properties [Rao and Gupta, 1977]. Rumalaya was tested and found to be useful in various orthopedic problems such as osteoarthritis of the knee joints, rheumatoid arthritis, ankylosing spondylitis, and cervical spondylosis. Ramalaya provided significant clinical improvement in the case of low backache, e.g., spondylolisthesis, spina bifida, and prolapsed intervertebral disc [Singh et al., 1984]. Dubey and co-workers noticed a significant decrease in duration and intensity of pain, more pronounced in fibrositis cases than in sacroiliitis and spondylitis ones [Dubey et al., 1984b]. This drug did not cause any side effects even after continuous oral administration [Dubey et al., 1984b]. Rumalaya is manufactured by Himalaya Drug Company.

VesPro GHS (Somatomed) is a patented drug that stimulates the organism to produce more of its own human growth hormone as well as other important

antiaging hormones. Each tablet contains a nonhormonal herbal base of mumie, licorice, schizandra, and *Tribulus terrestris*. VesPro GHS was found to be a safe and efficacious drug capable of improving many of the clinical signs and symptoms associated with the chronic fatigue syndrome.

CONSTITUENTS OF MUMIE, SAPROPEL, AND PEAT

The main substances that are formed in the process of plant humification are fulvic and humic acids. They comprise a chemical and physical heterogeneous group of high-molecular-weight hydroxylated polyphenolic compounds with colloidal, polydispersed, and polyelectrolyte characteristics and a mixed aliphatic and aromatic nature [Senesi and Loffredo, 1999]. These acids are found abundantly in peat, sapropel, mumie, and other humus matters, being of medicinal importance.

At present, three theories of plant humification exist. According to the lignin theory, lignin is incompletely utilized by microorganisms with the generation of *o*-hydroxyphenols and oxidation of aliphatic side chains to form COOH groups. The modified material is subjected to further unknown changes to yield first humic acids and then fulvic acids [Waksman, 1938]. In an aerobic microenvironment, lignin may be broken down into low-molecular-weight products. According to the polyphenol theory, phenolic aldehydes and acids released from lignin or nonlignin sources (e.g., cellulose) during microbiological attack undergo enzymatic conversion to quinones, the latter polymerize in the presence or absence of amino compounds to form dark-colored polymers [Kononova, 1966]. According to the sugar–amine condensation concept, reduced sugars and amino acids formed as byproducts of microbial metabolism undergo nonenzymatic polymerization to form brown nitrogenous polymers [Stevenson, 1994]. An attractive feature of this theory is that the reactants (sugars, amino acids, etc.) are produced in abundance through the activities of microorganisms. The drastic and frequent climatic changes in mountain regions (freezing and thawing, wetting and drying), together with the intermixing of reactants with mineral materials having catalytic properties, may facilitate condensation and mumie formation (Fig. 1). The composition of fulvic and humic acids in natural organic-mineral matter vary with geographic location. In particular, peat and mumie are exposed to a higher oxygen influence than sapropel, which is permanently submerged. This leads to intensification of oxidation processes and to changes in chemical and biological characteristics of humic and fulvic acids [Orlov, 1995; Esteves and Duarte, 2000].

It was hypothesized that the nonpolar groups in the molecules of humus substances formed micellar or double-layer structures that trap the nonpolar organic in a microscopic hydrophobic environment similar to the behavior of surface-active micelles [Wershaw, 1986]. The structure of the humin complex explains the stabilization of soil organic matter by binding functional biomarkers and encapsulation of small polar molecules [Lichtfouse, 1999]. In fact, the mumie extracts were shown to possess a lattice-like structure perforated by voids of varying dimensions (10–50 μm) [Ghosal et al., 1991a]. These are filled with organic molecules or metal complexes that may be responsible for therapeutic effects. Degrading the humus samples of mumie and analysis of the products spectroscopically shows that they are structurally similar to ordinary soil.

Although it is now widely accepted that mumie, peat, and sapropel extracts can be valuable in conventional medicine, the other way forward is to formulate drugs using the active constituents, rather than to use crude natural extracts. This makes it feasible to do research directed to isolation of active ingredients from natural organic–mineral matter and to study the opportunity of using them individually or in combinations with each other. In particular, a preliminary screening showed that a lipid concentrate extracted from a silt-sulphide sapropel exhibits the highest antioxidative activity [Pisareva et al., 1997]. The lipid concentrate from sapropel was shown to have an antimetastatic effect and it reduced hematotoxicity of cyclophosphane [Burkova et al., 1995].

Characteristics and Pharmacological Properties of Fulvic and Humic Acids

The therapeutic properties of fulvic and humic acid are similar to the properties of crude extracts from peat, sapropel, and mumie. Fulvic and humic acids are panaceas of oriental medicine where they continue to be used extensively for treatment for cold stress, diabetes, skin diseases, rheumatic pain, kidney stones, heart ailments, leprosy, and immune system diseases. Many reports on the beneficial use of fulvic acids for medicine have been published. These include reports documented in a Chinese pharmacological compendium dating from the 15th century. The reports deal with the drug "Wujinsan" containing humic and fulvic acids, implying that these substances are efficient antiinflammatory and blood-coagulating agents. Fulvic acids possess antiallergic action by the mechanism of mast cell protection. Experimental study testified that oxyfulvic acids are effective in the topical treatment of traumatic and chemical dermatitis in cats, dogs, and mice [Dekker and Medlen, 1999; Van Rensburg et al.,

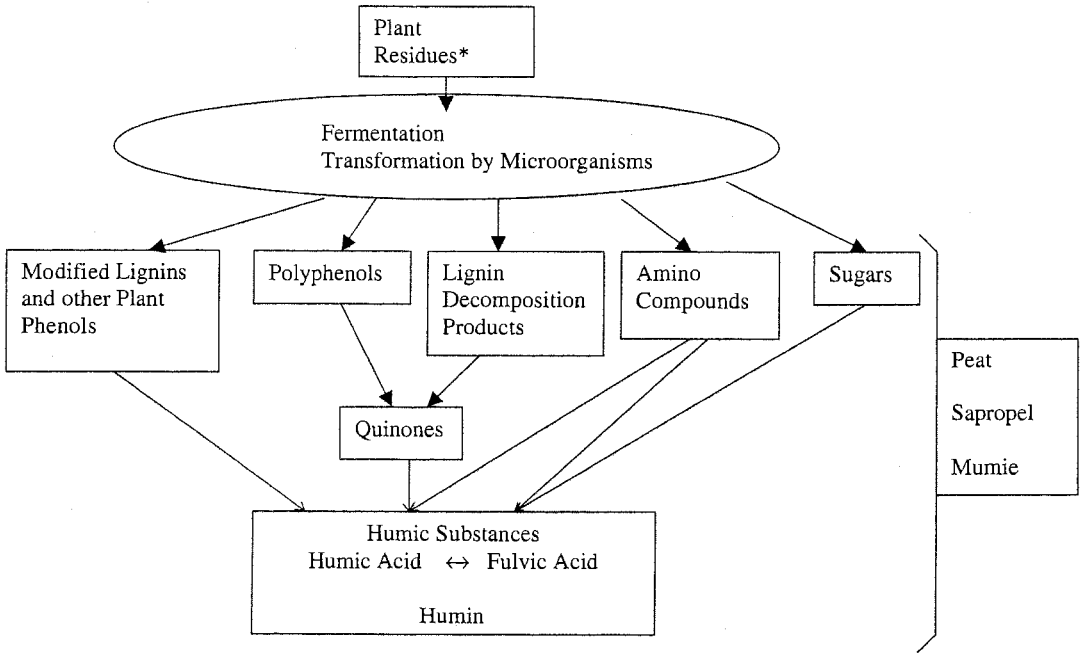


Fig. 1. Schematic diagram of humic substance formation for peat, sapropel, and mumie (*for the formation of sapropel humus matter, the phytozooplankton litter also plays an important role, together with plant residues).

2001]. Neither sensitizing nor irritating properties were detected in the concentrations of up to 10% in humic acid solution [Wiegleb et al., 1993]. Humic and fulvic acids were used as externally applicable drugs in the clinical treatment for hematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis, and osteochondrosis [Laub, 1999]. With respect to internal use, fulvic and humic acids have been shown to be particular useful in therapy for gastritis, diarrhea, stomach ulcers (antiulcerogenic and antistress activity), dysentery, colitis, and diabetes mellitus [Yudina et al., 1996, 1998b; Laub, 1999]. Experimental and clinical results demonstrate that fulvic and humic acids stimulate osteoclastic resorption of transplanted bones as well as hydroxyapatite used for bone substituent [Schlickewei et al., 1993].

The antimicrobial activity of fulvic acid was described by van Rensburg et al. [2000]. In that study, all eight microbial pathogens were tested (*Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, β-haemolytic streptococcus, Klebsiella pneumoniae, Proteus mirabilis*, and *Candida albicans*) and found to be sensitive to oxyfulvic acid at concentrations $\leq 1.5\%$. Ammonium humate isolated from peat water is a higher molecular polyphenolic compound with a strong antiviral activity against herpes simplex virus type 1 and type 2 (at humate concentrations 0.5–20 μg/ml) and influenza virus type A and B [Thiel et al., 1977; Schiller et al., 1979; Thiel et al., 1981; Hils et al., 1986]. Fulvic and humic acids also increase number and functional activity of macrophages, neutrophils, and killer T-cells [Riede et al., 1991; Laub, 1999].

Humic acids were found to inhibit the mutagenicity of benzo[a]pyrene, 2-aminoanthracene, 2-nitrofluorene, and 1-nitropyrene in the *S. typhimurium* test. Desmutagenic effect was caused by adsorption of mutagenes by humic acids [Sato et al., 1987]. Humic acids shortened prothrombin time, activated thromboplastin period [Lu et al., 1990], and inhibited plasma protein C activity [Yang et al., 1994]. Humic acid is involved in tissue factor induction and plasminogen activator inhibitor synthesis in human umbilical vein endothelial cells [Yang et al., 1996]. Sodium humate was found to raise the activity of plasminogen activator [Klocking et al., 1984]. In the experiments on the chronic effects of humic acid in vivo, it was found that humic acid caused peroxisome proliferation in mouse liver, increased the activities of hepatic acyl-CoA oxidase, carnitine palmitoyltransferase, and carnitine acetyltransferase in rats [Lu et al., 1994; Lee et al., 1999].

About 50 years ago, professor Khristeva first discovered the high efficiency of humates as fertilizers and anabolic substances, which was supported by the following investigations. Sodium humate introduced into chickens increased body mass by 5–7% on average and poultry safety by 3–5% [Khristeva, 1951; Stepchenko et al., 1991; Zhorina and Stepchenko, 1991].

Fulvic acids are powerful antioxidants and possess superoxide and hydroxyl radical scavenging properties [Wang et al., 1996]. They are excellent natural chelators and cation exchangers [Schnitzer and Khan, 1972]. Fulvic acids may also have a physiological role, acting as carrier molecules or chelating agents for the more bioactive smaller compounds [Ghosal et al., 1991a]. Fulvic acids can be accumulated in tissues as semiquinone radicals [Peng et al., 1992, 1999] and behave as electron donors or acceptors, depending on the redox state of the system [Senesi et al., 1977; Sposito et al., 1982; Pardoe et al., 1990]. Besides, fulvic and humic acids modified the toxic behavior of various organic xenobiotics, impacting their transport into the cell interior. A single administration of sodium humate 5–10 min after irradiation with the dose of 193.5 mCi/kg leads to 43.3% survival of animals after 60 days; with the dose of 232.2 mCi/kg, there is a trend toward an increase in the lifespan of exposed rats [Pukhova et al., 1987]. Either an increase or reduction in toxicity was observed in the presence of humic substances [Herzig et al., 1994; Perminova, 1999]. Humic acid at concentrations ranging from 10 to 100 μg/ml caused lipid peroxidation in a dose-dependent manner. Such changes were accompanied by a depletion of glutathione and a reduction in activities of the antioxidant enzymes including catalase, superoxide dismutase, and glucose-6-phosphate dehydrogenase [Cheng et al., 1999]. Thus, the final biological effect of humus substances can be determined by the quantity of fulvic and humic acids, and redox state of the test biological system.

Fulvic acids have lower molecular weights (0.5–2 kDa) and a smaller number of total and aromatic carbons than humic acids (2–5 kDa), which in turn have longer-chain fatty acid fragments and therefore possess higher hydrophobicity than fulvic acids. Humic and fulvic acids contain carboxyl substituents in aromatic rings. Their aromatic nuclei have a low degree of condensation and are alternated with parts that are nonaromatic. The presence of conjugated π-electrons in aromatic rings and various functional groups as substituents in combination with the centers of paramagnetic character allow the substances to form complexes, to participate in ionic exchange and oxidation-reduction processes, to react in numerous tautomeric forms—the properties of importance for biological action of these acids. At present, to predict the biological effects of humic and fulvic acids, the corresponding quantitative structure–activity relationships methodologies are applied [Steinberg et al., 2000].

The major functional groups of humic acids are carboxylic, phenolic, and alcoholic hydroxyls, and ketone and quinone groups. That is why fulvic and humic acids are natural metal-complexing compounds [Rouleau et al., 1994]. Thus, crude extracts from mumie, sapropel, and peat are a rich source of microelements. Biological and physicochemical properties of ions are changed dramatically as a result of complexation with humin substances [Senesi and Loffredo, 1999]. The adsorbing capacity of humic and fulvic acids for poisons and mutagen molecules may be a reason for the antitoxic and desmutagenic effects of these acids [Sato et al., 1987; Badaev et al., 1989; Ferrara et al., 2000].

Minor Components of Mumie

Besides humus substances (humin, humic acids, fulvic acids, hymatomelanic acid), mumie contains the following components: albuminoids, amino acids (0.23–0.25%), oxygenated biphenyls, coumarin derivatives (benzocoumarin, dibenzo-α-pyrones), fluorene, mycotoxins (trichothecenes, naptho-l-pyrones, and alternariol), organic acids (benzoic acid and its derivatives, hippuric acid, naphthenic acids), phenolic lipids, polymeric quinines, sterols, tannins, terpenes, and triterpenes [Ghosal et al., 1976; Acharya et al., 1988; Ghosal et al., 1991b]. Mumie is organomineral matter and, according to the results of microelement analysis, it contains (in μg%): Cu (0.02), Zn (0.01), Li (20.0), Al (0.025), Cr (0.001), Pb (0.02), Ag (0.001), Co (0.01), Hg (0.002), P (0.008), Cd (0.05), Br (0.03), V (0.0016), Fe (0.16); Ca (31–39 mM/l), Mg (7.5–10 mM/l), K (100.0–106.6 mEq/l), As, Na, Cl, I, Mn, Mo, S, Si [Mumie liophylisati, 1992; Peerzada et al., 1999]. The structures of some mumie constituents are shown in Fig. 2.

Dibenzo-α-pyrones are capable of permeating through the blood–brain barrier and act as a powerful antioxidant, protecting the brain and nerve tissue from free-radical damage. They also inhibit the enzyme acetylcholinesterase, which breaks down acetylcholine [Schliebs et al., 1997]. This leads an increase of the acetylcholine level. Low levels of acetylcholine are associated with Alzheimer's disease, and poor memory and concentration. The dihydroxybenzo-α-pyrones in mumie cause recycling of ascorbic acid. Mild hydrolysis of humic acids from mumie affords two new dibenzo-α-pyrones: 3-*O*-palmitoyl-8-hydroxydibenzo-α-pyrone and 3-*O*-β-D-glucosyl-8-hydroxydibenzo-α-pyrone, and additionally two new tirucallane-type triterpenic acids: 24(Z)-3 β-hydroxy-tirucalla-8,24-dien-26-oic acid and 24(Z)-3 β-hydroxy-tirucalla-7,24-dien-26-oic acid [Ghosal, 1989].

Mumie contains ellagic and tannic acids, which are natural polyphenolic antioxidants. Tannic acids are stronger inhibitors of superoxide anion radical production by cells than ellagic acid in in vivo and in vitro

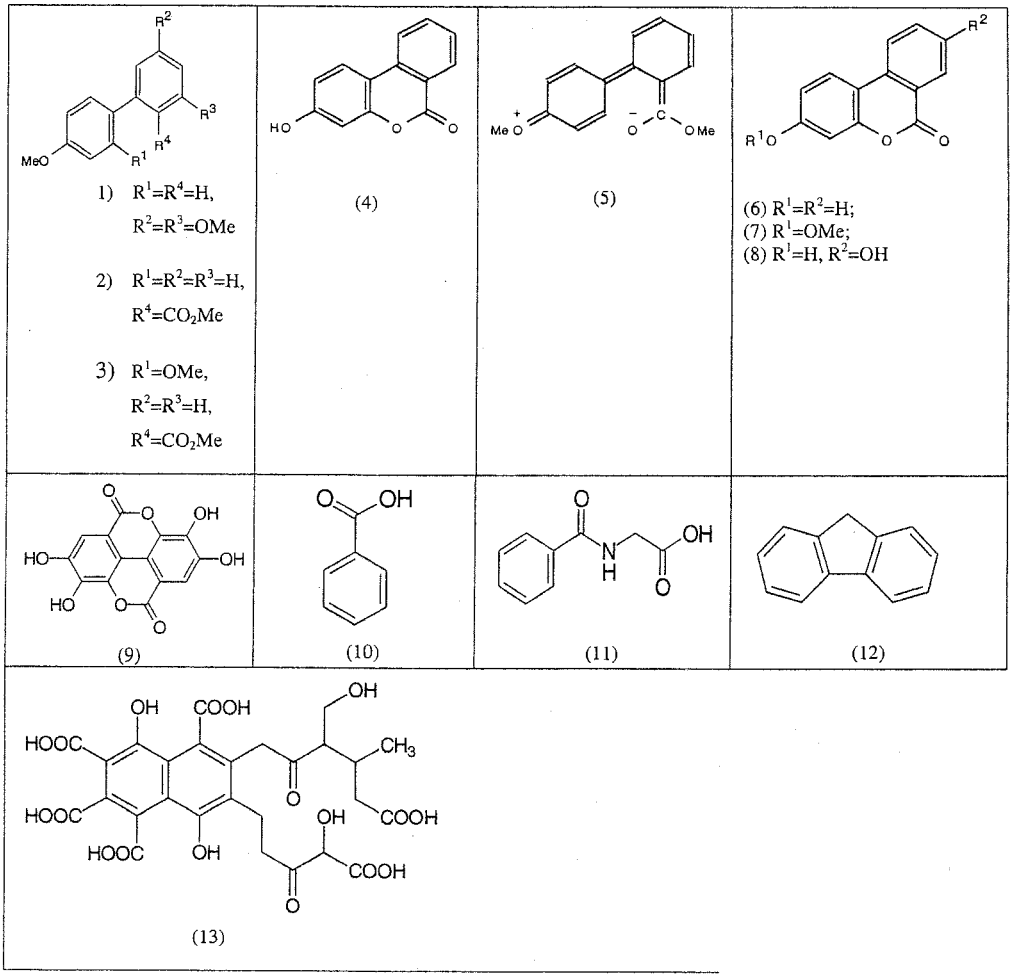


Fig. 2. Some of the compounds that are present in mumie samples: dibenzo-α-pyrones (1–8); ellagic acid (9), benzoic acid (10), hippuric acid (11), fluorene (12), and one of fulvic acids with low molecular weight (model structure by Buffe) (13).

conditions [Kaul and Khanduja, 1998]. Ellagic acid acts as an inhibitor of phospholipase A_2 and an activator of the Hageman factor. This acid is a power anticarcinogenic [Khanduja et al., 1999] and radioprotective [Thresiamma et al., 1998] compound. It induces a downregulation of insulin-like growth factor II, activates p21(waf1/Cip1), mediates a cumulative effect on G_1/S transition phase of the cell cycle, and causes apoptotic cell death of colon cancer cells (SW 480) [Narayanan and Re, 2001].

Some mumie samples contain varying levels of mycotoxins, such as trichothecenes, naptho-l-pyrones, and alternariol [DiCosmo and Straus, 1985]. These mycotoxins may contribute to biological activities of mumie. For example, the trichothecenes can both suppress and stimulate immune function [Bondy and Pestka, 2000].

Medical preparations from peat, sapropel, and mumie may contain halogenated aromatic nuclei as the products of natural degradation of fulvic and humic acids under the action of haloperoxidase enzymes. Some of these organohalogen metabolites demonstrate physiological importance as antibiotics and substances involved in lignin degradation [Dahlman et al., 1993]. The biological effect of halogenated aromatic compounds is mediated by interaction with the Ah receptor [DeVito and Birnbaum, 1995].

CONCLUSION

At present, on the basis of plant natural humification products, pharmacological drugs have been developed that have diverse application in medical practice. These drugs are complex medicines. They can be classified into two groups: (1) extracts and (2) the composites additionally containing extracts from some medicinal plants. The first type of drugs include extracts from peat and sapropel (Torfot, TPP, Peloidodistillate, Humisol, Peloidin, FiBS, Eplir), as well as

extracts from mumie (Mumie, Mumie-Vitas, Shilajit). The second type of preparations include the herbal formulas with mumie (Shilagen, Abana, Cystone, Diabecon 400, EveCare, Geriforte, Lukol, Pilex, Rumalava, Tentex forte, Nefrotec, Adrenotone, Siotone, La-Tone Gold, Andro-Surge, Solanova Libidoplex). Formulations of composite drugs, which include extracts from sapropel or peat, remains so far an undeveloped field in the pharmacology of products from humus material. Extracts from different kinds of humus material have already been applied in folk medicine for several millenniums; nevertheless, only in recent decades has a significant increase in the number of scientific publications been observed, dedicated to the study of extracts from humus material and their mechanisms of action in an organism. One of the problems in the development of the search for new medicines is connected with the difficulty of standardization of multicomponent extracts from humus origin. The modern methods of spectroscopy and identification of organic compounds in the multicomponent organic systems make this problem solvable. It should also be noted that up to now, insufficient attention was paid to the task of isolating individual biological constituents from humus material with the purpose of studying their biological activity. Today, more than 120 distinct chemical substances have been isolated from plants. These substances are considered very important drugs currently in use in one or more countries of the world [Duke, 1990]. The isolation of fractions from unique humificated products of plants for the purpose of detailed study of the pharmacological properties for each group of its constituents seems to be a very promising trend in the development of new drugs. It is reasonable to consider that mumie, as well as peat and sapropel, are very interesting humus materials that also contain unknown substances with high biological activity.

REFERENCES

Acharya SB, Frotan MH, Goel RK, Tripathi SK, Das PK. 1988. Pharmacological actions of Shilajit. Indian J Exp Biol 26:775–777.

Agzamov RA, Arifkhanova SI, Vakhidova GA. 1988. Mumie as a pathogenetic agent in the treatment of tuberculosis. Probl Tuberk 7:49–52.

Akhmedov NK, Aminov MK. 1979. Influence of Mumie, hyaluronidase and vitamin B_1 to the regeneration processes of damaged peripheral nerve. Med J Uzbek (9):33–35.

Alam MI, Gomes A. 1998. Viper venom-induced inflammation and inhibition of free radical formation by pure compound (2-hydroxy-4-methoxy benzoic acid) isolated and purified from anantamul (*Hemidesmus indicus R.* BR) root extract. Toxicon 36:207–215.

Almatov KT, Akhmerov AI. 1977. Mumie effect on oxidative phosphorylation and enzymes of mitochondrial respiratory chain of rat liver, small and large intestine. Transact Tashkent Med Inst 7:11–14.

Anisimov VE, Shakirzyanova RM. 1982. Application of Mumie in therapeutic practice. Kazan Med J 63:65–68.

Antani JA, Kulkarni RD, Antani NJ. 1990. Effect of Abana on ventricular function in ischemic heart disease. Jpn Heart J 31:829–835.

Anturlikar SD, Gopumadhavan S, Chauhan BL, Mitra SK. 1995. Effect of D-400, a herbal formulation, on blood sugar of normal and alloxan-induced diabetic rats. Indian J Physiol Pharmacol 39:95–100.

Badaev SA, Gichner T, Pospisil F, Veleminsky J. 1989. Humic acids inhibit the formation but not the mutagenicity of N-methyl-N-nitrosourea. Mutat Res 210:9–13.

Bhagwat SS. 1962. Lukol in the treatment of leucorrhoea. Curr Med Pract 3:145–147.

Bhatta MN. 1982. Clinical experience of Speman forte, Tentex forte and Himcolin cream. Probe 21:101–104.

Bhattacharya SK. 1995. Activity of Shilajit on alloxan-induced hyperglycemia in rats. Fitoterapia 66:328–330.

Bhattacharya SK, Ghosal S. 1992. Effects of Shilajit on rat brain monoamines. Phytother Res 6:1163–1164.

Bhattacharya SK, Kumar A. 1997. Effect of Trasina, an ayurvedic herbal formulation, on experimental models of Alzheimer's disease and central cholinergic markers in rats. J Altern Complement Med 3:327–336.

Bhattacharya SK, Dasgupta G, Bhaduri J, Mukhopadhyay M, Goel RK, Dey R. 1989. Mast cell protecting effects of Shilajit and its constituents. Phytother Res 3:249–252.

Bhattacharya SK, Sen AP, Ghosal S. 1995. Effects of Shilajit on biogenic free radicals. Phytother Res 9:56–59.

Bhattacharya SK, Satyan KS, Chakrabarti A. 1997. Effect of Trasina, an Ayurvedic herbal formulation, on pancreatic islet superoxide dismutase activity in hyperglycaemic rats. Indian J Exp Biol 35:297–299.

Bhattacharya SK, Bhattacharya A, Chakrabarti A. 2000. Adaptogenic activity of Siotone, a polyherbal formulation of Ayurvedic rasayanas. Indian J Exp Biol 38:119–128.

Bhaumik S, Chattopadhyay S, Ghosal S. 1993. Effect of Shilajit on mouse peritoneal macrophages. Phytother Res 7:425–427.

Blach-Olszewska Z, Zaczynska E, Broniarek E, Inglot AD. 1993. Production of cytokines by mouse peritoneal cells treated with Tolpa Peat Preparation (TPP): dependence on age and strain of the mice. Arch Immun Ther Exp 41:81–85.

Bondy GS, Pestka JJ. 2000. Immunomodulation by fungal toxins. J Toxicol Environ Health B Crit Rev 3:109–143.

Bose S, Gupta YK. 1999. Effect of CNS active herbal drugs on swim test in mice. Indian J Pharm 31:75.

Burkova VN, Pisareva SI, Matis EY. 1995. Antimetastatic substance. Russian Patent (Decision on 27.11.95 for Application 5065317/14).

Bushmich DG, Golatska IS. 1972. Curative effect of subconjunctival injections of various doses of Torfot in corneal opacities. Oftalmol Zh 27:103–107.

Chatterjee BN. 1982. Role of Cystone in various urinary disorders. Probe 1:27–30.

Cheng ML, Ho HY, Chiu DT, Lu FJ. 1999. Humic acid-mediated oxidative damages to human erythrocytes: a possible mechanism

leading to anemia in Blackfoot disease. Free Radic Biol Med 27:470–477.

Dabak SM, Burute PB, Dani SP. 1984. Lukol in the treatment of leucorrhoea and abnormal uterine bleeding following MTP, JUCD and tubectomy. Med Surg 5:17–20.

Dadkar VN, Tahiliani RR, Jaguste VS, Damle VB, Dhar HL. 1990. Double blind comparative trial of Abana and methyldopa for monotherapy of hypertension in Indian patients. Jpn Heart J 31:193–199.

Dahlman O, Morck R, Ljungquist P. 1993. Chlorinated structural elements in high molecular weight organic matter from unpolluted waters and bleached-craft mill effluents. Environm Sci Technol 27:1616–1620.

Degtiarenko TV. 1990. The effect of tissue preparations on the development of anaphylaxis. Oftalmol Zh (7):428–432.

Degtiarenko TV, Ivanova AS, Skvortsov VI, Masternak TV, Larin AS. 1989. Primary screening of the immunopharmacologic activity of Filatov tissue therapy preparations. Oftalmol Zh 1:34–39.

Dekker J, Medlen CE. 1999. Fulvic acid and its use in the treatment of various conditions. PTC Patent PCT/1B 99/01567.

DeVito MJ, Birnbaum LS. 1995. Dioxins: model chemicals for assessing receptor-mediated toxicity. Toxicology 102: 115–123.

DiCosmo F, Straus NA. 1985. Alternariol, a dibenzopyrone mycotoxin of *Alternaria spp.*, is a new photosensitizing and DNA cross-linking agent. Experientia 41:1188–1190.

Dubey GP, Agarwal A, Agarwal S, Pandey R. 1980. Clinical application of Geriforte in the management of certain psychosomatic disorders. Probe (1):18–21.

Dubey GP, Agarwal A, Agarwal S, Srivastava VK. 1984a. Antistress properties of an indigenous compound and its significance in the management of psychosomatic disorders. Asian Med J 11: 723–727.

Dubey GP, Ratogi V, Malviya PK, Agrawal A. 1984b. Management of chronic backache by an indigenous drug "Rumalaya". Indian Med J 78:82–86.

Dubey GP, Dixit SP, Singh A. 1994. Alloxan-induced diabetes in rabbits and effect of an herbal formulation D-400. Indian J Pharm 26:225–226.

Duke JA. 1990. Promising phytomedicinals. In: Janick J, Simon JE, editors. Advances in new crops. Portland: Timber Press, pp 491–498.

Dunaev MV, Korshikov AV, Mazunova TI, Maslennikov BI, Mayakova EF, Ostrerova LL. 1996. Application of biostimulator from peat in stomatology. Med Consult (3):22–25.

Esteves VI, Duarte AC. 2000. Differences between humic substances from riverine, estuarine, and marine environments observed by fluorescence spectroscopy. Acta Hydrochim Hydrobiol 28:359–363.

Ferrara G, Loffredo E, Simeone R, Senesi N. 2000. Evaluation of antimutagenic and desmutagenic effects of humic and fulvic acids on roof tips of *Vicia faba*. Environ Toxicol 15:513–517.

Filatov VP. 1961. Selected works in 4 volumes. Kiev: Ukraine Academy of Sciences Publishers.

Foigelman AY. 1972. The experience of Mumie application in the complex treatment of burn patients. Samarkand, PhD Thesis.

Garg SK, Singh RC. 1985. Role of Cystone in burning micturition. Probe 2:119–121.

Ghosal S. 1989. Shilajit. Chemistry of two bioactive benzopyrane metabolites. J Chem Res (Suppl)I:350–351.

Ghosal S. 1990. Chemistry of Shilajit, an immunomodulatory Ayurvedic rasayan. Pure Appl Chem 62:1285–1288.

Ghosal S. 1995. Interaction of Shilajit with free radicals. Ind J Chem 34B:596–602.

Ghosal S, Bhattacharya SK. 1996. Antioxidant defense by native and processed Shilajit: a comparative study. Indian J Chem 35B: 127–132.

Ghosal S, Reddy JP, Lal VK. 1976. Shilajit I: chemical constituents. J Pharm Sci 65:772–773.

Ghosal S, Singh SK, Kumar Y. 1988. Antiulcerogenic activity of fulvic acids and 4′-methoxy-6-carbomethoxybiphenyl isolated from Shilajit. Phytother Res 2:187–191.

Ghosal S, Lal J, Singh SK. 1991a. The core structure of Shilajit humus. Soil Biology Biochem 23:673–680.

Ghosal S, Lal J, Singh SK, Goel RK, Jaiswal AK, Bhattacharya SK. 1991b. The need for formulation of Shilajit by its isolated active constituents. Phytother Res 5:211– 216.

Ghosal S, Lal J, Jaiswal AK, Bhattacharya SK. 1993. Effects of Shilajit and its active constituents on learning and memory in rats. Phytother Res 7:29–34.

Goel RK, Banerjee RS, Acharya SB. 1990. Antiulcerogenic and antiinflammatory studies with Shilajit. J Ethnopharmacol 29: 95–103.

Gordievskii ID, Barulina NV. 1974. Application of Mumie asil after tonsillectomy. Zdrav Kazakhstan (6):62–63.

Gorgiladze TU, Marmur RK, Nepomiashchaia VM, Kolesnichenko AM, Kuksa VD. 1984. Therapeutic action of Torfot in corneal opacities. Oftalmol Zh (1):6–8.

Grechko VE, Stepanchenko AV, Sineva NA, Puzin MN, Bolonkina GD. 1985. Combined treatment of trigeminal neuralgia by lidocaine and Mumie electrophoresis. Stomatologiia 64:27–29.

Gupta K, Bhanot K. 1973. A clinical trial of Lukol in the treatment of non-specific leucorrhoea. Probe 13:25–29.

Gupta SS, Seth CB, Mathur VS. 1966. Effect of Gurmar and Shilajit on body weight of young rats. Indian J Physiol Pharmacol 9:87–92.

Habilov LL. 1971. The complex treatment of paradontosis patients with application of Mumie asil. Tashkent. PhD Thesis.

Herzig I, Hampl J, Docekalova H, Pisarikova B, Vlcek J. 1994. Effect of sodium huminate on cadmium deposition in the organs of chickens. Vet Med (Prague) 39:175–185.

Hils J, May A, Sperber M, Klocking R, Helbig B, Sprossig M. 1986. Inhibition of several strains of influenza virus type A and B by phenolic polymers. Biomed Biochim Acta 45:1173–1179.

Inglot AD, Zielinska-Jenczylik J, Piasecki E. 1993. Tolpa peat preparation (TPP) induces interferon and tumor necrosis factor production in human peripheral blood leukocytes. Arch Imm Ther Exp 41:73–80.

Ioshchenko SE, Zyrianova VV. 1991. Metabolic shifts in the modelling of pancreatitis and its experimental therapy with applications of sapropel. Vopr Kurortol Fizioter Lech Fiz Kult (4):45–48.

Ismailova VN. 1965. On the question of Mumie dosage and special features of bone fractures healing with its application. Med J Uzbek (9):69–70.

Jadhav SS, Bahga HS. 1971. Effect of Tentex forte and Speman individually and in combination on gonadal structure in rats. J Ind Med Prof 17:8055–8062.

Jagetia GC, Aruna R. 1997. The herbal preparation Abana protects against radiation-induced micronuclei in mouse bone marrow. Mutat Res 393:157–163.

Jaiswal AK, Bhattacharya SK. 1992. Effect of Shilajit on memory, anxiety and brain monoamines in rats. Ind J Pharm 24:12–17.

Juszkiewicz T. 1993. Studies of the embryotoxic and teratogenic effects of Tolpa peat preparation. Acta Pol Pharm 50:383–388.

Kaul A, Khanduja KL. 1998. Polyphenols inhibit promotional phase of tumorigenesis: relevance of superoxide radicals. Nutr Cancer 32:81–85.

Kaur G, Kulkarni SK. 2000. Comparative study of antidepressants and herbal psychotropic drugs in a mouse model of chronic fatigue. J Chronic Fatigue Syndrome 6:23–35.

Kelginbaev NS, Sorokina VA, Stefanidu AG, Ismailova VN. 1973. Treatment of long tubular bone fractures with Mumie asil preparations in experiments and clinical conditions. Exp Surg Anesthes 18:31–35.

Khakimov ZN. 1974. Mumie as a kind of natural resources and its signs. Tashkent. PhD Thesis.

Khan C. 1983. Efficacy of Cystone in urolithiasis. Capsule 2:37–41.

Khanduja KL, Gandhi RK, Pathania V, Syal N. 1999. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. Food Chem Toxicol 37:313–318.

Khristeva LA. 1951. The role of humic acid in plant nutrition and humus fertilizers. Trudi Pochv Inst VV Dokuchaeva. 38:108–184.

Klocking HP, Klocking R, Helbig B. 1984. Effect of phenol ring polymers on the release of plasminogen activators. Farmakol Toksikol 47:93–95.

Komarova LA, Egorova GI, Kirianova VV. 1991. The combined use of sinusoidal modulated currents, mud preparations and phytotherapy in peptic ulcer. Vopr Kurortol Fizioter Lech Fiz Kult (4):27–30.

Kononova MM. 1966. Soil organic matter. Oxford: Pergamon.

Korago AA. 1992. Introduction in biominerology. St. Petersburg: Nauka.

Koziorowska J, Chlopkiewicz B, Anuszewska E. 1993. Evaluation of mutagenic and genotoxic properties of TPP. Acta Pol Pharm 50:379–382.

Kozlovskaya VI. 1968. Treatment of peripheral nervous system diseases with Caucasian Mumie. Vrach Delo (6):88–92.

Kozlovskaya VI. 1971. Application of Caucasian Mumie in the treatment for experimental ulcers of gastric and duodenum. Vrach Delo (4):23–25.

Kozlovskaya VI. 1972. The stimulation effect of Caucasian Mumie on composition of peripheral blood. Vrach Delo (6):123–126.

Krylov OA, Nizkodubova SV, Nechai GM, Fedotov DS. 1990. The correction of disorders in the exocrine function of the liver by using sapropel lipids in tetrachloromethane poisoning (experimental research). Vopr Kurortol Fizioter Lech Fiz Kult (3):41–44.

Kuberger MB, Kalmanovskaia LA. 1966. On the problem of the treatment of diseases of the gallbladder and biliary tract in children with Peloidin and electrophoresis. Sov Med 29:91–93.

Kulkarni SK, Joseph P. 1998a. Anticonvulsant profile of Siotone granules, a herbal preparation. Indian J Exp Biol 36:658–662.

Kulkarni SK, Joseph P. 1998b. Psychopharmacological profile of Siotone granules, a herbal psychotropic preparation. Indian Drugs 35:536–554.

Kuzmenko DI, Sidorenko GN, Levitskii EF, Laptev BI, Dzhuraeva EI. 1998. A comparative evaluation of the effect of different types of sapropel on dynamic liver function in intact rats and in the modelling of toxic hepatitis. Vopr Kurortol Fizioter Lech Fiz Kult (2):37–38.

Laub RJ. 1999. Process for preparing synthetic soil-extract materials and medicaments based thereon. USA Patent 5,945,446.

Lee Y, Huang H, Yang M-L, Huang L-R, Chen C-H, Lu F-J. 1999. Peroxisome proliferation, adipocyte determination and differentiation of C3H10T1/2 fibroblast cells induced by humic acid: induction of PPAR in diverse cells. J Cell Physiol 179:218–225.

Lichtfouse E. 1999. A novel model of humin. Analusis 27:385–386.

Lotosh TD. 1991. Experimental bases and prospects for the use of humic acid preparations from peat in medicine and agricultural production. Nauchnye Dokl Vyss Shkoly Biol Nauki (10):99–103.

Lu FJ, Shih SR, Liu TM, Shown SH. 1990. The effect of fluorescent humic substances existing in the well water of Blackfoot disease endemic areas in Taiwan on prothrombin time and activated partial thromboplastin time *in vitro*. Thromb Res 57:747–753.

Lu FJ, Huang TS, Chen YS. 1994. Effects of humic acid-metal complexes on hepatic carnitine palmitoyltransferase, carnitine acetyltransferase and catalase activities. Environ Toxicol Chem 13:435–441.

Magidzon MD, Khmelevskii MG. 1982. Mental disorders as complications during the Mumie application. Vrach Delo (3):96–97.

Mamadjanov MM. 1975. Treatment of peripheral nervous system diseases by Mumie. Zdrav Tajik (4):29–31.

Mardicar BR. 1975. A preliminary report on clinical trials with Tentex forte and Speman. Probe 15:8–14.

Maslinski Cz, Wyczolkowska J, Czuwaj M, Michon T. 1993. Investigations of allergenic properties of Tolpa peat preparation. Acta Pol Pharm 50:469–474.

Mathur SP, Owen G, Dinel H, Schnitzer M. 1993. Determination of compost maturity. Biol Agricult Horticult 10:65–85.

Misger MS. 1982. Controlled trial in 100 cases with nephrourethrolithiasis with Cystone, an indigenous drug and other advanced methods. Curr Med Pract 5:26–29.

Misger MS, Mir MA, Wani NA. 1977. Pilex therapy in the treatment of haemorroids. Indian Med Gaz 16:353–356.

Mitra SK, Gopumadhavan S, Muralidhar TS, Anturlikar SD, Sujatha MB. 1995a. Effect of D-400, a herbomineral preparation on lipid profile, glycated haemoglobin and glucose tolerance in streptozotocin induced diabetes in rats. Indian J Exp Biol 33:798–800.

Mitra SK, Hemavathi TS, Prasana BM, Prasana Kumar KM. 1995b. Effect of D-400 on blood glucose profile in non-insulin dependent diabetes mellitus patients. Indian Pract 58:547–549.

Mitra SK, Gopumadhavan S, Muralidhar TS. 1996a. Effect of D-400, an Ayurvedic formulation on experimentally induced diabetes mellitus. Phytother Res 10:433–435.

Mitra SK, Gopumadhavan S, Muralidhar TS, Anturlikar SD, Sujatha MB. 1996b. Effect of a herbomineral preparation D-400 in streptozotocin-induced diabetic rats. J Ethnopharmacol 54:41–46.

Mitra SK, Gopumadhavan S, Muralidhar TS, Seshadri SJ. 1996c. Effect of D-400 a herbomineral formulation on liver glycogen

content and microscopic structure of pancreas and liver in streptozotocin-induced diabetes in rats. Indian J Exp Boil 34: 964–967.

Mitra SK, Sunitha A, Kumar VV. 1998. Evecare (U-3107) as a uterine tonic: pilot study. Indian Pract 51:269–272.

Mumie liophylisati. 1992. Pharmacopoeia Article. Republic of Kazakhstan.

Muratova KN, Shakirov DS. 1968. Clinical treatment of suppurative wounds with Mumie. Surgery (Moscow) 44:122–124.

Narayanan BA, Re GG. 2001. IGF-II down regulation associated cell cycle arrest in colon cancer cells exposed to phenolic antioxidant ellagic acid. Anticancer Res 21:359–364.

Nigam P, Mukhija RD, Gupta AK, Dayal SG, Goyal BM. 1984. Gonadal involvement in leprosy: study of gynaecomastia, testicular and epididymal involvement and therapeutic efficacy of indigenous drugs. Hansenol Int 9:10–20.

Nizkodubova SV, Fedotov DS, Pisanko NI. 1991. Correction of energy metabolism disorders in rat liver in tetrachloromethane poisoning using sapropel lipids. Vopr Med Khim 37:53–56.

Obminska-Domoradzka B, Switala M, Debowy J, Garbulinski T. 1993a. The effect of Tolpa peat preparation on the phagocytic and metabolic activity of neutrophils in normothermic rabbits and those with pyrogen-induced fever. Acta Pol Pharm 50:389–392.

Obminska-Domoradzka B, Switala M, Debowy J, Garbulinski T. 1993b. The dose-dependent effect of Tolpa peat preparation on the humoral response of mice immunized with sheep erythrocytes. Acta Pol Pharm 50:497–500.

Orlov DS. 1995. Humic substances of soils and general theory of humification. Rotterdam: AA Balkema Publishers.

Orlov DS. 1997. Humic substances in the biosphere. Soros Obraz Zh (2):56–63.

Pardoe HL, Townshend A, Clerc JT, Vender Linden, editors. 1990. Humic and fulvic compounds. Analyt Chim Acta 232:1–235.

Pasnani JS, Hemavathi KG, Gulati OD, Rajani AP. 1988. Effects of Abana, an Ayurvedic preparation, on rabbit atrium and intestine. J Ethnopharmacol 24:287–302.

Pathania V, Syal N, Hundal MK, Khanduja KL. 1998. Geriforte stimulates antioxidant defense system. Indian J Exp Biol 36: 414–417.

Peerzada N, Saramani S, Tariq SA. 1999. Silajit. Part II. Determination of metals in Silajit and biological significance of some metals. Sci Intern 11:79–82.

Peng A, Yang C, Rui H, Li H. 1992. Study on the pathogenic factors of Kashin-Beck disease. J Toxicol Environ Health 35:79–90.

Peng A, Wang WH, Wang CX, Wang ZJ, Rui HF, Wang WZ, Yang ZW. 1999. The role of humic substances in drinking water in Kashin-Beck disease in China. Environ Health Perspect 107: 293–296.

Perederko IG, Solenii VI, Tolubayev NS, Marchenko AA, Kirpa YuI. 1998. The organization of medical rehabilitation of patients, operated apropos of vertebral-cerebrospinal injury. Second Congress of Neurosurgeons of Ukraine Odessa. 3:Abstract 6.

Perminova IV. 1999. Size exclusion chromatography of humic substances: complexities of data interpretation attributable to non-size exclusion effects. Soil Sci 164:834–840.

Piotrowska D, Dlugosz A, Witkiewicz K, Pajak J. 2000. The research on antioxidative properties of Tolpa Peat Preparation and its fractions. Acta Pol Pharm 57:127–129.

Pisareva SI, Pynchenkov VI, Matis EY, Yudina NV, Moskvin VS. 1997. Antioxidative properties of organic matter occurring in medicinal muds. Proceedings of First Korea–Russia International Symposium on Science and Technology. pp 356–359.

Psakhis BI, Aizenberg SG. 1976. The use of Mumie for treatment of postoperative trepanized cavities of the middle ear. J Ear Nose Throat Dis (5):57–61.

Pukhova GG, Druzhina NA, Stepchenko LM, Chebotarev EE. 1987. Effect of sodium humate on animals irradiated with lethal doses. Radiobiologiia 27:650–653.

Rai SC. 1960. Clinical trials with Cystone in urolithiasis. Curr Med Pract 9:484–487.

Rajic A, Akihisa T, Ukiya M, Yasukawa K, Sandeman RM, Chandler DS, Polya GM. 2001. Inhibition of trypsin and chymotrypsin by antiinflammatory triterpenoids from Compositae flowers. Planta Med 67:599–604.

Rangnekar GV, Arora OP. 1975. Treatment of piles with indigenous drugs Pilex tablets and ointment along with styplon. Probe 14:214–219.

Rao M, Rao MN. 1998. Protective effects of Cystone, a polyherbal ayurvedic preparation, on cisplatin-induced renal toxicity in rats. J Ethnopharmacol 62:1–6.

Rao M, Praveen Rao PN, Kamath R, Rao MN. 1999. Reduction of cisplatin-induced nephrotoxicity by Cystone, a polyherbal ayurvedic preparation, in C57BL/6J mice bearing B16F1 melanoma without reducing its antitumor activity. J Ethnopharmacol 68: 77–81.

Rao PT, Gupta ML. 1977. Rumalaya in cervical and lumbar spondylosis. Probe (3):184.

Rasulov KR. 1964. Mumie as the medicinal means of Tibetan medicine. Med J Uzbek (8):80–84.

Riabtseva IT, Kiryanova KA, Andreeva TB. 1975. Treatment of inflammatory processes of the genital system with Peloidin. Akush Ginekol (5):65–66.

Riede UN, Zeck-Kapp G, Freudenberg N, Keller HU, Seubert B. 1991. Humate-induced activation of human granulocytes. Virchows Arch B Cell Pathol Incl Mol Pathol 60:27–34.

Rogozkin VD, Tukhtaev T. 1968. Effect of Mumie preparation on lymphopoiesis in acute radiation sickness. Bull Exp Biol Med 65:110–111.

Rohling EJ. 1994. Review and new aspects concerning the formation of eastern Mediterranean sapropels. Mar Geol 122:1–28.

Rouleau C, Tjalve H, Gottofrey J, Pelletier E. 1994. Effects of some chelating agents on the uptake and distribution of 54Mn(II) in the brown trout (Salmo trutta). Pharmacol Toxicol 74:271–279.

Rowland D. 1999. Vitamin/mineral composition. USA Patent 5,405,613.

Saratikov AS, Vengerovskii AI, Pauls OV, Sedykh IM. 1990. The effect of Eplir on an experimental toxic lesion of the liver. Farmakol Toksikol 53:42–45.

Sato T, Ose Y, Nagase H, Hayase K. 1987. Mechanism of the desmutagenic effect of humic acid. Mutat Res 176:199–204.

Schiller F, Klocking R, Wutzler P, Farber I. 1979. Results of an oriented clinical trial of ammonium humate for the local treatment of herpesvirus hominis (HVH) infections. Dermatol Monatsschr 165:505–509.

Schlickewei W, Riede UN, Yu J, Ziechmann W, Kuner EH, Seubert B. 1993. Influence of humate on calcium hydroxyapatite implants. Arch Orthop Trauma Surg 112:275–279.

Schliebs R, Liebman A, Bhattacharya SK, Kumar A, Ghosah S, Bigl V. 1997. System administration of defined extracts from *Withania somnifera* (Indian ginseng) and Shilajit differentially affects cholinergic but not glutamatergic and gabanergic markers in rat brain. Neurochem Int 30:181–190.

Schnitzer M, Khan SU. 1972. Humic substances in the environment. New York: Marcel Dekker.

Senesi N, Chen Y, Schnitzer M. 1977. The role of humic acids in extracellular electron transport and chemical determination of pH in natural waters. Soil Biol Biochem 9:397–403.

Senesi N, Loffredo E. 1999. The chemistry of soil organic mater. In: Sparks DL, editor. Soil physical chemistry. Boca Raton: CRC Press. p 239–370.

Sengupta S. 1987. Cystone in urinary tract complaints during pregnancy. Med Surg 27:7–11.

Sevostianova NV. 1998. The antimutagenic effect of humizol in cultures of blood T-lymphocytes exposed to cyclophosphane. Vopr Kurortol Fizioter Lech Fiz Kult) (6):46–47.

Shakirov AS. 1965a. Influence of Mumie on bone regeneration and blood alkaline phosphatase after bone fracture in an experiment. Ortop Travmatol Protez 26:24–27.

Shakirov AS. 1965b. Uptake of radioactive ^{32}P on the focus of bone fracture with Mumie treatment. Med J Uzbek (10):30–33.

Shakirov DS. 1967. A comparative evaluation of antibacterial properties of Mumie and penicillin. Antibiotiki 12:248–251.

Shakirov DS. 1969. Treatment of infected wounds with Mumie asil in experiment. Exp Surg Anesthes 6:36–39.

Sharma BM, Panagariya A, Jain K. 1983. Clinical trial of Cystone in various renal disorders. Probe (2):113–114.

Shmakova IP, Gorbatiuk AL, Gordienko OS, Dukova OR. 1990. The use of the vacuum phonophoresis of Peloidin on patients with the neurological syndromes of lumbar osteochondrosis. Vopr Kurortol Fizioter Lech Fiz Kult (4):30–33.

Shpak NI, Soloveva VP, Sotnikova EP. 1990. The combined use of Torfot and vitamin B6 in ophthalmology. Oftalmol Zh (2):122–123.

Shvetskii AG, Vorobeva LM. 1978. Effect of the nonspecific biogenic stimulators Pentoxyl and Mumie on metabolic processes. Vopr Med Khim 24:102–108.

Singh B, Nath R, Mishra N, Kohli RP. 1978. An experimental evaluation of antistress effects of Geriforte (an Ayuvedic drug). Q J Crude Drug Res 3:125–128.

Singh B, Sharma SP, Goyal R. 1994. Evaluation of Geriforte, an herbal geriatric tonic, on antioxidant defense system in Wistar rats. Ann N Y Acad Sci 717:170–173.

Singh K, Thakur SR, Handa R. 1984. Clinical trial of Ramalaya tablets and Rumalaya cream in cases of low backache. Curr Med Pract 11:691–694.

Singh PP, Vanita R, Gochar BL. 1985. Urinary mucoprotein excretion in stone formers and the effect of an indigenous formulation on its excretion. Asiana Med J 4:261–264.

Skopinska-Rozewska E. 1991. Immunomodulating effects of Tolpa peat preparation. Reumatologia 24:161–171.

Skopinska-Rozewska E, Polakowski I, Zukowska M, Radomska D, Malkowska-Zwierz W, Demkow V, Skopinski P, Pazdur J, Liszewska-Pfeifer D. 1993. The effect of Tolpa peat preparation (TPP) on human mononuclear leukocyte-induced angiogenesis. Herba Pol 34:41–51.

Soliev TS. 1983. The treatment of deforming osteoarthrosis by non-specific bio-stimulator Mumie. Med J Uzbek (8): 19–21.

Sposito G, Holtaclaw KM, LeVesque CS, Johnston CT. 1982. Trace metal chemistry in arid-zone filed soils amended with sewage sludge. II. Comparative study of the fulvic and fraction. Soil Sci Soc Am J 45:265–270.

Srivastava RK, Dayal SS, Singh RC. 1991. Role of Cystone in management of urinary tract infection. Curr Med Pract 35:89–94.

Steinberg CEW, Haitzer M, Bruggemann R, Perminova IV, Yashenko NY, Petrosyan VS. 2000. Towards a quantitative structure activity relationship (QSAR) of dissolved humic substances as detoxifying agents in freshwaters. Int Rev Hydrobiol 85:253–266.

Stepchenko LM, Zhorina LV, Kravtsova LV. 1991. The effect of sodium humate on metabolism and resistance in highly productive poultry. Nauch Dokl Vyss Shkoly Biol Nauki (10):90–95.

Stevenson FJ. 1994. Humus chemistry. Genesis, composition, reactions. New York: John Wiley.

Strelis AK, Zhivotiagina NA, Zhivotiagin VN. 1989. The effect of peloid therapy on cardiovascular function in patients with pulmonary tuberculosis. Vrach Delo (9):56–58.

Strelis AK, Zhivotiagina NA. 1991. The dynamics of the data on the hemogram and blood biochemical indices in pulmonary tuberculosis patients undergoing peloid therapy. Vopr Kurortol Fizioter Lech Fiz Kult (3):22–25.

Strelis AK, Zhivotiagina NA, Romanovskaia NI. 1991. The effect of pelotherapy on the indices of T- and B-system immunity in patients with infiltrative pulmonary tuberculosis. Vrach Delo (11):98–101.

Suleimanov I. 1972. Effects of Mumie on bone regeneration in patients subjected to surgery for osteoarticular tuberculosis. Ortop Travm Protez 33:64–66.

Sundaram R, Venkataranganna MV, Gopumadhavan S, Mitra SK. 1996. Interaction of a herbomineral preparation D-400, with oral hypoglycaemic drugs. J Ethnopharmacol 55:55–61.

Tazhimametov BT, Usmanov MU, Dzhuraev KA, Sharipov NI, Zulfikarov K. 1987. Effect of Mumie on the healing of suppurative wounds. Clin Surg (1):51–52.

Thiel KD, Klocking R, Schweizer H, Sprossig M. 1977. *In vitro* studies of the antiviral activity of ammonium humate against herpes simplex virus type 1 and type 2. Zentralbl Bakteriol A 239:304–321.

Thiel KD, Helbig B, Klocking R, Wutzler P, Sprossig M, Schweizer H. 1981. Comparison of the *in vitro* activities of ammonium humate and of enzymically oxidized chlorogenic and caffeic acids against type 1 and type 2 human herpes virus. Pharmazie 36: 50–53.

Thresiamma KC, George J, Kuttan R. 1998. Protective effect of curcumin, ellagic acid and bixin on radiation induced genotoxicity. J Exp Clin Cancer Res 17:431–434.

Tiwari AK, Gode JD, Dubey GP. 1993. Influence of Abana on experimental atherogenesis in hypercholesterolemic rabbits. Jpn Heart J 34:451–458.

Tiwari P, Ramarao P, Ghosal S. 2001. Effects of Shilajit on the development of tolerance to morphine in mice. Phytother Res 15:177–179.

Tiwari VP, Tiwari KC. 1973. Joshi: an interpretation of Ayurvedica findings on Shilajit. J Res Indigen Med 8:57.

Tkachenko SS, Rutskii VV, Grachev IR. 1979. Reparative regeneration of the bone tissue under the effect of Mumie asyl. Ortop Traum Protez 40:49–52.

Tripathi YB, Shukla S, Chaurasia S. 1996. Antilipid peroxidative property of Shilajit. Phytother Res 10:269–270.

Vaishwanar I, Kowale CN, Jiddewar GG. 1976. Effect of two Ayurvedic drugs Shilajit and Eclinol on changes in liver and serum lipids produced by carbon tetrachloride. Indian J Exp Biol 14:57–58.

van Rensburg CE, van Straten A, Dekker J. 2000. An *in vitro* investigation of the antimicrobial activity of oxifulvic acid. J Antimicrob Chemother 46:853.

van Rensburg CE, Malfeld SCK, Dekker J. 2001. Topical application of oxifulvic acid suppresses the cutaneous immune response in mice. Drug Dev Res 53:29–32.

Vengerovskii AI, Golovina EL, Burkova VN, Saratikov AS. 2001. Enteric sorbents potentiate hepatoprotective effect of Eplir in experimental toxic hepatitis. Eksp Klin Farmakol 64:46–48.

Vereshchagin IA, Golosnoi EV. 1994. The treatment of salmonellosis in children. Antibiot Khimioter 39:49–51.

Volkova O. 2000. Artificial feeding of chinchilla (*Chincilla laniger Molinal*, 1828, reprint). St. Petersburg: Leningarskii Zoopark.

Waksman SA. 1938. Humus. London: Bailliere, Tindall and Cox.

Wang C, Wang Z, Peng A, Hou J, Xin W. 1996. Interaction between fulvic acids of different origins and active oxygen radicals. Sci China C Life Sci 39:267–275.

Wershaw RI. 1986. A new model for humic materials and their interactions with hydrophobic organic chemicals in soil-water or sediment-water systems. J Contam Hydrol 1:29–45.

Wiegleb K, Lange N, Kuhnert M. 1993. The use of the HET-CAM test for the determination of the irritating effects of humic acids. Dtsch Tierarztl Wochenschr 100:412–416.

Yang HL, Tu SC, Lu FJ, Chiu HC. 1994. Plasma protein C activity is enhanced by arsenic but inhibited by fluorescent humic acid associated with Blackfoot disease. Am J Hematol 46:264–269.

Yang HL, Chiu HC, Lu FJ. 1996. Effect of humic acid on the viability and coagulant properties of human umbilical vein endothelial cells. Am J Hematol 51:200–206.

Yudina NV, Burkova VN, Pisareva SI, Saratikov AS. 1996. The method for extraction of anti-ulcer remedy. Russian Patent 2055587.

Yudina NV, Pisareva SI, Pinchenkov VI, Loskutova YuV. 1998a. Parameters of estimation for biological activity of organic substances of sapropel. Chem Plant Mater 4:33–38.

Yudina NV, Pisareva SI, Saratikov AS. 1998b. Antiulcerogenic activity of phenol compounds of peat. Chem Plant Mater 4:29–32.

Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B. 1996. Studies on the immunomodulatory effects of Ashwagandha. J Ethnopharmacol 50:69–76.

Zhorina LV, Stepchenko LM. 1991. The content of free amino acids in the tissues of broiler chicks administered sodium humate in the ration. Nauchnye Dokl Vyss Shkoly Biol Nauki (10):147–150.

Biopolymers 1 / 69CJVR 2001

13 Medical Aspects and Applications of Humic Substances

Prof. Dr. Renate Klöcking[1]
Dr. rer. nat. Björn Helbig[2]
[1] Institute for Antiviral Chemotherapy, Friedrich Schiller University Jena, Winzerlaer Str. 10, 07745 Jena, Germany, Tel: +49 (0) 361–7411482, Fax: +49 (0) 361–7411166, E-mail: "rkloeck@zmkh.ef.uni-jena.de" and Nordhäuser Str. 78, 99089 Erfurt, Germany
[2] Institute for Antiviral Chemotherapy, Friedrich Schiller University Jena, Winzerlaer Str. 10, 07745 Jena, Germany, Tel: +49 (0) 3641–657307, Fax: +49 (0) 3641–657301

AA	Arachidonic acid
BFD	Blackfoot disease
BOP	Catechol oxidation product
CC_{50}	Half-maximal cytotoxic concentration

CMV	Cytomegalovirus
2,5-DHBQOP	2,5-Dihydroxybenzoquinone oxidation product
2,5-DHPOP	2,5-Dihydroxyphenylacetic acid oxidation product
3,4-DHPOP	3,4-Dihydroxyphenylacetic acid oxidation product
2,5-DHTOP	2,5-Dihydroxytoluene oxidation product
3,4-DHTOP	3,4-Dihydroxytoluene oxidation product
CHOP	Chlorogenic acid oxidation product
DNA	Deoxyribonucleic acid
GENOP	Gentisinic acid oxidation product
HA	Humic acids
HS	Humic substances
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HYDROP	Hydroquinone oxidation product
HYKOP	Hydrocaffeic acid oxidation product
IC_{50}	Half-maximal inhibition concentration
IL-1	Interleukin-1
KOP	Caffeic acid oxidation product
M.W.	Molecular weight
MX	Mutagen 'X'
POP	Protocatechuic acid oxidation product
RSV	Respiratory syncytial virus
TNF-α	Tumor necrosis factor-alpha
UV	Ultraviolet

1 Introduction

Humic substances (HS) comprising one of the largest reservoirs of carbon in nature may originate from different sources. For example, they can be formed as final products of biosynthetic pathways in micro-organisms, degradation and transformation products in plants, synthetic oxidation products of phenolic compounds and as polymers resulting from roasting processes, e.g. coffee roasting. Surprisingly, besides their brown color, which is responsible for the high UV absorption of HS, polymers of the HS type have several features in common that enable them to interact with other biopolymers as well as with low-molecular weight organic and inorganic compounds and, in particular, with metals, thus forming chelate complexes. Fractal structures, neighboring carboxyl and hydroxyl groups, reduction-oxidation and association-dissociation potentials are some of the most important features of HS as they cause HS to be important biogeochemical components of the Earth's surface. Besides their traditional use as fuel and organic fertilizers, HS are substrates for medical preparations, and also starting materials in the synthesis of specialized industrial products.

In this chapter, we will focus on medical and veterinary-medical applications of HS, and follow this with a discussion of several aspects of environmental health. Finally, we will look ahead to the possibilities of preparing novel biopolymers of the HS type, and to their potential use and application.

2 Historical Outline

The balneotherapeutic use of peat represents the most significant medical application of HS with regard to volume, therapeutic spectrum and tradition. Heavily degraded high moor peat, which is abundant in HS, has been used therapeutically long ago in Babylonia and the Roman Empire, where the inhabitants already recognized the healing effects of mud (Priegnitz, 1986). As health clinics' specialties, mud baths were offered in Europe in the early 19th century. Traditional indications for mud therapy are gynecological and rheumatic diseases (Baatz, 1988; Kleinschmidt, 1988; Kovarik, 1988; Lent, 1988). Beside mud baths which consist of peat pulp, baths with suspended peat material as well as drinking cures were also applied, the latter especially in case of gastric, intestinal or hepatic diseases (Kallus, 1964). The most frequent indications of peat therapy currently offered by health clinics in Germany are summarized in Table 1.

These conditions comprise various disorders of the musculoskeletal and gynecological systems, as well as skin diseases. Acute inflammatory and infectious diseases as well as malign tumors are usually regarded as contraindications. The primary effect of high-temperature peat therapy is the unique depth hyperthermia, which improves blood circulation and regeneration processes in the patient being treated. Depth warming is caused by the special physical consistency of the mud bath. As therapeutic effects have also been reported for low-temperature peat therapy (Balasheva and Gadzhi, 1971), HS (and possibly other chemical compounds) are also strongly suggested to participate in the healing effect by both chemical and biochemical effects. In a recent study, Bellometti et al. (1997) were able to show a favorable influence of mud bath therapy on osteoarthritis, a rheumatic condition characterized by the progressive destruction of cartilage. It was shown that peat therapy influenced the state of chondrocytes as well as the level of inflammation markers interleukin-1 (IL-1)

Tab. 1 Selected indications of peat therapy in the effect of which humic substances are probably involved

Diseases	*Indications*	*Major therapeutic effects*
Musculoskeletal diseases	Degenerative and deforming arthroses Gout Spondylopathies, e.g. Morbus Bechterew, Osteoporosis Muscular rheumatism Rheumatoid arthritis (polyarthritis) Rehabilitation after operations and accidents	Depth hyperthermia improves blood circulation and regeneration processes
Gynecological diseases	Chronic inflammatory diseases Hormonal imbalances Low back pain Adhesions Sterility Climacteric complaints	Depth hyperthermia Estrogenic effect and/or support of endogenous estrogen production Prophylaxis of thrombosis through release of tissue-type plasminogen activator (profibrinolytic effect)
Skin diseases	Chronic eczema Neurodermatitis Psoriasis	Activation of skin metabolism and regeneration processes, improvement of blood circulation

and tumor necrosis factor alpha (TNF-α). Iubitskaia and Ivanov (1999) demonstrated clearly a substantial contribution of HA to the balneotherapy of osteoarthritis patients. Using sodium humate (instead of peat) they observed analgesic, anti-inflammatory and lipid modulatory effects. Moreover, due to the low concentration of the HA preparation and the lack of effects of other mud factors, sodium humate procedures proved to be well tolerated by the patients.

The question of whether, and to what extent, HS are transferred to the patient during the mud bath has still to be answered. Application conditions such as temperature, ionic strength and pH value are thought to influence the balneotherapeutic effect.

3 Pharmacological Effects of Humic Substances with Potential Use in Medicine

In spite of the predominantly positive experience with balneological peat therapy, only limited knowledge is available of the physiologic and pharmacological effects of peat components. Nevertheless, our understanding of the biologic effects of HS with regard to their antiviral activity, interactions with isolated enzymes, effects on blood coagulation and fibrinolysis, estrogenic activity as well as toxicologically significant interactions with environmentally harmful substances has been considerably extended during the last decades. In this section, we will discuss some of these effects with regard to their potential therapeutic uses.

3.1 Antiviral Activity

Studies on the antiviral effect of HS were initiated after the successful combat against foot-and-mouth disease by means of peat dust-containing litter (Schultz, 1962, 1965). Preliminary in-vitro studies with Coxsackie A9 virus, influenza A virus and herpes simplex virus type 1 (HSV-1) have already shown that HS are effective against both naked and enveloped DNA viruses (Klöcking and Sprößig, 1972, 1975; Thiel et al., 1977). The same is true for synthetic HA derived from polyphenolic compounds, which in part are superior to natural HA in their effect (Thiel et al., 1976, 1981; Klöcking et al., 1983; Eichhorn et al., 1984; Hils et al., 1986). One of the antivirally most active synthetic polymers is the oxidation product of caffeic acid, KOP, the effect of which on HSV-1 *in vitro* compared with that of naturally occurring peat HA is shown in Figure 1.

Further investigations corroborate the ability of HA-like polymers to inhibit selectively viruses for human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), cytomegalovirus (CMV) and vaccinia virus (Schols et al., 1991; Neyts et al., 1992). No inhibition was found against poliovirus type 1, Semliki forest virus, parainfluenza virus type 3, reovirus type 1 and Sindbis virus. Adenovirus

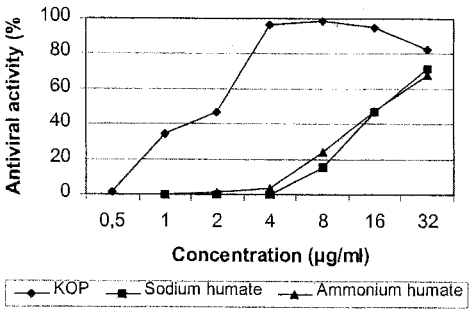


Fig. 1 Antiviral activity to HSV-1 of the synthetic HA-like polymer KOP and naturally occurring peat water HA (as sodium and ammonium salts, respectively). Test substances were added to Vero cells immediately before virus infection. After incubation at 37 °C for 120 h, cell viability was detected using the XTT-based tetrazolium reduction assay EZ4U according to Klöcking et al. (1995).

type 2 and ECHO virus type 6 showed little or no response to natural HA. Half-maximum Anti-HSV-1 inhibition concentrations (IC_{50}) and half-maximum cytotoxic concentrations (CC_{50}) of HA and HA-like polymers are summarized in Table 2, indicating the selective antiviral effect of the polymers tested.

With most viruses, the inhibitory effect of HA and HA-like polymers is directed specifically against an early stage of virus replication, namely virus attachment to cells (Klöcking and Sprößig, 1975; Schols et al., 1991; Neyts et al., 1992). As for CMV, it appears likely that the polyanionic HA occupy positively charged domains of the viral envelope glycoproteins, which are necessary for virus attachment to the cell surface (Neyts et al., 1992).

The effect of HA and HA-like polymers on an early stage of herpesvirus replication has been confirmed by the results of animal experiments. The number of lesions in the cornea of HSV-1-infected rabbits was strongly reduced when a solution of the synthetic HA-like polymer KOP (1%) was applied into the conjunctival sac of the eye along with or immediately after the infectious agent. However, KOP had no effect on the developing lesions when applied 1 and 24 hours later, respectively (Klöcking, 1994). Current interest is directed to the prophylactic effect of HA-type substances on recurrent HSV infection. It is known that topical application of KOP may significantly reduce or even completely suppress experimentally induced herpes in the mouse ear (Dürre and Schindler, 1992), though the mechanistic basis of this effect remains to be elucidated.

A low-molecular weight HA-like polymer (HS 1500, M.W. = 1500 Daltons), synthesized from hydroquinone was found to strongly inhibit HIV-1 *in vitro* (Schneider et al., 1996). Studies on the mechanism of action revealed virus penetration into host cells as the target of the anti-HIV-1 activity. HS 1500 has passed a panel of preclinical tests including eye irritation according to Draize, as well as pregnancy risk in rats. Neither sensitizing nor irritating effects were detectable in concentrations of up to 10% HA (Wiegleb et al., 1993; Lange et al., 1996a,b). SP-303 is another phenolic polymer with antiviral activity, which was isolated from a

Tab. 2 Half-maximal cytotoxic concentrations (CC_{50}) and half-maximal antiviral inhibitory concentrations (IC_{50}) of humic acids and humic acid-like polymers

Test substance	*M.W. (Da)*	*Starting compound*	CC_{50} *(µg/mL)*	IC_{50} *(µg/mL)*
BOP	5300	Catechol	69	26
3,4-DHTOP	3800	3,4-Dihydroxytoluene	>128	42
POP	8000	Protocatechuic acid	70	9.6
3,4-DHPOP	6000	3,4-Dihydroxyphenylacetic acid	>128	9.6
HYKOP	6000	Hydrocaffeic acid	>128	8.0
KOP	6000	Caffeic acid	>64	2.3
CHOP	14000	Chlorogenic acid	>128	6.4
HYDROP	5200	Hydroquinone	>128	3.7
2,5-DHTOP	4700	2,5-Dihydroxytoluene	>128	1.6
GENOP	5500	Gentisinic acid	>128	2.2
2,5-DHPOP	5500	2,5-Dihydroxyphenylacetic acid	>256	0.7
2,5-DHBQOP	3400	2,5-Dihydroxybezoquinone	>512	322
Sodium humate	7500	not known	>128	18.2
Ammonium humate	7900	not known	108	17.8

Euphorbiaceae shrub. The polymer inhibits a panel of respiratory viruses, such as parainfluenza virus type 1, respiratory syncytial virus, influenza A viruses, and influenza B viruses (Gilbert et al., 1993; Wyde et al., 1993). Hemagglutination and other studies suggested that SP-303 at least partially inactivates viruses by direct interaction with virus or host cell lipid membranes. SP-303 at antiviral concentrations did not induce interferon or inhibit virus attachment; however, it abolished RSV penetration into host cells (Barnard et al., 1993). Administered as a small-particle aerosol to influenza A/HK virus-infected mice and RSV-infected cotton rats, SP-303 at 0.5–9.4 mg/kg/day for 3–4 days increased both the percentage and duration of survival of mice. Taken together, results so far show that HS are promising candidates for prophylactic rather than therapeutic use in the treatment of viral diseases.

3.2 Anti-inflammatory Effect and Pro-inflammatory Properties

Various healing effects of peat therapy were attributed to the anti-inflammatory activity of HS. Taugner (1963) showed in the rat paw edema model that sodium humate significantly inhibits the development of various edemas compared with untreated controls. As found by Klöcking et al. (1968) in the same model, ammonium humate isolated from peat water exceeds the anti-inflammatory effect of sodium humate and is twice as effective as acetylsalicylic acid and aminophenazone, respectively. Amosova et al. (1990), in evaluating the biologic activity of HA from Tambukan therapeutic mud in animals, found HA (10 mg/kg) to suppress both phases of the inflammatory process: the exudation (by 44 %) and the proliferation process (by 50–55%).

The anti-inflammatory effect of HS has been supported by a plausible biochemical explanation. As demonstrated by Schewe et al. (1991), naturally occurring HA, and even more synthetic HA-like polymers, inhibit the lipoxygenase pathway of the arachidonic acid (AA) cascade. AA is an integral part of the cell membrane, and the substrate for the synthesis of eicosanoid-based inflammation mediators such as leukotrienes, thromboxane and prostacyclin. Recently, HA (sodium humate) as well as various synthetic HA-like polymers were also found to suppress the heat-induced (42 °C, 6 h) AA release of human promonocytic U937 cells (Dunkelberg et al., 1997; Klöcking et al., 1997). The inhibition of AA release was most pronounced in cells treated with nontoxic concentrations (20 µg/mL) of 3,4-DHPOP (96%) and 3,4-DHTOP (92%), respectively (Table 3). CHOP, sodium humate, KOP and BOP protected cells from membrane damage at 65–90%. These findings may be indicative for membrane-protective activities of HA type substances.

Unlike 5-lipoxygenase, phospholipase A_2 (porcine pancreas), the rate-limiting key enzyme of the AA cascade, is strongly activated at low HA concentrations (0.1–

Tab. 3 Influence of naturally occurring humic acids (sodium humate) and of synthetic humic acid-like polymers on the heat-induced (42 °C, 6 h) [^{3}H]arachidonic acid (AA) release of U937 cells. AA release of untreated control cells = 100%. MEC, Maximum effect concentration; SD, Standard deviation; *Significant, $p \leq 0.05$.

Polymer	*MEC µg/mL*	*[^{3}H]AA release % of controls ± SD*
Sodium humate	80	26.5 ± 8.9*
BOP	160	10.1 ± 14.2*
3,4-DHTOP	20	8.1 ± 10.1*
3,4-DHPOP	20	4.3 ± 2.4*
KOP	40	18.1 ± 7.1*
CHOP	80	34.9 ± 12.6*

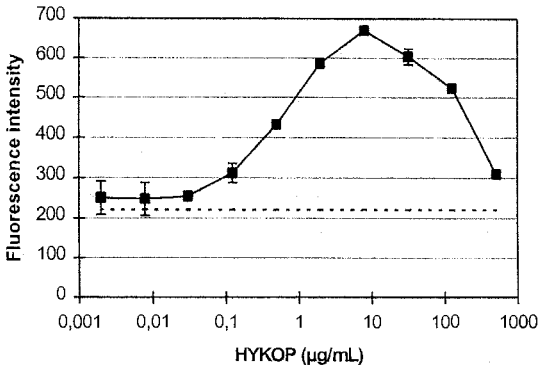


Fig. 2 Influence of the synthetic HA-like polymer HYKOP on the PLA_2-catalyzed hydrolysis of NBD-C_6-HPC using the method of Bennett et al. (1991). Dotted line = Reference value (without HYKOP).

1 µg/mL) and normalized or slightly inhibited at high HA concentrations (Klöcking et al., 1999). Figure 2 shows the typical concentration-dependent course of the dose–response curve for HYKOP, the oxidation product of hydrocaffeic acid. The shape of the dose–response curve suggests HS to have a regulatory function on PLA_2 activity.

Little is known about the influence of different molecular weight fractions of HS on inflammation. Pro-inflammatory activity has found to be associated with the synthetic low-molecular weight HS 1500, which activates human neutrophiles similar to TNF-α (Zeck-Kapp et al., 1991). Liang et al. (1998), while investigating rabbit articular chondrocytes, revealed an inhibitory effect of the ethyl acetate fraction of the commercial Aldrich HA (100–500 µg/mL) on the survival of chondrocytes. Cell injuries were attributed primarily to $O_2^{\bullet -}$ production, which is converted into H_2O_2, thus initiating lipid peroxidation followed by cell necrosis.

In referring to the function of HS as electron donor–acceptor system, Jurcsik (1994) discussed the behavior of HS as the consequence of a 'buffering effect'; this means that HA are able to produce as well as to bind activated oxygen species. This regulatory system is assumed to be important for the favorable influence of HS on wound healing and killing of cancer cells (Jurcsik, 1994).

3.3 Influence on Blood Coagulation and Fibrinolysis

Prophylaxis and therapy of fusions after tubal or ovarian inflammations as well as the postoperative treatment of sterility operations in order to prevent secondary adhesions and repeated occlusions of the ovarian duct are important indications of peat therapy. Fusions are caused by postoperatively reduced degradation of fibrin to soluble fibrin degradation products. As shown by Mesrogli et al. (1988) in laparotomied rats, postoperative baths in peat extract, peat pulp or HA have a clear adhesion-inhibiting effect. A possible explanation of this effect could be the activated fibrin degradation due to the HA-induced release of tissue-type plasminogen activator (t-PA). t-PA is regarded as the regulator of the antithrombotic defense mechanism. It activates plasminogen to plasmin, which splits insoluble fibrin to soluble fibrinogen degradation products (Klöcking, 1991). In addition, HA inhibit the coagulation enzyme thrombin, thereby suppressing the formation of fibrin monomers from fibrinogen (Klöcking, 1994; Klöcking et al., 1999).

Compared with other polyanionic compounds (heparin, pentosanpolysulfate), the anticoagulant effect of HA was found to be less pronounced.

3.4 Estrogenic Activity

Since the first detection of estrogenic substances in the bitumen fraction of peat by Aschheim and Hohlweg (1933), attempts were made to identify the nature of these substances. The assumption that steroid hormones are responsible for the colpotropic effect of peat could hardly be confirmed by chemical analysis. Therefore, the question arose whether, in addition to the lipid-soluble hormones, other peat components might contribute to the estrogenic activity of peat. Studies in castrated ICR (Institute of Cancer Research, USA) mice showed both naturally occurring peat humic acids and synthetic HA-like polymers to be positive in the Allen-Doisy test (Klöcking et al., 1992). The estrogenic activity of sodium humate was found to be 1/3000 of the estriol standard preparation. Referring to the HA content of dry peat, the estrogenic activity of the high-moor peat studied was 5000 times as high as has been supposed to date. Although the components responsible for the estrogenic activity of peat remain under discussion, the results suggest that HA – provided that they can penetrate the skin – may contribute substantially to the estrogenic effect of peat. These findings may have also implications for the use of HS in dermatology and cosmetics.

4 Veterinary-Medical Applications of Humic Substances

In veterinary medicine, HS are successfully applied as drugs for prophylaxis and therapy of gastrointestinal diseases in small animals. Furthermore, HS are utilized as antidotes to prevent intoxication (Kühnert et al., 1989). In order to bind and possibly metabolize as-yet resorbed poisons in the stomach-gastrointestinal tract, HA are given orally as a 20–30% watery solution or suspension in a dosage of 0.5–1.0 g/kg twice daily for 3–5 days (Kühnert, 1996). As observed by Ridwan (1977), a HA concentration of 0.1% is sufficient to reduce significantly the incorporation of lead and cadmium in rats, thus minimizing the risk of heavy metal intoxication. Experiments in mice have shown that orally administered lead humate is less toxic than lead acetate (Klöcking, R., 1980). Opposing results have been obtained following parenteral application of the same compounds. Clearly, the application route is an important factor in deciding whether a metal bound to HS is toxic or detoxified.

5 Humic Substances and Environmental Health

5.1 Mutagenicity

As HS are naturally widespread in the environment and present in surface water, studies on their genotoxic potential are justified, particularly as by-products of chlorination and ozonization in HS containing drinking water are known to be extremely active in bacterial genotoxicity tests (Meier et al., 1987; Meier, 1988). Mutagenesis studies carried out on fractions of drinking water samples have shown that 3-chloro-4-(dichloromethyl)-5-hydroxy-(5H)-furanone (MX) is one of the main chlorination intermediate products, responsible for more than 20% of observed mutagenicity (Holmbom, 1984; Kronberg et al., 1985, 1991). However, in-vivo mutagenicity tests have provided con-

flicting results, possibly due to the great reactivity and instability of the furanones formed (Dayan, 1993). Furanones occur also in foods, where they appear mainly as a result of Maillard reactions between sugars and amino acids during heating. Furthermore, they play an important role in the flavor of fruits and as an essential antioxidant food component (ascorbic acid, vitamin C) for humans (Colin-Slaughter, 1999). Although furanones are mutagenic to bacteria and cause DNA damage in laboratory animals, these compounds are, in practice, very effective anti-carcinogenic agents in the diets of animals which are being treated with known cancer-inducing compounds such as benzo[a]pyrene or azoxymethane. Evidence for the desmutagenic activity of HA has also been reported by Cozzi et al. (1993), De Simone et al. (1993), and Ferrara et al. (2000).

5.2 Protection against Ionizing Irradiation

Oris et al. (1990) were able to show that dissolved humic materials at concentrations in the range of 1 to 7 μg/mL significantly reduced acute photo-induced toxicity in fish (*Pimephales promelas*) and daphnia (*Daphnia magna*). The phenomenon is explained by selective attenuation of the active wavelengths of solar UV radiation by dissolved HS.

A protective effect of HA to injuries caused by an external whole-body ^{60}Co gamma irradiation in female Wistar rats has been reported in a World Patent Application (WO 9858655). HA were extracted from a 3000- to 7000-years-old fen peat standardized by topographic and paleobotanical characterization. The HA preparation (240 mg/animal/day) was applied by gastric intubation to female Wistar rats of 190–220 g bodyweight for 7 days before irradiation (7 Gy), followed by an additional 4-week treatment with the same dose after irradiation. No injury of the hemopoietic system occurred in the HA-treated animals. Lower dosages of HA (90 mg/animal/day) were also effective, albeit to a lesser extent. The HA-containing preparation is intended to improve the regeneration of the hemopoietic system in case of accidental radiation effects, and possibly to mitigate against injuries due to chemotherapy.

A therapeutic effect of sodium humate given as a single dose to experimental mongrel rats 5–10 min following irradiation with lethal doses of ^{60}Co led to 43% survival of animals after 60 days (Pukhova et al., 1987).

In addition to the protection against radiation-induced injuries and the supporting effect on tissue regeneration, HA show indirect detoxifying effects, e.g. by preventing the photoactivation of polycyclic aromatic hydrocarbons. As demonstrated by Nikkila et al. (1999), HA reduced the toxicity of UV-B-irradiated pyrene to *Daphnia magna* in a dose-dependent fashion. The effect was assumed to be due to the decrease in the photomodification of the dissolved pyrene by diminishing the light penetration into the water, and possible interaction with the intact parent compound.

5.3 Blackfoot Disease

Artesian drinking water containing a high concentration of greenish-blue fluorescent HS and/or arsenic has been implicated as one of the etiological factors of Blackfoot disease (BFD), which occurs endemically in the southwest coast of Taiwan (Lu, 1990). Clinically, BFD is a peripheral vascular disorder with symptoms similar to those of arteriosclerosis obliterans and thrombotic vasculopathy. The disease can be induced experimentally in mice receiving fluorescent HS at a daily dose of 5 mg per 20 g body mass for at least 22 days. The pathogenesis of the disease has not yet fully established. In-vitro studies

with purified commercial HA revealed a destruction of human erythrocytes at (relatively high) HA concentrations of 50 – 100 μg/mL, probably due to the generation of oxidative stress (Cheng et al., 1999). Recently, Gau et al. (2000) demonstrated an inhibition of lipopolysaccharide-induced expression of NF-κB in HA-pretreated cultured human umbilical vein endothelial cells.

6 Outlook and Perspectives

The impact of HS on the quality of human health is increasingly recognized as an important subject of future research work. Investigations of HS aimed at the molecular structure and mechanism of action encompass specialized investigations within such diverse fields as physical, analytical, environmental and food chemistry, cell biology, molecular genetics, pharmacology and toxicology.

As outlined in this chapter, some of the naturally occurring or synthetically prepared biopolymers of the HA type have the potential of highly effective drugs. Therefore, in addition to the classic use of peat in balneotherapy and veterinary medicine, the application of isolated HS as well as synthetic HA-like polymers may play a considerable role in future. There are a large number of phenolic compounds which can be transformed into HA-like substances targeted for special functions such as antivirally active agents, heavy metal-chelating compounds, toxic chemical-binding polymers and substances protecting against ionizing radiation. However, the use of HS as therapeutic drugs make high demands on pharmacologically evidenced efficacy, toxicological safety standards and a clearly defined chemical composition of the preparation used.

To elucidate the chemical structure of synthetic HA that originate from comparatively simple individual starting compounds remains an important goal for the near future. The results will definitely stimulate and facilitate the much more complicated exploration of natural HS.

7 References

Amosova, Ya. M., Kosyanova, Z. F., Orlov, D. S., Tikhomirova, K. S., Shinkarenko, A. L. (1990) Humic acids in the therapeutic muds with a special reference to their physiological activity, *Kurortol. Fizioter.* 27 (4), 1–6.

Aschheim, S., Hohlweg, W. (1933) Über das Vorkommen östrogener Wirkstoffe in Bitumen, *Dtsch. Med. Wochenschr.* 59, 12–14.

Baatz, H. (1988) Moortherapie in der Frauenheilkunde, in: *Moortherapie – Grundlagen und Anwendungen* (Flaig, W., Goecke, C., Kauffels, W., Eds.), pp. 161–168. Wien, Berlin: Ueberreuter.

Balasheva, L. I. and Gadzhi, F. I. (1971) Experience in the treatment of patients with rheumatism in inactive phase using low-temperature diluted mud baths, *Vopr. Kurortol. Fizioter. Lech. Fiz. Kult.* 36, 240–242.

Barnard, D. L., Huffman, J. H., Meyerson, L. R., Sidwell, R. W. (1993) Mode of inhibition of respiratory syncytial virus by a plant flavonoid, SP-303, *Chemotherapy (Basel)* 39, 212–217.

Bellometti, S., Giannini, S., Sartori, L., Crepaldi, G. (1997) Cytokine levels in osteoarthrosis patients undergoing mud bath therapy, *Int. J. Clin. Pharmacol. Res.* 17, 149–153.

Bennett, E. R., Yedgar, S., Lerer, B., Ebstein, R. P. (1991) Phospholipase A_2 activity in Epstein-Barr virus-transformed lymphoblast cells from schizophrenic patients, *Biol. Psychiatry* 29, 1058–1062.

Cheng, M. L., Ho, H. Y., Chiu, D. T. Y., Lu, F. J. (1999) Humic acid-mediated oxidative damages to human erythrocytes: a possible mechanism leading to anemia in Blackfoot disease, *Free Radic. Biol. Med.* 27, 470–477.

Colin-Slaughter, J. (1999) The naturally occurring furanones: formation and function from pheromone to food, *Biol. Rev. Camb. Philos. Soc.* 74, 259–276.

Cozzi, R., Nicolai, M., Perticone, P., De Salvia, R., Spuntarelli, F. (1993) Desmutagenic activity of natural humic acids: inhibition of mitomycin C and maleic hydrazide mutagenicity, *Mutat. Res.* 299, 37–44.

Dayan, A. D. (1993) Carcinogenicity and drinking water, *Pharmacol. Toxicol.* 72, 108–115.

De Simone, C., Piccolo, A., De Marco, A. (1993) Effects of humic acids on the genotoxic activity of maleic hydrazide, *Fresenius Environ. Bull.* 2, 157–161.

Dunkelberg, H., Klöcking, H.-P., Klöcking, R. (1997) Suppression of heat-induced [^{3}H]arachidonic acid release in U937 cells by humic acid-like polymers, *Pharmacol. Toxicol.* 80 (Suppl. III), 175–176.

Dürre, K., Schindler, S. (1992) Austestung antiviraler Substanzen an der rezidivierenden kutanen Herpes-simplex-Virus-Infektion der Maus unter Berücksichtigung des Einflusses von Penetrationsvermittlern. Dissertation, Medizinische Akademie Erfurt.

Eichhorn, U., Klöcking, R., Helbig, B. (1984) Anwendung von ^{51}Cr-markierten FL-Zellen zur Testung der antiviralen Aktivität von Phenolkörperpolymerisaten gegen Coxsackieviren *in vitro*, *Dtsch. Gesundh. wes.* 39, 1514–1519.

Ferrara, G., Loffredo, E., Simeone, R., Senesi, N. (2000) Evaluation of antimutagenic and desmutagenic effects of humic and fulvic acids on root tips of *Vicia faba*, *Environ. Toxicol.* 15, 513–517.

Gau, R.-J., Yang, H.-L., Chow, S.-N., Suen, J.-L., Lu, F.-J. (2000) Humic acid suppresses the LPS-induced expression of cell surface adhesion proteins through the inhibition of NF-κB activation, *Toxicol. Appl. Pharmacol.* 166, 59–67.

Gilbert, B. E., Wyde, P. R., Wilson, S. Z., Meyerson, L. R. (1993) SP-303 small-particle aerosol treatment of influenza A virus infection in mice and

respiratory syncytial virus infection in cotton rats, *Antiviral Res.* **21**, 37–45.

Hils, J., May, A., Sperber, M., Klöcking, R., Helbig, B., Sprößig, M. (1986) Hemmung ausgewählter Influenzavirusstämme der Typen A und B durch Phenolkörperpolymerisate, *Biomed. Biochim. Acta* **45**, 1173–1179.

Holmbom, B., Voss, R. H., Mortimer, R. D., Wong, A. (1984) Fraction, isolation and characterization of Ames mutagenic compounds in Kraft chlorination effluents, *Environ. Sci. Technol.* **18**, 333–337.

Iubitskaia, N. S., Ivanov, E. M. (1999) Sodium humate in the treatment of osteoarthrosis patients, *Vopr. Kurortol. Fizioter. Lech. Fiz. Kult.* 1999(5), 22–24.

Jurcsik, I. (1994) Possibilities of applying humic acids in medicine (wound healing and cancer therapy), in: Humic Substances in the Global Environment (Senesi, N., Miano, T.M., Eds), pp. 1331–1336. Amsterdam, London, New York, Tokyo: Elsevier.

Kallus, J. (1964) Gastritis, das gastroduodenale Ulkus und Neydhartinger Moor, in: *Bericht über den 8. Internationalen Kongreß für universelle Moor- und Torfforschung*, pp.111–114. Linz: Länderverlag.

Kleinschmidt, J. (1988) Moortherapie bei rheumatischen Erkrankungen, in: *Moortherapie – Grundlagen und Anwendungen* (Flaig, W., Goecke, C., Kauffels, W., Eds.), pp. 216–224. Wien, Berlin: Ueberreuter.

Klöcking, H.-P. (1991) Influence of natural humic acids and synthetic phenolic polymers on fibrinolysis, in: *Humic Substances in the Aquatic and Terrestrial Environment* (Allard, B., Boren, H: Grimvall, A., Eds.), Vol. 33 of Lecture Notes in Earth Sciences (Bhattacharji, S., Friedmann, G. M., Neugebauer, H. J., Seilacher, A., Eds.), pp. 423–428. Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong, Barcelona: Springer-Verlag.

Klöcking, H.-P. (1996) Anti-factor IIa–activity of humic acid-like polymers derived from p-diphenolic compounds. In: *Humic Substances and Organic Matter in Soil and Water Environments: Characterization, Transformations and Interactions*: Proceedings of the 7th International Conference of the International Humic Substances Society at the University of the West Indies, St. Augustine, Trinidad and Tobago, 3–8 July 1994 (Clapp, C. E., Hayes, M. H. B., Senesi, N., Griffith, S. M., Eds), pp. 411–415. Birmingham: Dawn Printers.

Klöcking, H.-P., Dunkelberg, H., Klöcking, R. (1997) Substances of the humic acid type prevent U937 cells from heat-induced arachidonic acid release, in: *Modern Aspects in Monitoring of Environmental Pollution in the Sea* (Müller, W. E. G., Ed.), pp. 156–158. Erfurt: Akademie gemeinnütziger Wissenschaften.

Klöcking, H.-P., Helbig, B., Klöcking, R. (1999) Antithrombin activity of synthetic humic acid-like polymers derived from o-diphenolic starting compounds, *Thromb. Haemost.* Suppl. 299–300.

Klöcking, R. (1980) Giftung und Entgiftung von Schwermetallen durch Huminsäuren, *Arch. Exper. Veterinärmedizin* **34**, 389–393.

Klöcking, R. (1994) Humic substances as potential therapeutics, in: *Humic Substances in The Global Environment* (Senesi, N., Miano, T. M., Eds.) pp. 1245–1257. Amsterdam, London, New York, Tokyo: Elsevier.

Klöcking, R., Helbig, B. (1999) Report on the Workshop of DGMT, Section IV, October 7–8, 1999, Bad Elster, Germany, *Telma*, **29**, 239–243.

Klöcking, R., Sprößig, M. (1972) Antiviral properties of humic acids, *Experientia* **28**, 607–608.

Klöcking, R., Sprößig, M. (1975) Wirkung von Ammoniumhumat auf einige Virus-Zell-Systeme, *Z. Allg. Mikrobiol.* 15, 25–30.

Klöcking, R., Hofmann, R., Mücke, D. (1968) Tierexperimentelle Untersuchungen zur entzündungshemmenden Wirkung von Humaten, *Arzneim. Forsch.* **18**, 941–942.

Klöcking, R., Sprößig, M., Wutzler, P., Thiel, K.-D., Helbig, B. (1983) Antiviral wirksame Huminsäuren und huminsäureähnliche Polymere, *Z. Physiother.* **33**, 95–101.

Klöcking, R., Fernekorn, A., Stölzner, W. (1992) Nachweis einer östrogenen Aktivität von Huminsäuren und huminsäureähnlichen Polymeren, *Telma* **22**, 187–197.

Klöcking, R., Schacke, M., Wutzler, P. (1995) Primärscreening antiherpetischer Verbindungen mit EZ4U, *Chemotherapie J.* **4**, 141–147.

Kovarik, R. (1988) Über die Anwendung von Präparaten aus Torf, bzw. Huminstoffen bei gynäkologischen Erkrankungen, in: *Moortherapie – Grundlagen und Anwendungen* (Flaig, W., Goecke, C., Kauffels, W., Eds.), pp. 177–197. Wien, Berlin: Ueberreuter.

Kronberg, L., Holmbom, B., Tikkanen, L. (1985) Mutagenic activity in drinking water and humic water after chlorine treatment, *Vatten* **41**, 106–109.

Kronberg, L., Christman, R. F., Singh, R., Ball, L. M. (1991) Identification of oxidized and reduced forms of the strong bacterial mutagen (Z)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (MX) in extracts of chlorine-treated water, *Environ. Sci. Technol.* **25**, 99–104.

Kühnert, M. (1996) Vergiftungen, in: *Lehrbuch der Pharmakologie und Toxikologie für die Veterinärmedizin* (Frey, H. H., Löscher, W., Eds.), p. 675. Stuttgart: Ferdinand Enke.

Kühnert, M., Fuchs, V., Golbs, S. (1989) Pharmakologisch-toxikologische Eigenschaften von Huminsäuren und ihre Wirkungsprofile für eine veterinärmedizinische Therapie, *DTW Dtsch. Tierärztl. Wochenschr.* 96, 3–10.

Lange, N., Faqi, A. S., Kühnert, M., Haase, A., Hoke, H., Seubert, B. (1996a) Influences of a low molecular humic substance on pre- and postnatal development of rats, *DTW Dtsch. Tierärztl. Wochenschr.* 103, 6–9.

Lange, N., Kühnert, M., Haase, A., Hoke, H., Seubert, B. (1996b) The resorptive behavior of a low-molecular weight humic substance after a single oral administration to the rat, *DTW Dtsch. Tierärztl. Wochenschr.* 103, 134–135.

Lent, W. (1988) Bericht über die Moorforschung und -anwendung in der DDR, Polen, Tschechoslowakei und UdSSR, in: *Moortherapie – Grundlagen und Anwendungen* (Flaig, W., Goecke, C., Kauffels, W., Eds.), pp. 169–176. Wien, Berlin: Ueberreuter.

Liang, H. J., Tsai, C. L., Lu, F. J. (1998) Oxidative stress induced by humic acid solvent extraction fraction in cultured rabbit articular chondrocytes, *J. Toxicol. Environ. Health* 54, 477–489.

Lu, F. J. (1990) Fluorescent humic substances and blackfoot disease in Taiwan, *Appl. Organomet. Chem.* 4, 191–195.

Mesrogli, M., Maas, A., Schneider, J. (1988) Stellenwert der Moortherapie in der Sterilitätsbehandlung, in: *Moortherapie: Grundlagen und Anwendungen* (Flaig, W., Goecke, C., Kauffels, W., Eds.), pp. 225–235. Wien, Berlin: Ueberreuter.

Meyer, J. R. (1988) Genotoxic activity of organic chemicals in drinking water, *Mutat. Res.* 196, 211–245.

Meyer, J. R., Knohl, R. B., Coleman, W. E., Ringhand, H. P., Munch, J. W., Kaylor, W. H., Streicher, R. P., Kopfler, F. C. (1987) Studies on the potent bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, *Mutat. Res.* 189, 363–373.

Neyts, J., Snoeck, R., Wutzler, P., Cushman, M., Klöcking, R. Helbig, B., Wang, P., De Clercq, E. (1992) *Antiviral Chem. Chemother.* 3, 215–222.

Nikkila, A., Penttinen, S., Kukkonen, J. V. (1999) UV-B-Induced acute toxicity of pyrene to the waterflea *Daphnia magna* in natural freshwaters, *Ecotoxicol. Environ. Safety* 44, 271–279

Oris, J. T., Hall, A. T., Tylka, J. D. (1990) Humic acids reduce the photo-induced toxicity of anthracene to fish and *Daphnia*, *Environ. Toxicol. Chem.* 9, 575–583.

Priegnitz, H. (1986) *Wasserkur und Badelust.* Leipzig: Koehler & Amelang.

Pukhova, G. G., Druzhina, N. A., Stepchenko, L. M., Chebotarev, E. E. (1987) Effect of sodium humate on animals irradiated with lethal doses, *Radiobiologiia* 27, 650–653.

Ridwan, F. N. J. (1977) Untersuchungen zum Einfluß von Huminsäuren auf die Blei- und Cadmium-Absorption bei Ratten. Dissertation, Universität Göttingen.

Schewe, C., Klöcking, R., Helbig, B., Schewe, T. (1991) Lipoxygenase-inhibitory action of antiviral polymeric oxidation products of polyphenols, *Biomed. Biochim. Acta* 50, 299–305.

Schneider, J., Weis, R., Manner, C., Kary, B., Werner, A., Seubert, B. J., Riede, U. N. (1996) Inhibition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: mechanism of inhibition, *Virology* 218, 389–395.

Schols, D., Wutzler, P., Klöcking, R., Helbig, B., De Clercq, E. (1991) *J. Acq. Immun. Defic. Synd.* 4, 677–685.

Schultz, H. (1962) Die viricide Wirkung der Huminsäuren im Torfmull auf das Virus der Maul- und Klauenseuche, *Dtsch. tierärztl. Wochenschr.* 69, 613–616.

Schultz, H. (1965) Untersuchungen über die viricide Wirkungsweise der Huminsäuren im Torfmull, *Dtsch. tierärztl. Wochenschr.* 72, 294–297.

Taugner, B. (1963) Tierexperimentelle Untersuchungen über ein Natriumhuminat-Salicylsäure-Bad, *Arzneimittelforschung* 13, 329–333.

Thiel, K.-D., Klöcking, R., Helbig, B. (1976) In-vitro-Untersuchungen zur antiviralen Aktivität enzymatisch oxidierter o-Diphenolverbindungen gegenüber Herpes simplex-Virus Typ 1 und Typ 2, *Zbl. Bakt. Hyg., I. Abt. Orig,* A 234, 159–169.

Thiel, K.-D., Klöcking, R., Schweizer, H., Sprößig, M. (1977) Untersuchungen *in vitro* zur antiviralen Aktivität von Ammoniumhumat gegenüber Herpes simples Virus Typ 1 und Typ 2, *Zbl. Bakt. Hyg., I. Abt. Orig,* A 239, 304–332.

Thiel, K.-D., Helbig, B., Klöcking, R., Wutzler, P., Sprößig, M., Schweizer, H. (1981) Vergleich der In-vitro-Wirkung von Ammoniumhumat und enzymatisch oxidierter Chlorogen- und Kaffeesäure gegenüber Herpesvirus hominis Typ 1 und Typ 2, *Pharmazie* 36, 50–53.

Wiegleb, K., Lange, N., Kühnert, M. (1993) The use of the HET-CAM test for the determination of the irritating effects of humic acids, *DTW Dtsch. Tierärztl. Wochenschr.* 100, 412–416.

Wyde, P. R., Meyerson, L. R., Gilbert, B. E. (1993) In vitro evaluation of the antiviral activity of SP-303, an Euphorbiaceae shrub extract, against a panel of respiratory viruses, *Drug Dev. Res.* **28**, 467–472.

Zeck-Kapp, G., Nauck, M., Riede, U. N., Block, L., Freudenberg, N., Seubert, B. (1991) Low-molecular humic substances as pro-inflammatory cell signals, *Verh. Dtsch. Ges. Path.***75**, 504.

Display from CAplus

Title

The general survey of research & development on the application of **humic** substances in china

Author

Jin, Fenghe; Nishizaki, Yasushi; Yamaguchi, Tatsuaki

Organization

Environmental Sciences Corporation, Japan

Publication Source

Chiba Kogyo Daigaku Kenkyu Hokoku, Riko-hen (**1999**), 46, 123-130

Identifier-CODEN

RPCTAL

ISSN

0385-7026

Publisher

Chiba Kogyo Daigaku

Abstract

A review with 66 refs. on the utilization of **humic** acid resources in China as surveyed from the treatises in the Chinese journal, "Fuzhisuan", published by China **Humic** Acid Industry Assocn. (CHAIA). This is the only journal focusing specifically on **humic** acid in the world, publishing three issues a year. Sources of **humic** acid in China, agricultural applications such as soil ameliorants, fertilizers, and agrochems., including animal feeds are discussed. Industrial applications have developed mainly from the 1970s, aiming at the higher cost return from the product, e.g. additive of battery, reducing water agent of concrete, slurry treatment material, heavy metal adsorbent, pottery and others. Medicinal use originated from peat bath in the 1950s. Pharmacol. studies are testing effectiveness against inflammations, tumors and others. China has plenty of resources for **humic** substances, maintaining a high focus on the study for its utilization.

Document Type

Journal; General Review

Language

Japanese

Accession Number

1999:407932 CAPLUS

Reference Number

131:73047

DRUG DEVELOPMENT RESEARCH 57:34–39 (2002)

DDR

Research Article

Phase I Trial With Oral Oxihumate in HIV-Infected Patients

Mariëtte E. Botes,[1] J. Dekker,[2] and C.E.J. van Rensburg[3]*

[1]Department of Internal Medicine, Kalafong Hospital, University of Pretoria, Pretoria, South Africa
[2]Enerkom (Pty) Ltd, Pretoria, South Africa
[3]Department of Pharmacology, University of Pretoria, Pretoria, South Africa

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Oxihumate is formed through conversion of bituminous coal by controlled wet oxidation. The objective of the study, based on in vitro studies, was to evaluate the safety and toxicity profile of oxihumate at various doses in HIV-1-infected individuals. Thirty-seven antiretroviral-naïve adults were randomized in a double-blind study to dosages of either 2, 4, 6, or 8 g oxihumate or placebo per day and treated for 2 weeks. The baseline CD4-positive lymphocyte count was 213–570 × 10^6/L and baseline viral load 675–275,000 copies/ml. Patients were reviewed at days 5, 10, and 15, as well as 1 week posttreatment. Of the 37 patients, 35 were followed up until the end of the study. None of the adverse events was thought to be related to the investigative drug. Efficacy parameters were compared between treatment groups at week 2 using analysis of covariance (with baseline value for a patient as the covariant). Strong evidence ($P = 0.018$) was found that the posttreatment weight differed between treatment groups (adjusted for initial weight). All active treatment groups gained weight compared to the placebo. No significant difference in the viral load or CD4-positive lymphocyte count between the active ingredient and the placebo was found. None of the biochemical and hematological parameters differed significantly from the baseline at end of treatment. In conclusion, oxihumate is well tolerated, with an excellent safety profile when tested over a period of 2 weeks. This period was too short to establish efficacy or optimum dosage levels. Drug Dev. Res. 57:34–39, 2002.

Key words: oxihumate; HIV; phase I trial

INTRODUCTION

Humic acids are formed during the decomposition of organic matter and can therefore be found in practically all natural environments in which organic materials and microorganisms are, or have been, present [Visser, 1973].

Antiviral properties, at a concentration of 100 μg/ml of ammonium humate in vitro, have been described by Thiel et al. [1977], resulting in the successful use of this agent as a topical treatment for herpes virus-induced skin diseases [Klöcking et al., 1983]. Schneider et al. [1996] reported on the in vitro anti-HIV activity of synthetic humate analogs derived from hydroquinone. These compounds inhibited HIV-1 infection of MT-2 cells with an impressively low IC_{50} of 50–300 ng/ml. The infectivity of HIV particles was inhibited by interference with the CD4-induced proteolytic cleavage of the V3 loop of virion gp120SU.

Contract grant sponsors: Enerkom (Pty) Ltd.; Technology and Human Resources for Industry Programmes (THRIP) of the National Research Foundation; Department of Trade and Industry.

*Correspondence to: C.E.J. van Rensburg, Department of Pharmacology, P.O. Box 2034, Pretoria 0001, South Africa. E-mail: cmedlen@postillion.up.ac.za

Received 19 May 2002; Accepted 12 July 2002

Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/ddr.10117

Humic acids (sold under the trade name of Huminit) have been used in medicines for the treatment of hyperacidity and other gastric disturbances in humans [Gramsch, 1961; Reichert, 1966]. Humic acids have also been used as antiinflammatory agents because of their local antiinflammatory, hyperemic, and analgesic properties [Brandt, 1964; Eichelsdörfer, 1976] and in the treatment of anemia, hypercholesterolemia [Soloveyva and Lotosch, 1984], and Von Willebrand's disease [Lopez-Fernandez et al., 1992]. No unfavorable side effects have been observed at dosages of up to 1.8 g per day.

Oxihumate, produced from coal, has not been used to treat humans before, but has been tested extensively on animals (Progress Report: Biocon (Pty) Ltd, Pretoria, South Africa, July, 1999). No major toxicity was detected. According to subchronic toxicity studies in animals, only slight alterations in blood chemistry have been noted at dosages above 100 mg/kg. Twelve percent of oxihumate, administered orally to a baboon, appears in the blood pool (pharmacokinetic study with oxihumate on baboons: I Dormehl, Atomic Energy Corporation Institute for Life Sciences, Pretoria, South Africa, December, 1997). Thus, a dosage of 2,000 mg, i.e., 28.5 mg/kg, would theoretically produce a serum level of 48 mg/ml in human adults. This level is well within the concentration range where in vitro anti-HIV activity was observed [Van Rensburg et al., 2002]. The half-life of oxihumate, indicated by pharmacokinetic studies, appears to be 24 hr.

A South African company (Enerkom (Pty) Ltd) has developed a unique process to convert bituminous coal by controlled wet oxidation, followed by base treatment to form water-soluble oxihumates [Bergh et al., 1997].

Oxihumate possesses immunostimulatory properties (data submitted), as well as antiviral activity, as was the case with synthetic humate analogs [Scheider et al., 1996], by inhibiting HIV-1 infection of MT-2 cells by interference with a V3 loop-mediated step of virus entry [Van Rensburg et al., 2002].

Antiretroviral therapy, which is the standard of care in the Western world, is not accessible for most patients in Africa and more affordable treatment options have to be explored. A drug that shows antiretroviral properties should therefore be investigated in clinical studies in HIV-positive patients.

Based on previous studies, we hypothesized that oxihumate possesses immunostimulatory and anti-HIV properties and should therefore be therapeutically effective in HIV-infected patients. The objective of this study was to investigate the safety and toxicity profile of oxihumate at various doses up to 8 g/day in HIV-1-infected patients.

SUBJECTS AND METHODS

Study Design

In this prospective, double-blind, randomized study, oxihumate was compared with placebo for safety during a 2-week period on a cohort of patients from the Immunology Clinic at Kalafong Hospital, Pretoria, South Africa.

The study was approved by the Ethics Committee of the University of Pretoria, as well as the Medicines Control Council of South Africa. The study was conducted in accordance with the Declaration of Helsinki and ICH-GCP Guidelines. Before enrollment, all patients signed an informed consent form that had been approved by the Ethics Committee of the University of Pretoria.

Study Population

The key eligibility criteria were serologically proven HIV-1 infection, age 18 years or older, CD4-positive lymphocyte counts between 200–500 $\times 10^6$/L, and HIV-1 RNA between 500 and 500,000 copies/ml. Patients had to have a life expectancy of more than 3 months, a World Health Organization (WHO) performance status score of <3, and a Karnofsky index of >80.

Patients had to be in a stable clinical condition, with no opportunistic infection at onset or 4 weeks prior to entry into the study. Exclusion criteria were prior exposure to antiretroviral agents, immunomodulatory agents, immunosuppressive agents, and/or radiotherapy. Patients with a history of drug or alcohol abuse or in need of psychiatric treatment were excluded. Patients with liver enzymes >3 times upper limit of normal (ULN) or renal functions >2 ULN were also excluded.

Women of childbearing potential were required to use barrier contraception during the study. Pregnant or lactating women were excluded.

Study Medication

Oxihumate is available in 500 mg capsules. There were four treatment groups in the study: 2, 4, 6, and 8 g oxihumate per day. Patients were enrolled into one treatment group at a time, starting with the lowest dosage. In every group, seven patients were randomized to the active ingredient and two to the placebo. The patients took the capsules under supervision of the study staff twice daily with balanced meals for 2 weeks. The study period was limited to 2 weeks since oxihumate had not been tested before in a clinical

study. Patients would be removed from the study if intolerance or toxicity occurred. There was no alternative treatment available.

Study Procedures

Eligibility screening included a history and complete physical examination, performance status evaluation, CD4-positive lymphocyte count, viral load (NucliSense NASBA amplification method), full blood count, electrolytes, urea, creatinine, and tests of liver function. At day 0 (baseline) the CD4-positive lymphocyte count and viral load were repeated. The CD4-positive lymphocyte and viral load pretreatment values were calculated as the mean of the screening and baseline values. Follow-up visits were scheduled for days 5, 10, 15, and 21 (1 week posttreatment). At follow-up the physical examination, performance status evaluation, CD4-positive lymphocyte count, viral load, and hematological and serum chemistry panels were repeated.

Statistical Analysis

All data were recorded into case report forms. The blind was kept throughout the study. Study endpoints were new clinical AIDS-defining conditions, death, and development of drug-related toxicities. The safety analysis included all patients who initiated treatment (intent-to-treat population).

RESULTS

Demographic and Clinical Characteristics of Patients

The baseline characteristics of the patients in each of the dosage groups are presented in Table 1. Fifty-seven patients were screened and 37 patients, 11 males and 26 females, were enrolled between February and October 1999. In all of the groups nine patients were enrolled, except for the 6-g group, where one extra patient was enrolled to replace one patient that dropped out after work-related circumstances prevented her from continuing in the study. The median age was 33 (range 18–58). The baseline median CD4-positive lymphocyte count was 327 (range 213–569) $\times 10^6$/L and baseline median viral load 21,500 copies HIV-1 RNA/ml plasma (range 675–275,000). Karnofsky score index was 80–100 and WHO performance status 0–1.

Subject Accountability

Of the 37 patients, 35 (94%) were followed until the end of study (1 week posttreatment). One patient (3%) in the 6-g group dropped out during the first week of treatment for reasons unrelated to oxihumate treatment and one patient in the 2-g group (3%) had a serious adverse event (death) that was unrelated to study medication use.

Safety Parameters

The study focused on safety and tolerability. Safety parameters (chemistry and hematology) were

TABLE 1. Baseline Characteristics of the Patients in Each of the Dosage Groups

Baseline characteristic	Placebo	2g/day	4g/day	6g/day	8g/day
Number	8	7	7	8	7
Male:Female	2:6	2:5	3:4	2:6	2:5
Mean age	32	30	39	30	26
Age range	25–58	20–40	25–45	19–45	18–43
Median Karnofsky performance	85	90	90	95	100
Range Karnofsky performance	80–100	80–100	90–100	80–100	90–100
Median CD4	335	327	341	312	321
Range CD4	225–475	213–570	250–477	248–410	274–486
Median HIV viral load (log)	4.61	4.07	4.33	4.62	4.31
Range HIV viral load (log)	3.08–5.41	2.83–5.18	3.63–5.44	3.67–5.4	4.08–5.13
Time since first HIV-1 antibody detection (months)	1–51	0–37	2–53	1–44	0–25
Median time since first HIV-1 antibody detection (months)	30	8	16	14	16
History of HIV-related disease					
Herpes simplex mucocutaneous ulceration			1	1	
Mycobacterium tuberculosis, pulmonary	2		1	2	
Candidiasis, oral	2	2			1
Candida vaginitis/perineal Candidiasis	1	2	1	2	1
Herpes zoster (shingles)	1		2		
Chronic diarrhea	1	1			
HIV-related neuropathy				2	
Bacterial pneumonia				2	

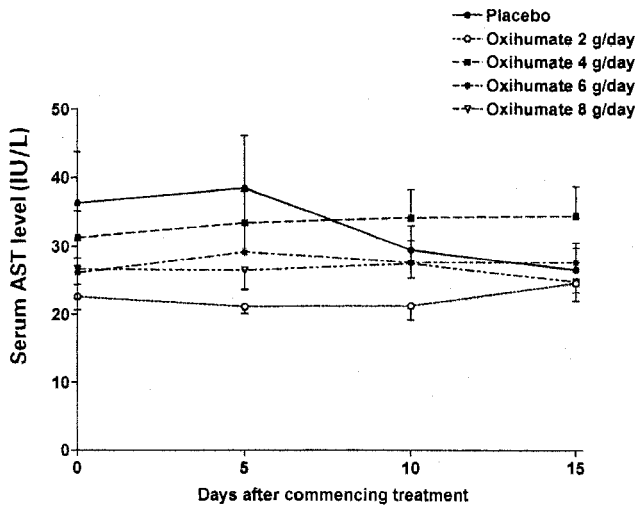


Fig. 1. Serum-AST level of HIV positive patients during a 2-week treatment with oxihumate.

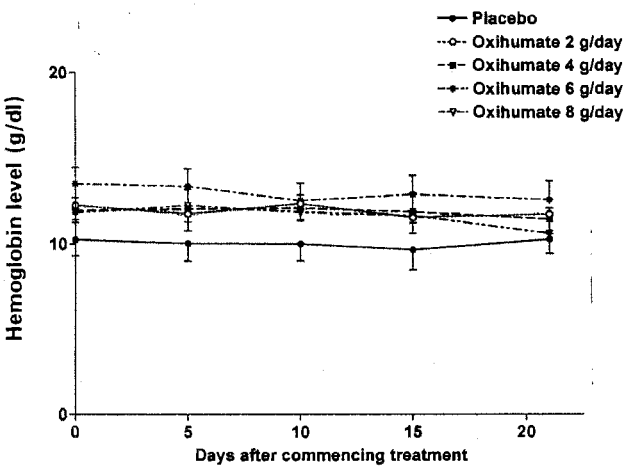


Fig. 3. Hemoglobin level of HIV-positive patients during a 2-week treatment with oxihumate.

compared between treatment groups at week 2 using analysis of covariance (with baseline value for a patient as the covariant). Chemistry included urea, creatinine, uric acid, electrolytes, liver enzymes, bilirubin, lactate dehydrogenase, glucose, and cholesterol. None of the biochemical parameters such as serum-AST (Fig. 1), and urea levels (Fig. 2), or the hematological parameters such as hemoglobin (Fig. 3) differed significantly from the baseline at end of treatment.

Tolerability

Treatment was well tolerated. One patient in the oxihumate 4 g/day group and one patient in the oxihumate 8 g/day group developed headache, but both patients had a background history of headache. One patient in the placebo group experienced vomiting. One patient in the oxihumate 2 g/day group experienced an episode of diarrhea that was probably not related to the study medication.

Progression to Study Endpoints

No AIDS-defining conditions occurred during the study or 1-week follow-up. One death occurred on day 8 in the oxihumate 2 g/day group due to *S. pneumoniae* septicemia.

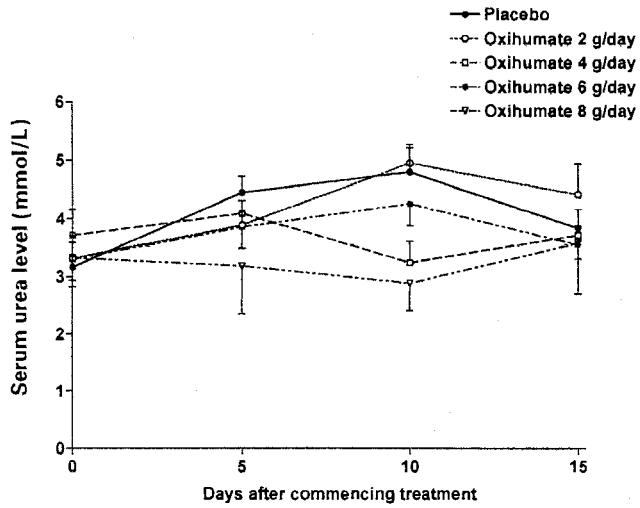


Fig. 2. Urea level of HIV-positive patients during a 2-week treatment with oxihumate.

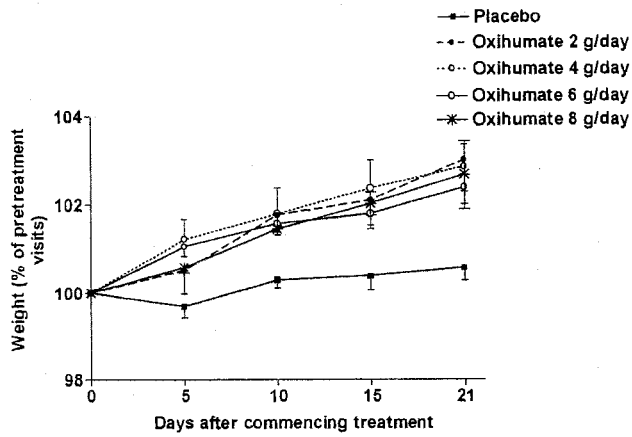


Fig. 4. Weight of HIV-positive patients on a 2-week treatment of oxihumate, expressed as a percentage of their average weight before treatment.

Preliminary Efficacy Results

Efficacy parameters were compared between treatment groups at week 2 using analysis of covariance (with baseline value for a patient as the covariant). Strong evidence ($P = 0.018$) was found that the posttreatment weight differed between treatment groups (adjusted for initial weight). All four of the active treatment groups gained weight compared to the placebo (Fig. 4). There was no clinical difference in the viral load or CD4-positive lymphocyte count between the active ingredient and the placebo (results not shown).

Intercurrent Infections

All the intercurrent infections that occurred during the study are listed in Table 2. One death occurred during the study due to *S. pneumoniae* pneumonia and septicemia.

TABLE 2. Intercurrent Infections That Occurred During the Study

Endpoint	Placebo	2g/day	4g/day	6g/day	8g/day
Oral candidiasis		1			
Pneumonia		1*			
Upper respiratory tract infection			2		
Perineal ulceration	1				

*Serious adverse event (hospital admission and death).

DISCUSSION

Oxihumate, a water-soluble humate derived from coal, increases the activity of the IL-2-producing TH_1 cells, while decreasing TH_2-associated cytokine production in vitro (data submitted elsewhere). Furthermore, it has been shown that oxihumate, as was the case for synthetic humate [Scheider et al., 1996], inhibited the infectivity of HIV particles by interference with a V3 loop-mediated step of virus entry [Van Rensburg et al., 2002]. The antiviral property, together with lack of resistant build-up [Van Rensburg et al., 2002], and the very positive results obtained with toxicity studies in animals, motivated us to proceed with a Phase I study with oxihumate in HIV-infected individuals.

Although humic acids have previously been used as a treatment in various medical conditions with proven safety [Gramsch, 1961; Reichert, 1966; Brandt, 1964; Eichelsdörfer, 1976; Soloveyva et al., 1984; Lopez-Vernandez et al., 1992], this is the first clinical study that was undertaken with oxihumate.

This is the first report regarding the safety, tolerability, and preliminary efficacy of oxihumate in patients with HIV infection. In view of the small number of patients involved, the study should primarily be considered a safety and tolerability study.

The short-term safety profile of oxihumate was established in this study without any doubt. Oxihumate had no detrimental effects on any of the biochemical or hematological parameters up to a dosage of 8 g per day for 2 weeks. A significant increase in weight was seen at all four dosage levels in the active drug group as compared to the placebo group. Larger clinical trials should be undertaken to confirm these very preliminary, yet encouraging, results. However, no change in either viral load or CD4-positive lymphocyte count was observed. An expanded study involving an increase in patient numbers with a longer follow-up time to observe any significant changes in these markers will be necessary.

ACKNOWLEDGMENTS

The authors thank Dr. D. Martin of Toga Laboratories, Edenvale, South Africa, for advice during the study and in the writing of the manuscript, and Dr. J. Levin of the Statistical Department of the Medical Research Council of South Africa in Pretoria for statistical analyses. Without the participation and cooperation of the patients, the study would not have been possible.

REFERENCES

Bergh JJ, Cronje IJ, Dekker J, Dekker TG, Gerritsma LM, Mienie LJ. 1997. Non-catalytic oxidation of water-slurried coal with oxygen: identification of fulvic acids and acute toxicity. Fuel 76:149–154.

Brandt H. 1964. Die Behandlung degenerativer Erkrankungen der Wirbelsäule und der Gelenke mit salizylierten Huminsäurebädern. Fortschr Med 82:110.

Eichelsdörfer D. 1976. Moor in der Heilkunde. In: Göttlich K, editor. Moor- und Torfkunde. Stuttgart: Schweizerbart.

Gramsch H. 1961. Ein Beitrag zur Behandlung der Gastropathien. Med Monatsschr 15:658–687.

Klöcking R, Sprössig M, Witzler P, Thiel KD, Helbig B. 1983. Antiviral wirksam Huminsäureähnliche Polymere. Z Physiother 33:95–101.

Lopez-Fernandez MF, Blanco-Lopes MJ, Castineira MP, Batlle J. 1992. Further evidence for recessive inheritance of Von Willebrand disease with abnormal binding of Von Willebrand factor to factor VIII. Am J Hematol 40:20–27.

Reichert B. 1966. Huminsäuren und ihre Derivate in der modernen Therapie. Dtsch Apoth 18:204–206.

Scheider J, Weis, R, Manner C, Kary B, Werner A, Seubert BJ, Riede UN. 1996. Inhibition of HIV-1 in cell culture by synthetic

humate analogues derived from hydroquinone: mechanism of inhibition. Virology 218:389–395.

Soloveyva VP, Lotosh TD. 1984. Biologically active peat substances-body resistance stimulators. Proc 7th Int Peat Congress, Dublin, 4. p 420–434.

Thiel KD, Klöcking R, Schweizer H, Sprössig M. 1977. Untersuchungen in vitro zur antiviralen Aktivität von Ammoniumhumat gegenüber Herpes simplex-Virus Typ I und Typ 2. Zbl Bakteriol Parasiten kd Infekt Kr u Hyg Abt 1 Org A 301–321.

Van Rensburg CEJ, Dekker J, Weis R, Smith T-L, Jansen van Rensburg E and Schneider J. 2002. Investigation of the anti-HIV properties of oxihumate. Chemotherapy 48:138–143.

Visser SA. 1973. Some biological effects of humic acids in the rat. Acta Biol Med Germ Band 31:569–581.

US005284651A

United States Patent [19]

Riede et al.

[11] Patent Number: 5,284,651

[45] Date of Patent: Feb. 8, 1994

[54] HUMINATES, PROCESS AND USES

[75] Inventors: Urs N. Riede, Freiburg; Bernhard Seubert, Edingen, both of Fed. Rep. of Germany

[73] Assignee: Rutgerswerke Aktiengesellschaft AG, Fed. Rep. of Germany

[21] Appl. No.: 960,898

[22] Filed: Oct. 14, 1992

[30] Foreign Application Priority Data

Oct. 17, 1991 [DE] Fed. Rep. of Germany 4134379

[51] Int. Cl.[5] A61K 31/74; C08G 65/38; C07G 17/00

[52] U.S. Cl. 424/78.06; 528/212; 528/219; 522/47; 522/181

[58] Field of Search 424/78.06; 514/734, 514/730; 522/47, 181; 528/212, 219

[56] References Cited

U.S. PATENT DOCUMENTS

4,921,840 5/1990 Seubert et al. 514/33
4,942,181 7/1990 Riede et al. 514/730

OTHER PUBLICATIONS

Chem. Ab. vol. 104: 6219r.
Chimika Chronika New Series, 1, 203–209 (1972).
Chem. Ab. vol. 103: 98707d.
Chem. Ab. vol. 105: 96590c.

Primary Examiner—Thurman K. Page
Assistant Examiner—Peter F. Kulkosky
Attorney, Agent, or Firm—Bierman and Muserlian

[57] ABSTRACT

Physiologically active, non-toxic, non-teratogenic and non-mutagenic huminates produced by oxidation of polyvalent phenols in aqueous alkaline medium at a reaction temperature under 40° C., the pH value of the reaction medium always being over 9.0 during the oxidation and the supply of oxidant being proportioned so that the content of the quinones corresponding to the polyvalent phenols is always less than 0.5%, based on the polyvalent phenols used, and that the oxidation reaction is stopped when the quinone concentration decreases despite oxidant supply.

5 Claims, No Drawings

HUMINATES, PROCESS AND USES

STATE OF THE ART

U.S. Pat. No. 4,914,059 describes alkali metal and ammonium salts of humic acids of low molecular weight as having a wound healing action, and that the said huminates, in contrast to humins previously known, or respectively their salts, are much less toxic. These low-molecular weight humins can either be isolated from naturally occurring humins or produced synthetically as taught in U.S. Pat. No. 4,921,840 in which polyvalent phenols are oxidized in alkaline solution with the pH of the reaction mixture of 8.8 to 9.0. This reaction condition ensures that only low-molecular weight huminates are formed whose mean molecular weight is about 1000 with a range of 300 to 1500. Only these huminates are regarded to be physiologically active but non-toxic, non-mutagenic and non-teratogenic.

Adjusting and maintaining a pH value in the narrow range of 8.8 to 9.0 and the pH value in the range of 6.2 to 7.2 to be adjusted during processing are unfavorable inasmuch as the buffer capacity of aqueous solutions is extremely low in the range from pH 6.0 to 9.0, so that due to small changes in the concentration of acid or base, pH values of the reaction medium occur which lie outside the specified range.

OBJECTS OF THE INVENTION

It is an object of the invention to provide a simple and easy-to-control process for the preparation of physiologically active, non-toxic, non-mutagenic huminates and the huminates produced thereby.

It is another object of the invention to provide novel methods of treating various conditions.

These and other objects and advantages of the invention will become obvious from the following detailed description.

THE INVENTION

The novel process of the invention for the production of physiologically active, non-toxic, non-teratogenic and non-mutagenic huminates comprises oxidizing polyvalent phenols in an aqueous alkaline medium at a pH greater than 9.0 and at a temperature less than 40° C. while regulating the oxidant to maintain the amount of quinones to less than 0.5% based on the polyvalent phenols, stopping the oxidation when the quinon concentration begins to decrease and recovering the huminate.

Contrary to the prior art belief, the desired physiological properties of a medicament with low toxicity and non-mutagenicity

Result

In basin a, a mortality rate of 16% (Guppy) or respectively 50% (red phantom salmon fish) and a generally poor vitality of the fish was observed. In basin b, the mortality had fallen to 10.5% and 19%, respectively, and the vitality of the surviving fish was moderate. In basin c, the mortality was 5% and by and large, the vitality was good.

Detoxification of water

The toxicity of the huminate produced in the example was determined by Daphnia magna (test according to OECD guidelines). The LC_{50} designated the concentration of a substance in water which after the stated time was lethal in 50% of the tested animals. The LC_{50} of the huminate of the invention was 2,700 mg/1000 ml.

	Action in contaminated water			
Contaminant	Amount of contaminant	Quant. of huminate	Survival time in hours	LC_{50} mg/1000 ml
Cadmium	1 mg/lt	0 ppm	0.2	0.5
chloride		20 ppm	3	2.5
Lead acetate	5 mg/lt	0 ppm	0.5	2.5
		20 ppm	6	7.5
Mercury	3 mg/lt	0 ppm	0.5	0.5
1-chloride		20 ppm	1.5	2.8
Parathion	4 mg/lt	0 ppm	0.2	0.5
		20 ppm	24	10.0
Atrazine	4 mg/lt	0 ppm	0.5	2.0
		20 ppm	4.5	10.0

or teratogenicity of humins, it is not the molecular weight range of 300 to 1500 that is necessary for their activity but other criteria.

It has now been found that additional huminates of higher molecular weight, i.e. up to about 50,000 D, also possess the healing properties with very low toxicity and non-mutagenic and non-teratogenic properties. The common characteristic of these desired huminates is that their aqueous solutions show no Tyndall effect and do not fluoresce. Tests for these properties are carried out with aqueous solutions at a concentration wherein they still have about 50% transmission of the irradiated light.

The huminates may be simply produced by oxidation of polyvalent phenols in an aqueous alkaline medium at temperatures below 40° C. if the pH of the reaction medium is always more than 9.0 and the amount of oxidant is regulated so that the amount of the quinones corresponding to the polyvalent phenols is always less than 0.5% based on the polyvalent phenol used and then stopping the oxidation when the quinone concentration decreases despite the supply of oxidant. Preferably, the oxidation is halted when the quinone concentration falls below 0.5% by weight based on the polyvalent phenol.

Examples of suitable starting materials are all known polyvalent phenols such as pyrocatechol, resorcinol, hydroquinone, orcinol, gallic acid, protocatechuic acid, pyrogallol, 2-oxyhydroquinone, phloroglucinol or tetraoxybenzoles. The polyvalent phenols may be used in pure form or as mixtures thereof. The preferred starting material is hydroquinone.

Preferably, the polyvalent phenols are dissolved in aqueous alkaline solution and examples of suitable bases are alkali metal hydroxides or carbonates, ammonia and strong amines, preferably sodium hydroxide or potassium hydroxide for economical reasons. The amount of the base should be more than 1.6 times the stoichiometric amount necessary for phenolate formation of the polyvalent phenols to ensure neutralization of all the phenolic hydroxy groups. The reaction medium will then have a pH greater nine.

The alkalinity of the reaction solution may be greater but since the reaction medium has to be neutralized for processing, it is preferred for economical reasons not to use too highly an alkaline solution. It is preferred to keep the pH slightly greater than 9 and to add more base during the reaction to prevent a decreae in the pH value.

Obviously, the compounds used should be as pure as possible to avoid undesired secondary reactions, or

respectively, the end products can be obtained without any further chemical processing which, too, would involve undesired chemical modifications of the end products and can be used in the reaction medium obtained. Generally it is sufficient to use as water, demineralized water of a conductivity of 6 to 10/μS/cm and a pH value in the range from 5 to 7, and as the alkali metal hydroxide either a "chemically pure" grade or of the purity per DAB of 9. As polyvalent phenol, likewise a "chemically pure" grade of a polyvalent phenol content of more than 98% should be used.

The oxidation of the polyvalent phenol can occur either electrochemically or plasmachemically, or respectively chemically by causing oxygen or an oxygen-containing gas mixture to pass over or through the reaction mixture. The electrochemical oxidation occurs in an electrochemical reaction, with the rate of oxidation being determined by setting the anodic voltage and the current density. The anodic voltage can be varied in the range from 4 to 16 volts and the current density in the range from 0.5 to 4 A/cm^2. The reaction time is then in the range of 1 to 3 days.

The plasmachemical oxidation occurs in an apparatus known per se for corona discharge with the oxidation rate being determined by setting the operating voltage and the field strength. The voltage may be varied in the range of 20 to 250 kv at frequencies of 16⅔ to 400 Hz, and the field strength from 80 kv/cm to 200 kv/cm. The reaction time is then in the range of 15 to 1000 minutes.

For chemical oxidation, the alkaline solution of the polyvalent phenolate is placed in a reaction vessel which prevents uncontrolled access of air, that is, in a closed reaction vessel, but which is provided with a device for letting gases in or through. While stirring, oxygen or an oxygen-containing gas mixture is passed in a continuous gas stream either over or through the reaction solution or is conducted onto the reaction solution under pressure, the temperature of the reaction mixture being in the range from 10° to 40° C., preferably from room temperature to 30° C.

As oxygen-containing gas mixture, air can be used which, however must first be conducted over an alkaline filter for adsorption of CO_2 and for the removal of dust particles. The chemical oxidation can occur also by reaction with mild oxidants such as hydrogen peroxide, its addition compounds, or persulfates.

During the oxidation reaction, the pH value of the reaction mixture and the content of quinone corresponding to the polyvalent phenol in the reaction mixture are monitored. As soon as one of he two parameters is fallen short of or exceeded, the reaction must be stopped. The oxidation reaction is controlled through the amounts of oxidant used, the term oxidant to be understood, depending on the process employed, as chemical reagent or as applied voltage and current stength.

As a rule, the reaction time is 10 to 15 days. The reaction is terminated, if despite additional supply of oxidant, the quinone content decreases, preferably when the quinone concentration has fallen to a value below 0.05%, referred to the polyvalent phenols used.

Thereafter, the reaction mixture is acidified in a manner known in itself to a pH value in the range from 4 to 5, and is processed by methods known in themselves, as e.g. ultra-centrifugation, ultrafiltration or electrophoresis. Thus, for example, the dark brown solution resulting from the chemical, plasmachemical or electrochemical oxidation reaction is adjusted to a pH in the range from 4 to 5, preferably 45 and possibly buffered. This is done either by addition of acid or by action of acid ion exchanger and/or subsequent addition of a corresponding buffer solution.

If the neutralized and buffered solution contains undesired suspended substances, these substances are removed by a separating process such as centrifuging (10,000 to 30,000×g). Although for many applications, this solution can be used directly, it should be purified and freed of undesired secondary products by purification processes known in themselves, such as preparative chromatography methods, ultrafiltration, ultra-centrifugation or electrodialysis, if the products are used as medicaments.

Thereafter, the brown solution can be concentrated to 3 to 5% huminate (to about 40% huminate) or dried by means of a suitable dryer to a residual moisture of 2%. By drying, a readily crumbling, crystalline, hygroscopic substance of a lustrous black color is obtained. The dilute as well as the concentrated solution and also the solid obtained are stable.

In stability tests, after 60 days of load cycles 66/4° C. in the 12/12 hour rhythm, no alteration of the parameters content, pH value, redox potential and microdialysis test beyond accidental fluctuations is observable. Toxicity tests show that the huminates are little toxic, but it was found that the huminates produced also have the same healing actions and detoxifying effects observed in the mentioned low-molecular weight humins.

The huminates of the invention are therefore suitable for all applications that are known of the natural or synthetic low-molecular weight humins. In particular, they are useful as wound medicaments, for the preparation of wound-healing products, for the production, of highly effective moor baths, nose medicines against pollen allergies, anti-dandruff shampoos, as well as agents for the treatment of fishes, particularly fish under stress. In addition, they are suitable for detoxification of water as well as of surfaces or solid objects contaminated with cholinesterase-inhibiting substances of the organophosphate type and/or with dermatotoxic substances of the dichlorodiethylsulfide type.

In the following examples, there are described several preferred embodiemnts to illustrate the invention. However, it is to be understood that the invention is not intended to be limited to the specific embodiments.

EXAMPLE

95 kg of potassium hydroxide (DAB 9 quality) were dissolved in 1,000 liters of demineralized water with a pH of 5 to 7 and a conductivity under 5 μS/cm in an alkali-resistant vessel. After cooling the solution to below 40° C., 55 kg of hydroquinone 99% chemical purity) were added thereto over 45 minutes with cooling to ensure the temperature did not exceed 40° C. The pH adjusted itself to about 10. The vessel was closed and air purified of dust, carbon dioxide and other contaminants through an alkaline filter system was passed over the surface of the stirred mixture. The air stream was regulated so that the content of 1,4-benzoquinone was always less than 0.5% by weight following LD_{50} values were obtained: based on the hydroquinone added. The reaction temperature was always below 40° C. and the pH was always above 9.

After 10 to 15 days, the hydroquinone content of the brown reaction solution dropped to below 2% and despite the further supply of air, the content of 1,4-benzoquinone fell to values below 0.05%. At this moment,

the reactin was stopped and the supply of air was halted. The reaction mixture was adjusted to a pH value of 4.5 by addition of an acid ion exchanger which was separated by centrifuging. The acid solution was subjected to ultrafiltration, using a filtration device of 0.5/yum purity and the ultrafiltrate was concentrated under a weak vacuum to a huminate concentration of 40% by weight. The solution can still be handled easily and contained huminates with molecular weights in the range of 1,000 to 50,000. The solution did not show a Tyndall effect and contained no fluorescing components.

Toxicity

When test mice were injected with the 1% solution, the following LD_{50} values were obtained:

1370 mg/kg subcutaneously 920 mg/kg intraperitoneally

840 mg/kg intraveinously

Stability

No changes were observable after storage for 6 months under exclusion of air at 23° C.

Healing effects

A culture of L cells (mouse fibroblasts) suspended after treatment with thrypsine was mixed with 50 ppm of huminate and the culture was incubated for 48 hours at 37° C., using a commercial culture medium. At the same time, a similar culture without huminates was incubated in the same manner as a control. Then, the number of living cells was determined in both cultures. In the culture mixed with huminate of the invention, the number of living cells was 30% higher than in the control culture.

Healing of wounds

On 2 groups of 10 hairless mice, superficial wounds affecting only the topmost epithelium layers were inflicted with a size of about 50 mm^2, using a microdermatome. In ten of these mice, the wound was wetted once with a 1% huminate solution and the other 10 mice remained untreated. During the observation period of 7 days, the following were observed: As compared with the untreated mice, the wound area decreased quicker in the treated test animals and the wound dried up earlier, the granulation set in earlier, and the wound became clean earlier. On the whole, healing was observable 2 to 3 days earlier than in the control animals.

Action in stressed fish

Groups of 50 fish of different species were placed after a transport into three different basins, each containing 70 liters and they were observed and compared for 7 days. The conditions of keeping and feeding were the same in each instance. Only the water, identical in principle, had been changed by additions: Basin a: no addition; Basin b: 20 ml (prescribed dose) of a commercial humic acid-containing prophylactic; and Basin c: 3 ml of a 2% aqueous solution of the huminate of the invention. After 2 days, an additional 2 ml of the 2% solution were added.

Activity as nose medicine

A test group of 10 persons suffering from allergic reactions caused by pollen, especially in the nose and eye region, used a 0.5% aqueous solution of the huminate of the example as a nose spray by spraying a dose of the solution into the nose when the hay fever symptoms appeared, using a conventional bottle as normally used for nose sprays. The discomforts diminished in all patients within minutes. Their noses became free, the sneeze irritation disappeared and the swelling up of the eyes was reduced. Undesirable side effects were not observed even after prolonged use of the product. Six of the test persons reported that after using the product for several days, the number of necessary applications was substantially reduced. On days of low pollen count, these patients were without discomfort even without the nose spray of the invention.

Action of the anti-dandruff product

To 100 ml portions of a commercial shampoo, there were added 50 ml of a 5% aqueous solution of the huminate of the above example. Various test persons suffering from dandruff used one of the dilute shampoos they had obtained twice with 5 ml each for their daily hair and scalp wash. The results were the same in all cases: After a period of application of about one week, the dandruff problem was no longer acute. Evem after using the shampoo for several weeks, no increase of sebaceous gland activities was observable on the scalp. In some cases, a reduction of the hair greasing was observed subjectively.

Various modifications of the compositions and method of the invention may be made without departing from the spirit or scope thereof and it is to be understood that the invention is intended to be limited only as defined in the appended claims.

What we claim is:

1. In a process for the production of physiologically active, non-toxic, non-teratogenic and non-mutagenic huminates by oxidation of polyvalent phenols in alkaline, aqueous medium at a reaction temperature under 40° C. and subsequent isolation, the improvement comprising the pH of the reaction medium is always over 9.0 during the oxidation and the supply of the oxidant is proportioned so that the content of the formed quinones corresponding to the polyvalent phenol is always less than 0.5% based on the polyvalent phenols used, and that the oxidation reaction is stopped when despite the supply of oxidant, the quinone concentration decreases, the aqueous solutions of the huminate show no Tyndall effect and do not fluoresce.

2. The process of claim 1 wherein the oxidation reaction is stopped when, despite the supply of oxidant, the quinone concentration has dropped below 0.05%, based on the polyvalent phenols used.

3. The process of claim 1 wherein hydroquinone is used as the polyvalent phenol.

4. Huminate produced by the process of claim 1.

5. A method of increasing wound healing comprising applying to a wound of a warm-blooded animal a wound healing amount of a huminate of claim 4.

* * * * *

PHARMACOKINETICS OF SODIUM HUMATE IN CHICKENS

J. Hampl[1], I. Herzig[1], J. Vlček[2]

[1]*Veterinary Research Institute, Brno*
[2]*Pharmaceutical Faculty, Charles University, Hradec Králové*

Free or liposome-encapsulated sodium humate was administered to chickens intracardially, orally or subcutaneously and the following principal pharmacokinetic parameters were determined using the MW Pharm software for two-compartment models: elimination half-life (t 1/2 el), steady state distribution volume (Vd_{ss}), blood clearance (Cl), maximal drug concentration (C_{max}), time required for appearance of C_{max} (t_{max}), area under the curve (AUC) and bioavailability (F). Blood clearance of liposome-encapsulated sodium humate was higher than that of free sodium humate regardless of the way of administration. On the other hand, the elimination half-life was longer after the extravascular than after the intracardial administration. C_{max} values indicate that the penetration of sodium humate from the injection site into blood circulation is very slow. Biological availability of sodium humate also depended on the way of administration and dosage form. Aside from the intracardial administration, the highest bioavailability was found after subcutaneous administration of free sodium humate.

^{125}I labelled sodium humate; intracardial application; oral application; subcutaneous application; distribution volume; elimination half-life; clearance; bioavailability; liposomes

Growing interest in the application of humins in biology and medicine has become apparent in recent years. Natural and synthetic humic acids are characterized by a set of properties including antiviral, profibrinolytic, anti-inflammatory and oestrogenic activities and production of chelate compounds with heavy metals (K l ö c k i n g , 1992). Favourable effects of humic acids and their salts are explained by the enhancement of metabolic cell wall activity, particularly by acceleration of oxidations which can result in stimulation of biological processes provided that the supply of nutrients into the extracellular compartment is adequate.

* This work has been supported by grant No. 322/3110/02/93 from the Ministry of Industry and Trade of Czech Republic.

Humic acids are weak organic acids with chelate structure, binding with a number of toxic compounds to form insoluble and unabsorbable complexes which are eliminated with faeces (Kühnert, 1982). The discovery of the binding capacity of humic acids instigated a number of studies (Beveridge and Pickering, 1980; Marinsky et al., 1982; Herzig et al., 1992) focused on the reduction of the content of heavy metals (e.g. cadmium) in or their elimination from water sediments and live organisms.

One of the ways of reducing the heavy metal content in live organisms is the administration of chelating agents specific for heavy metals. Frequently used for this purpose are for instance EDTA, DTPA (diethylene triamine pentaacetic acid), DF (desferrioxamine) and HBDE (N-N' -bis/2-hydroxybenzyl/-ethylene diamine-N, N'-diacetic acid). Such substances can be encapsulated into liposomes as a biologically degradable excipient (Rahman et al., 1973, 1988; Rosenthal et al., 1975; Guilmette et al., 1978; Blank et al., 1988; Herzig et al., 1994).

The aim of our investigations of principal pharmacokinetic characteristics of free and liposome-encapsulated sodium humate in chickens was to recognize its prospective applications.

MATERIALS AND METHODS

PREPARATION OF LIPOSOMES AND ENCAPSULATION OF SODIUM HUMATE

Multilamellar liposomes (MLV) were prepared from egg phosphatidyl choline (PC), cholesterol (C) and stearyl amine (SM) at the molar ratio 0.45 : 0.45 : 0.1 as described by Kirby and Gregoriadis (1984).

Sodium humate labelled with ^{125}I by the oxidation method with chloramine T (Hunter and Greenwood, 1962) was used to determine the rate of encapsulation into liposomes (Hampl et al., 1992) and also as a tracer for the determination of pharmacokinetic parameters in chickens. Radioactivity was measured using the Nuclear Enterprises NE 1600 counter. Freeze-dried liposomes were rehydrated always immediately before administration.

STABILITY OF ENCAPSULATION

The stability of encapsulation of sodium humate and tracer was checked during a 9-day period. MLV were suspended in chicken blood serum preserved with 0.01% of sodium azide at 24 °C, separated into a pellet by ultracentrifugation (Beckman, rotor SW 55 Ti, 40 000 r.p.m. for 30 min) at pre-set intervals, and radioactivity was measured in aliquots of the supernatant. Then the aliquot was returned to the supernatant and the liposome pellet was resuspended.

EXPERIMENTS IN CHICKENS

Sixty day-old sexed meat-type Hybro chicks were selected for the experiment. The birds were divided into six equal groups and the treatment began when their mean liveweight reached 800 g.

Treatment scheme:

Group 1 - intracardial administration of free sodium humate
Group 2 - intracardial administration of liposome-encapsulated sodium humate
Group 3 - oral administration of free sodium humate
Group 4 - oral administration of liposome-encapsulated sodium humate
Group 5 - subcutaneous administration of free sodium humate
Group 6 - subcutaneous administration of liposome-encapsulated sodium humate

Each bird in each group received 500 μg of sodium humate with the radioactivity $3.5.10^6$ cpm. Blood samples were collected from *v. ulnaris* 3 (only Groups 1 and 2), 30, 60 and 120 min, 4, 10, 24, 48 and 72 h, and 5, 7 and 9 (only Groups 2, 4 and 6 at the latter term) days after the administration and radioactivity was measured in 0.2 ml volumes.

CALCULATION OF PHARMACOKINETIC PARAMETERS

Principal pharmacokinetic parameters were determined by non-linear regression of a two-compartment model using the MW Pharm software. Regression curves were made up from mean values of blood radioactivity in groups of 3 to 10 chickens. The pharmacokinetic behaviour of sodium humate was characterized by the following values:

– elimination half-life (t 1/2 el)
– steady state distribution volume (Vd_{ss})
– blood clearance (Cl)
– maximal drug concentration (C_{max})
– time required for appearance of C_{max} (t_{max})
– area under the curve (AUC)
– bioavailability (F)

RESULTS

Stability tests demonstrated that the liposomal phospholipid bilayer was damaged and ^{125}I labelled sodium humate was released into the incubation medium as soon as the liposomes came into contact with blood serum. The large part of the encapsulated drug (24.3%) was released within the first 45 min, but the subsequent release was very slow (Tab. I).

Principal pharmacokinetic parameters of sodium humate after single intracardial, oral and subcutaneous treatments are given in Tab. II.

I. Release of sodium humate from liposomes during incubation in chicken blood serum

	0 min	45 min	4 hours	1 day	2 days	6 days	9 days
Percentage of encapsulated sodium humate	100.0	75.7	72.5	71.6	71.0	68.6	68.3

II. Pharmacokinetic behaviour of a single dose of sodium humate in chickens

Parameter		Administration					
		intracardial		oral		subcutaneous	
		HuNa	LHuNa	HuNa	LHuNa	HuNa	LHuNa
t 1/2 el	(hours)	14.8	27.0	78.1	76.2	83.0	85.5
Vd_{ss}	(ml/pc)	1 834	4 669	9 674	12 663	8 735	14 805
Cl	(ml/min)	1.4	2.0	1.4	1.9	1.2	2.0
C_{max}	(cpm/ml)	-	-	1 755	1 114	1 985	305
t_{max}	(min)	-	-	39	95	33	88
AUC_{0-7}	(min/cpm/ml)	23.8	17.0	7.1	7.0	17.2	8.3
F	(%)	100.0	100.0	29.8	41.3	72.3	49.2

HuNa = free sodium humate
LHuNa = liposome encapsulated sodium humate

The data show a considerable difference in elimination half-lives between the intracardial and both extravascular treatments. Similar values were found for oral and subcutaneous treatments, although those for both dosage forms were somewhat longer in the latter.

Vd_{ss}, as a parameter characterizing two-compartment models, was highest in birds treated subcutaneously with liposome-encapsulated humate and lowest in those receiving intracardial doses of free humate. Vd_{ss} and Cl were higher in birds treated with liposome-encapsulated humate irrespective of the way of administration. F of free sodium humate was higher after subcutaneous than after oral treatment. F of subcutaneously administered liposome-encapsulated sodium humate was higher than those of any of the orally administered dosage forms.

DISCUSSION

No data concerning pharmacokinetics of free or liposome-encapsulated sodium humate in chickens were found in the available literature.

MLV used in our experiments had a positive surface charge and the rate of encapsulation of sodium humate was higher than in chargeless or negatively charged liposomes (H a m p l et al., 1992). The labelling of sodium humate with

^{125}I was very stable as documented by the results of radiochromatographical analyses carried out during 35 days of incubation in chicken blood serum and PBS (H a m p l et al., 1992).

Approximately 25 per cent of liposome-encapsulated sodium humate were released during the first 45 min of incubation in chicken blood serum. The tests were performed at room temperature, because turbidity is known to develop in blood serum when incubated at the physiological temperature (40 to 42 °C) (S e n i o r et al., 1991). It is evident from our results that a part of the liposome--encapsulated sodium humate was released and its distribution and elimination behaviour was identical with that of free humate. This statement applies particularly to intracardial administration.

Intravenously administered liposomes are rapidly catched up by RES cells, particularly in the liver, spleen and bone marrow (L i u et al., 1992; M o g h i m i et al., 1991). The rate of catching depends on the size, surface charge, amount and phospholipid composition of liposomes (I l l u m et al., 1982; S e n i o r et al., 1985; K a t o et al., 1993).

The objective of the subcutaneous administration was to produce a depot from which liposomes and the encapsulated sodium humate would be slowly released (P e r e z - S o l e r et al., 1985). Low C_{max} values are an evidence that this objective was fulfilled in our experiments. As stated by K a d i r (1993), liposomes are exposed to enzymatic digestion at the site of subcutaneous administration, and interactions of lipids of the phospholipid bilayer with endogenous lipids and proteins, various types of liposome-cell interactions (absorption, phagocytosis, fusion) and other events result in destabilization of liposomes.

Liposome size is an important factor associated with their penetration ability (A l l e n et al., 1993). Liposomes with a diameter not exceeding 120 nm penetrate from the injection site into lymphatic and blood vessels and accumulate in lymph nodes and other tissues (P e r e z - S o l e r et al., 1985). Larger liposomes, such as MLV used in our experiments, remain at the injection site. This may be an explanation for the lower biological availability (F) of subcutaneously administered liposome-encapsulated sodium humate.

Oral administration is a simple method and liposome-encapsulated substances are protected from the action of proteolytic enzymes of the gastrointestinal tract (W e i n e r and C h i a - M i n g C h i a n g , 1988; C l a r k e and S t o k e s , 1992a, b; A r i e n et al., 1992, 1993).

Pharmacokinetic studies of the administration of a single dose of sodium humate by three different ways have shown that only approx. one third of the oral dose will reach the system, which is less than the corresponding value for a subcutaneous dose. The supply of sodium humate from the gastrointestinal tract was enhanced by its encapsulation into liposomes.

Sodium humate elimination rate, expressed by blood clearance values, was independent of the way of administration. Distribution volumes were largest after subcutaneous and lowest after intracardial administrations. The differences were probably due to different persistence of sodium humate at the injection site. Due to slowed-down release, blood concentrations were lower than those which would result from the administration of a dose corrected for biological availability after intracardial administration. Distribution volume size also depends on the rate of catching-up of liposomes by RES. Comparisons of mean group live-weights and of sizes of steady state distribution volumes allow us to conclude that sodium humate accumulates markedly in some of the compartments.

It can be concluded from the results of the 7-day experiment that subcutaneous administration of free sodium humate is the most effective way as far as its biological availability is concerned.

References

ALLEN, T. M. - HANSEN, C. B. - GUO, L. S. S.: Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection. Biochim. Biophys. Acta, *1150*, 1993: 9-16.

ARIEN, A. - GOIGOUX, C. - BASSE-CATHALINAT, B. - DUPUY, B.: Orally administered liposomes prepared with egg phosphatidylcholine and sterylamine: possible destruction in rat intestinal wall. Horm. metab. Res., *24*, 1992: 597-598.

ARIEN, A. - GOIGOUX, C. - BAQUEY, Ch. - DUPUY, B.: Study of *in vitro* and *in vivo* stability of liposomes loaded with calcitonin or indium in the gastrointestinal tract. Life Sci., *53*, 1993: 1279-1290.

BEVERIDGE, A. - PICKERING, W. F.: Influence of humate-solute interactions on aqueous heavy metal ion levels. Water, Air, Soil Pollut., *14*, 1980: 171-185.

BLANK, M. L. - BYRD, B. L. - CRESS, E. A. - WASHBURN, C. C. - SNYDER, F.: Use of DTPA in heavy metal detoxification. In: Liposomes as Drug Carriers. John Wiley and Sons Ltd., 1988: 497-506.

CLARKE, C. J. - STOKES, C. R.: The intestinal and serum humoral immune response of mice to systemically and orally administered antigens in liposomes: I. The response to liposome--entrapped soluble proteins. Vet. Immunol. Immunopath., *32*, 1992a: 125-138.

CLARKE, C. J. - STOKES, C. R.: The intestinal and serum humoral immune response of mice to orally administered antigens in liposomes: II. The response to liposome-entrapped bacterial proteins. Vet. Immunol. Immunopath., *32*, 1992b: 139-148.

GUILMETTE, R. A. - CERNY, E. A. - RAHMAN, Y. E.: Pharmacokinetics of the iron chelator desferrioxamine as affected by liposome encapsulation: potential in treatment of chronic hemosiderosis. Life Sci., *22*, 1978: 313-320.

HAMPL, J. - ŠTĚPÁNEK, J. - NEZVAL, J. - ČERNÍK, J. - ŠKROBÁK, F: Incorporation of sodium humate into liposomes and their penetration through the skin in piglets. In: Abstracts of

invited and volunteered papers „Humic substances in the global environment and implications in human health". Monopoli 1992. 332 p.

HERZIG, I. - DOČEKALOVÁ, H. - MAŠEK, J. - HAMPL, J.: Effect of humin substances on excretion of inorganic xenobiotics and digestibility of nutrients. In: Abstracts of invited and volunteered papers „Humic substances in the global environment and implications in human health". Monopoli 1992. 334 p.

HERZIG, I. - HAMPL , J. - DOČEKALOVÁ, H. - PÍSAŘÍKOVÁ, B. - VLČEK, J.: Ukládání kadmia v orgánech kuřat. při aplikaci humátu sodného. Vet. Med. - Czech, *39*, 1994: 175-185.

HUNTER, W. M. - GREENWOOD, F. C.: Preparation of iodine 131-labelled growth hormone of high specific activity. Nature, *194*, 1962: 495-496.

ILLUM, L. - DAVIS, S. S. - WILSON, C. G. - THOMAS, N. W. - FRIER, M. - HARDY, J. G.: Blood clearance and organ deposition of intravenously administered colloidal particles. The effects of particle size, nature and shape. Int. J. Pharm., *12*, 1982: 135-146.

KADIR, F.: Intramuscular and subcutaneous drug delivery: encapsulation in liposomes and other methods to manipulate drug availability. Pharm. World Sci., *15*, 1993: 173-175.

KATO, Y. - WATANABE, K. - NAKAKURA, M. - HOSOKAWA, T. - HAYAKAWA, E. - ITO, K.: Blood clearance and tissue distribution of various formulations of α-tocopherol injection after intravenous administration. Chem. Pharm. Bull., *41*, 1993: 599-604.

KIRBY, Ch. - GREGORIADIS, G.: Dehydration-rehydration vesicles - a simple method for high yield drug entrapment in liposomes. Biotechnology, *2*, 1984: 979- 984.

KLÖCKING, R.: Humic substances as potential therapeutics. In: Abstracts of invited and volunteered papers "Humic substances in the global environment and implications in human health". Monopoli 1992. 129 p.

KÜHNERT, M. - FUSCH, V. - GOLBS, S.: Pharmakologisch- toxikologische Untersuchungen an Huminsäuren unter der Zielstellung ihrer oralen Anwendung bei Magen-Darm- Erkrankungen der Nutztiere. In: Proc. Int. Symp., Minsk, 1982: 232-243.

LIU, D. - MORI, A - HUANG, L.: Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochim. Biophys. Acta, *1104*, 1992: 95-101.

MARINSKI, J. A. - GUPTA, S. - SCHINDLER, P.: The interaction of copper (II) with humic acid. J. Colloid Interface Sci., 1982: 401-411.

MOGHIMI, S. M. - PORTER, C. J. - MUIR, I. S. - ILLUM, L. - DAVIS, S. S.: Non-phagocytic uptake of intravenously injected microspheres in rat spleen: influence of particle size and hydrophilic coating. Biochem. Biophys. Res. Commun., *177*, 1991: 861-866.

PEREZ-SOLER, R. - LOPEZ-BERESTEIN, G. - JAHNS, M. - WRIGHT, K. - KASI, L. P.: Distribution of radiolabelled multilamellar liposomes injected intralymphatically and subcutaneously. Int. J. Nucl. Med. Biol., *12*, 1985: 261-266.

RAHMAN, Y. E.: Use of liposomes in metal poisonings and metal storage disease. In: Liposomes as Drug Carriers. John Wiley and Sons Ltd. 1988: 485-495.

RAHMAN, Y. E. - ROSENTHAL, M. W. - CERNY, E. A.: Intracellular plutonium: removal by liposome-encapsulated chelating agent. Science, 1980, 1973: 300-302.

ROSENTHAL, M. W. - RAHMAN, Y. E. - MORETTI, E. S. - CERNY, E. A.: Removal of polymetric plutonium by DTPA directed into cells by liposome encapsulation. Radiat. Res., *63*, 1975: 262-274.

SENIOR, J. - CRAWLEY, J. C. W. - GREGORIADIS, G.: Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection. Biochim. Biophys. Acta, *839*, 1985: 1-8.

SENIOR, J. H. - TRIMBLE, K. R. - MASKIEWICZ, R.: Interaction of positively-charged liposomes with blood: implications for their application *in vivo*. Biochim. Biophys. Acta, *1070*, 1991: 173-179.

VLČEK, J. - MACEK, K. - SUCHOPÁR, J. - RUDIŠAR, L.: Klinická farmakokinetika. Charles University Prague 1991. 61 pp.

WEINER, N. - CHIA-MING CHIANG: Gastrointestinal uptake of liposomes. In: Liposomes as Drug Carriers. John Wiley and Sons Ltd. 1988: 599-607.

Arrived on 15th March 1994

HAMPL, J. - HERZIG, I. - VLČEK, J. (Výzkumný ústav veterinárního lékařství, Brno; Farmaceutická fakulta University Karlovy, Hradec Králové):

Farmakokinetika humátu sodného u kuřat.

Vet. Med. - Czech, *39*, 1994 (6): 305-313.

U šesti skupin kuřat byly stanoveny základní farmakokinetické parametry humátu sodného ve volné formě nebo enkapsulovaného do liposomů, a to po intrakardiální, perorální a subkutánní aplikaci.

Velké multilamelární liposomy byly připraveny metodou dehydratace-rehydratace. Ke stanovení účinnosti enkapsulace humátu sodného byl jako radioindikátor použit humát sodný značený ^{125}I. Každému kuřeti bylo aplikováno 500 μg humátu sodného buď ve volné formě nebo v liposomech, a to vždy s konstantní aktivitou radioindikátoru. Z naměřených hodnot radioaktivity krve odebírané v určených intervalech byly pomocí software MW Pharm vypočteny tyto farmakokinetické parametry: poločas eliminační fáze (t 1/2 el), distribuční objem za ustáleného stavu (Vd_{ss}), krevní clearance (Cl), maximální koncentrace aplikované látky (C_{max}) a doba potřebná k jejímu dosažení (t_{max}), plocha pod křivkou (AUC) a biologická dostupnost aplikované látky (F).

Testováním stability liposomů s enkapsulovaným humátem sodným inkubací v kuřecím séru bylo prokázáno poškození liposomální fosfolipidní dvojvrstvy a tím uvolňování enkapsulovaného humátu sodného do média. Tato ztráta má jen omezený rozsah.

Farmakokinetická analýza po podání humátu sodného třemi aplikačními cestami ukázala, že po perorálním podání se do systému dostává pouze asi 1/3 podaného množství, přičemž podání v liposomální formě přísun humátu sodného z gastrointestinálního traktu zvýšilo. Způsob podání neovlivňuje rychlost eliminace humátu sodné-

ho vyjádřenou hodnotami krevní clearance. Po podání liposomálních preparátů byly zaznamenány vyšší hodnoty clearance. Způsob podání naopak ovlivnil délku eliminačních poločasů, které jsou u extravaskulárních podání delší.

Vyšší distribuční objem za ustáleného stavu po perorálním podání než po intrakardiálním a nejvyšší po subkutánní aplikaci je zapříčiněn dlouhodobým setrváváním humátu sodného v místě aplikace, resp. hromaděním v některém kompartmentu. Při srovnání obou extravaskulárních aplikací byla maximální koncentrace (C_{max}) dosažena rychleji při podání volného humátu sodného.

Hodnoty AUC a z nich vyplývající biologická dostupnost (F) v průběhu sedmi dní po aplikaci byla z obou extravaskulárních způsobů podání vyšší po subkutánní aplikaci volného humátu sodného. Dostupnost humátu sodného aplikovaného subkutánně v liposomální formě je vyšší než dostupnost z obou druhů preparátů v případě podání *per os*.

^{125}I humát sodný; aplikace intrakardiální, perorální, subkutánní; poločas eliminace; distribuční objem; clearance; biologická dostupnost; liposomy

Contact Address:

RNDr. Jaroslav H a m p l , Výzkumný ústav veterinárního lékařství, Hudcova 70, 621 32 Brno, Czech Republic
Tel.: 05 41321241, fax 05 41211229

HealthGate Document

Record from database: MEDLINE

Title
[Penetration kinetics of potential virustatics into the human skin]
Original Title
Penetrationskinetik eines potentiellen Virustaticums in die menschliche Haut.
Author
Wohlrab W; Helbig B; Klöcking R; Sprössig M
Source
Pharmazie, 39: 8, 1984 Aug, 562-4
Abstract
Caffeic acid oxidation product (KOP), a substance effective against various human viruses, penetrates quickly from a 1% W/O-emulsion into the skin and forms a reservoir in the horny layer. In the epidermis and dermis approximately 30 min after external application KOP concentrations of 1 to 3% of the applied total quantity are achieved, which remain nearly unchanged even after longer penetration time. In addition to references for therapy derivable from this the results permit to draw conclusions about drug resorption that can be proved in animal experiments under in-vivo conditions.
Language of Publication
German
Unique Identifier
85064066

MeSH Heading (Major)
Antiviral Agents [*ME]
Caffeic Acids [*ME]
Cinnamates [*ME]
Skin Absorption
MeSH Heading
Emulsions
English Abstract
Human
Kinetics

Publication Type
JOURNAL ARTICLE
ISSN
0031-7144
Country of Publication
GERMANY, EAST
RAS Registry/EC Number
0 (Antiviral Agents)
0 (Caffeic Acids)
0 (Cinnamates)

ACCESSION NUMBER: **113:65270** CA
TITLE: Topical agent for treatment of vesicular rash caused by herpes virus
INVENTOR(S): Wagner, Hermann
PATENT ASSIGNEE(S): Fed. Rep. Ger.
SOURCE: Ger., 2 pp.
CODEN: GWXXAW

	NUMBER	DATE
PATENT INFORMATION:	DE 3830333 C1	900315
APPLICATION INFORMATION:	DE 88-3830333	880907

DOCUMENT TYPE: Patent
LANGUAGE: German

AB A topical agent for transdermal treatment of a herpesvirus-induced vesicular rash contains colloidal S, K2S or Na2S, humic acid or its salts, and/or (an ext. of) peat soil. Thus, a therapeutic agent contained, per 100 mL liq. phase, K2S or Na2S 1.0, humic acid 2.4, colloidal S 1.4, NaCl 2.2 g, preservatives, and perfumes.

(19) BUNDESREPUBLIK DEUTSCHLAND

DEUTSCHES PATENTAMT

(12) **Patentschrift**

(11) **DE 3830333 C1**

(51) Int. Cl. 5:
A61K 33/04
A61K 31/185
A61K 35/10

(21) Aktenzeichen: P 38 30 333.7-41
(22) Anmeldetag: 7. 9. 88
(43) Offenlegungstag: —
(45) Veröffentlichungstag der Patenterteilung: 15. 3. 90

DE 3830333 C1

(73) Patentinhaber:
Wagner, Hermann, 7450 Hechingen, DE

(72) Erfinder:
gleich Patentinhaber

(56) Für die Beurteilung der Patentfähigkeit in Betracht gezogene Druckschriften:

CH 6 60 971
FR 21 28 187

(54) Pharmazeutikum zur Behandlung des durch Herpes Viren verursachten Bläschenausschlages

Zur transdermalen Therapie der Bläschenkrankheit werden Pharmazeutikas eingesetzt, die in einer der üblichen pharmazeutischen Grundlagen, wie Creme, Salbe, Lösung oder Puder, feinteiligen Schwefel enthalten.
Zur Erhöhung der Wirksamkeit schlägt die Erfindung vor, den kolloidalen Schwefel enthaltenden Pharmazeutikas zusätzlich Kal.- bzw. Na-Sulfurat sowie Huminsäure bzw. entsprechende Anteile an Moorerde bzw. Moorextrakt zuzufügen.
Die transdermale Anwendung dieses erfindungsgemäß aktivierten Therapeutikums bewirkt neben einer raschen Abheilung eine wesentliche Verzögerung des Wiederauftretens der Herpesbläschen.

DE 3830333 C1

Beschreibung

Gegenstand der Erfindung ist eine pharmazeutische Zusammensetzung, die bei externer Anwendung bei der Behandlung des im Gefolge einer Herpesinfektion entstehenden Bläschenausschlages bemerkenswert gute Erfolge aufweist.

Die nach einer Infektion mit Herpesviren, Herpes simplex oder Variella Zoster Virus, um nur die zwei wichtigsten zu nennen, sichtbaren, kleinen, mit Flüssigkeit gefüllten Bläschen, treten in unregelmäßigen Zeitabständen besonders an den Lippen (Herpes labialis), im Bereich der Geschlechtsorgane (Herpes genitalis) oder als Windpocken sowie als Gürtelrose auf. Das Auftreten der Bläschen kündigt sich durch Jucken, Brennen oder Rötung der Haut- bzw. der betroffenen Schleimhautbezirke an, wonach dann innerhalb weniger Stunden oder Tage die Ausbildung erfolgt.

Nach einigen Tagen brechen die flüssigkeitsgefüllten Bläschen auf, wobei die große Gefahr der Infektion weiterer eigener Körperteile wie z. B. der Augen, oder gesunder Personen besteht. Die Bläschenerkrankung ist sehr schmerzhaft und aus kosmetischen Gründen sehr lästig.

Da in der Therapie der Herpeserkrankung bis heute kein Medikament bekannt ist, das die Herpesviren im Körper vollständig und sicher vernichten könnte, und diese die Eigenschaft haben, sich unter Umständen jahrelang im Körper inaktiv zu verbergen, kommt es unter einem geeigneten äußeren Anlaß wie z. B. Streß, Erkältung, übermäßige Sonnenbestrahlung usw. immer wieder zu einem rezidivierenden, erneuten Ausbruch des Bläschenausschlags.

Die bis heute bekannten Behandlungsmethoden beschränken sich deshalb im wesentlichen auf die Heilung des Bläschenausschlags, die diesen zu einem raschen Abheilen bringt, die Schmerzen lindert oder beseitigt und eine Infektion anderer Körperteile oder Personen verhindert.

Zu diesem Zweck sind eine Reihe von galenischen Zubereitungen, wie Crems, Gels, Lösungen und Puder bekannt und in der praktischen Anwendung. Diese enthalten als therapeutisch wirksame Substanzen z. T. pflanzliche Bestandteile wie Extrakte aus Melissenblättern, Echinaceae, Kamillen, Arnika usw. oder pharmazeutisch wirksame Substanzen wie Benzocain, Sulfonamide, Tetracain, Allantoin und andere. Während jedoch bei den Therapeutika mit pflanzlichen Wirkstoffen die Heilwirkung sehr wenig ausgeprägt ist und sich deren Anwendung mehr auf eine begleitende und lindernde Wirkung während der Zeit der Selbstheilung beschränkt, können die mittels chem. Wirkstoffe wirksamen Therapeutika nur sehr beschränkt zum Einsatz kommen, da sie unerwünschte Nebenwirkungen haben und insbesondere auch allergische Reaktionen hervorrufen können. Aus der FR 21 28 187 ist ein Pharmazeutikum zur externen Behandlung von virusbedingten Erkrankungen der Haut auf der Grundlage von Schwefelpulver bekannt.

Es wurde nun gefunden, daß eine externe Behandlung der im Entstehen begriffenen oder bereits entstandenen Herpesbläschen, mit einer Salbe, Creme oder Lösung auf der üblichen galenischen Grundlage, die als therapeutischen Wirkstoff Kalium- bzw. Natriumsulfid enthalten, eine außerordentlich rasche Abheilung der Bläschen und damit eine rasche Beseitigung der damit verbundenen Beschwerden bewirkt.

Weiterhin wurde gefunden, daß eine gelbe Salbe, Creme oder Lösung auf üblicher galenischer Grundlage, mit einem therapeutisch wirksamen Zusatz von Huminsäure bzw. deren Salze, einen guten Heileffekt gegen Herpesbläschen besitzt.

Eine wesentliche Erhöhung der therapeutischen Wirksamkeit gegen Herpesbläschen kann erfindungsgemäß dadurch erreicht werden, daß die Therapeutika sowohl Na- bzw. Kaliumsulfid als auch Huminsäure bzw. deren Salze enthalten.

Eine weitere Verstärkung der therapeutischen Wirkung kann durch den erfindungsgemäßen Zusatz von kolloidalem Schwefel zu dem Na- bzw. Kaliumsulfid bzw. Huminsäure oder beide Substanzen enthaltendem Therapeutikum gegen Herpesbläschen erzielt werden.

Weiterhin kann erfindungsgemäß ein Teil der Huminsäure bzw. deren Salze durch natürliche Huminsäure enthaltenes Moor bzw. Moorextrakte ersetzt werden.

Eine Behandlung mit dem erfindungsgemäß ausgebildeten Therapeutikum zeigte neben der raschen Abheilung und Beseitigung der Beschwerden eine wesentliche Verzögerung des Wiederauftretens der Bläschen. Unerwünschte Nebenwirkungen wie insbesondere allergische Symptome konnten in keinem Falle beobachtet werden.

Beispiel

Die Zusammensetzung des erfindungsgemäßen Therapeutikums ist z. B. wie folgt

1,0 g Kal- bzw. Natriumsulfurat
2,4 g Huminsäure
1,4 g kolloidaler Schwefel
2,2 g NaClorid

auf 100 ml Flüssigkeit, Zusätze an Konservier- und Parfümierungsmittel wie i.: der Galenik üblich.

Patentansprüche

1. Pharmazeutische Zusammensetzung zur externen Behandlung der durch Herpes Viren verursachten Bläschenkrankheit, enthaltend feinteiligen Schwefel in einer üblichen pharmazeutischen Grundlage, dadurch gekennzeichnet, daß sie zusätzlich ca. 1% (G/V) Kalium- oder Natriumsulfid und 2,4% (G/V) Huminsäure, deren Salze, oder entsprechende Anteile an Moorerde oder Moorextrakt in flüssiger Phase enthält.

2. Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß sie zusätzlich 2,2% (G/V) Natriumchlorid enthält.

DRUG DEVELOPMENT RESEARCH 57:40–43 (2002)

DDR

Research Article

Pilot Study to Evaluate the Safety and Therapeutic Efficacy of Topical Oxifulvic Acid in Atopic Volunteers

J.R. Snyman,[1,*] J. Dekker,[2] S.C.K. Malfeld,[3] and C.E.J. van Rensburg[1]

[1]*Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa*

[2]*Enerkom (Pty) Ltd, Pretoria, South Africa*

[3]*Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa*

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT The study objectives were to establish first the safety and second the therapeutic efficacy of topically applied oxifulvic acid compared to 1% hydrocortisone and placebo creams. Oxifulvic acid has established antiinflammatory properties in vitro. It also inhibits elicited ear inflammation in mice at concentrations of 4.5 and 9%. In this double-blind cross-over study, 23 healthy volunteers allergic to grass or house dust mite allergen were recruited and included after signing informed consent. During the initial run-in period all volunteers were randomized to apply either 4.5 or 9% oxifulvic acid for 2 weeks on the volare aspect of one forearm (100 mm diameter) and rechallenged 21 days later to establish sensitization. Thereafter, volunteers were randomized to either placebo. 1% hydrocortisone, 4.5 or 9% oxifulvic acid creams. Creams were applied under occlusions 1 h prior to intradermal allergen challenge and every 8 h thereafter for 3 days (21-day intervals). The surface areas of the immediate and late phase skin reactions were calculated. Liver and kidney function tests as well as full blood counts were done at screening and thereafter weekly for the first 2 weeks and then at each follow-up visit. Topically applied oxifulvic acid had no significant effect on any of the safety parameters and also did not induce sensitization when applied on the skin. Oxifulvic acid (4.5%) caused inhibition of the elicited inflammatory reaction at 15 min and differed significantly from the 9% cream at 24 h. These changes were similar to that caused by hydrocortisone. No other significant changes were detected. Drug Dev. Res. 57:40–43, 2002.

Key words: oxifulvic acid; atopy; safety

INTRODUCTION

Fulvic acid is one of the components of the so-called humic substances which are naturally formed during the decay of plant and animal residues [MaCarthe et al., 1985]. Humic substances can be divided into humic acid, fulvic acid, and humin, based on their solubility in water. Humic acids are known to have some therapeutic benefit and have been used for the treatment of various diseases such as inflammation, hypercholesterolemia, and Von Willebrand's disease [Salz, 1974; Solovyeva and Lotosh, 1984; Lopez-Fernandez et al., 1992]. The evidence for therapeutic use of fulvic acids is, however, sparse. In vitro experiments [Wang et al., 1996] found that fulvic acid from peat possesses free radical scavenging properties.

Contract grant sponsors: Enerkom (Pty) Ltd.; Technology and Human Resources for Industry Programmes (THRIP) of the National Research Foundation; Department of Trade and Industry.

*Correspondence to: J.R. Snyman, Dept. of Pharmacology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa.

Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/ddr.10116

A unique process was created to convert bituminous coal by controlled wet oxidation in high yield to high-quality humic and fulvic acids. To distinguish these synthesized products from the naturally occurring substances they are called oxihumic and oxifulvic acid [Berg et al., 1997].

Oxifulvic acid has been shown in vitro to have antimicrobial activity [Van Rensburg et al., 2000] and to suppress superoxide production by neutrophils. Topical application of oxifulvic acid to laboratory mice demonstrated clear antiinflammatory properties [Van Rensburg et al., 2001]. Extended safety and toxicity testing on experimental animals have also shown no acute or chronic toxic reactions (J.R. Snyman, unpubl.).

The aim of the present study was to investigate the safety and tolerability of topically applied oxifulvic acid (4.5% and 9%) in healthy human volunteers (history of allergy to grass pollens and or house dust mite but otherwise healthy) with efficacy as a secondary outcome, i.e., inhibition of the cutaneous hypersensitivity reaction after intradermal antigen administration.

MATERIALS AND METHODS

In a double-blind cross-over study, 23 atopic volunteers allergic to grass pollen were randomized to receive placebo UEA cream or 1% hydrocortisone, or 4.5% oxifulvic acid or 9% oxifulvic acid cream on alternating forearms in each application, applied over an elicited cutaneous hypersensitivity reaction. The volunteers were all otherwise healthy with no prior history of drug allergy and were enrolled after signing informed consent. The study protocol was approved by the University of Pretoria Faculty of Health Sciences Research Ethics Committee.

In order to investigate the possible development of sensitization to oxifulvic acid, volunteers received either 4.5% or 9% oxifulvic acid on intact, non-inflammed forearm skin over a 14-day period (i.e., three applications of 100 cm^2/day). Rechallenge 21 days later was done with 9% oxifulvic acid cream on the same skin area to test for sensitization. During this period kidney and liver functions were evaluated at baseline and thereafter weekly for the first 2 weeks and then again at the next rechallenge. The same safety bloods were again collected on each of the following four tests sessions.

After the initial sensitization testing, the volunteers received an intradermal administration of antigen (i.e., 0.05 ml Southern Grass Mix® or house dust mite antigen; Bayer DHS division, Bayer (Pty), Isando, South Africa; equal to 10 PNU) on alternate forearms at 21-day intervals for a total of four episodes, corresponding with the four different study cream applications. Study creams were applied under occlusion for 1 h prior to antigen administration and thereafter every 8 h for 48 h.

The surface areas of the immediate wheal and flare and late phase skin reactions were calculated by marking the perimeters of these reactions on transparent plastic film at 0.25, 1, 6, and 24 h after intradermal antigen administration. This is an established method for evaluating the immediate cutaneous hypersensitivity reaction [Snyman et al., 1995].

Statistical Analysis

An ANOVA technique was used to detect differences within groups over time as well as to test for significant changes between groups on the various treatment regimens. Significance was established at the 5% level throughout the study.

RESULTS

There were no clinically significant changes in any of the safety parameters evaluated over the entire study period. Two volunteers withdrew from the study due to systemic reactions following the first intradermal antigen challenge and a further two stopped participation due to a lack of compliance with the study medication.

None of the volunteers developed sensitization to the oxifulvic acid and tolerated the cream well. There was no difference in side effects reported from placebo and no side effects due to cream application were reported in any of the study arms. See Figs. 1 and 2 for a summary of data on kidney and liver functions and full blood counts.

Oxifulvic acid resulted in slight inhibition of the elicited inflammatory reaction at 15 min and differed significantly from the 9% cream at 24 h. These changes were similar to that caused by hydrocortisone cream. No other significant changes were detected (see Fig. 3 for details).

DISCUSSION

In this study topically applied 4.5% and 9% oxifulvic acid did not differ from placebo (UEA) cream with regard to their side-effect profile. The test creams were also shown to be well tolerated and nonirritating and nonallergenic, confirming in vivo animal data [Van Rensburg et al., 2001].

The 4.5% oxifulvic acid cream also demonstrated some antiinflammatory properties similar to that demonstrated by 1% hydrocortisone cream. The fact that it was not possible to show the same results with the 9% cream possibly is reflective of its physicochemical properties, i.e., being more acidic in the cream. This low pH may explain poor skin penetration and therefore poor efficacy. The mechanism for oxifulvix acids in vivo activity was not investigated in this study

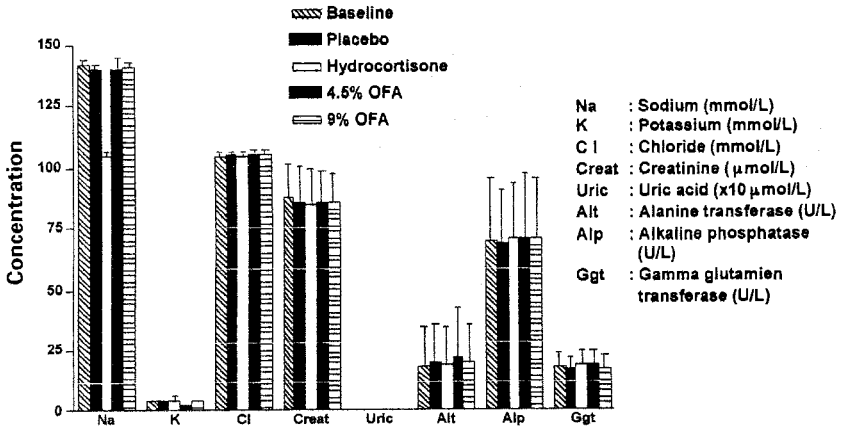


Fig. 1. Kidney and liver functions of volunteers during the trial.

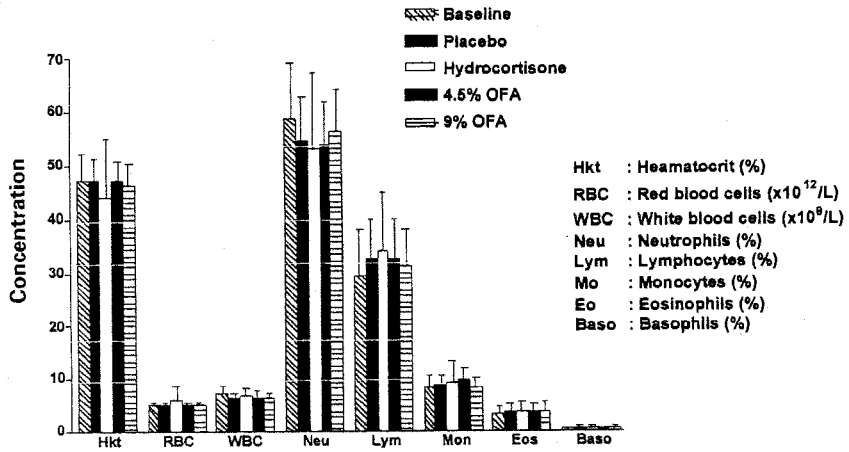


Fig. 2. Full blood counts of volunteers during the trial.

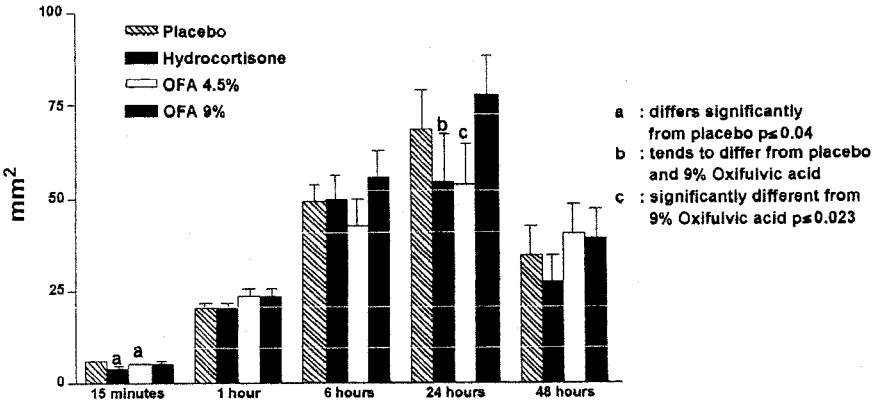


Fig. 3. Effects of a 24-h treatment of oxifulvic acid on elicited inflammatory reaction.

but in vitro studies have documented free radical scavenging properties [Wang et al., 1996] as well as inhibition of interleukin 2 production (J.R. Snyman, unpubl.). The contributions of these properties to oxifulvic acid's antiinflammatory effects need to b further explored before speculation on its pharmaco dynamics is possible. The fact that oxifulvic acid crean in an animal model had similar antiinflammator

properties as betamethasone (0.1%) and diclophenac (1%) creams [Van Rensburg et al., 2001] are perhaps indicative of it inhibiting specific immune modulators rather than only a nonspecific scavenging of free radicals.

In conclusion, oxifulvic acid is safe and well tolerated when applied topically on uninflammed and inflamed skin. This study also confirmed its antiinflammatory properties seen in animal studies [Van Rensburg et al., 2001]. The latter finding needs to be further explored utilizing different vehicles for active substance delivery to the skin and in various other models of inflammation in larger studies to reconfirm the above findings in humans.

ACKNOWLEDGMENTS

The authors thank Ms. J. Bekker for secretarial assistance and Ms. M. Steinmann for technical support.

REFERENCES

Bergh JJ, Cronje IJ, Dekker J, Dekker TG, Gerritsma LM, Mienie LJ. 1997. Non-catalytic oxidation of water-slurried coal with oxygen: identification of fulfic acid and acute toxicity. Fuel 76:147–154.

Lopez-Fernandez MF, Blanco-Lopez MJ, Castineria MP, Batlle J. 1992. Further evidence for recessive inheritance of von Willebrands disease with abnormal binding of von Willebrand factor to factor VIII. Am J Hematol 40:20–27.

MaCarthe P, Clapp CE, Malcolm RL, Bloom PR. 1985. Humic substances in soil and crop sciences; selected readings. Proceedings Symposium International Humic Substances Society of America, Chicago, IL, 2 December.

Salz H. 1974. Salhumin-Gel; ein lokaltheropeutikum mit hyperonisierender entzindungshemmender und analgetischer Wirkung. Med Monatsschr 28:548–530.

Snyman JR, Sommers De K, Van Wyk M, Geregorowski MD. 1995. The influence of betahistine on the dynamics of the cutaneous hypersensitivity reactions in patients with grass pollen allergy. Immunopharmacology 30:71–78.

Solovyeva VP, Lotosh TD. 1984. Biologically active peat substances as body resistance stimulators. Proc 7th Int Peat Congress. 4: 428–434.

van Rensburg CEJ, Van Straten A, Dekker J. 2000. An in vitro investigation of the antimicrobial activity of oxifulvic acid. J Antimicrob Chemother 46:853.

van Rensburg CEJ, Malfeld SCK, Dekker J. 2001. Topical application of oxifulvic acid suppresses the cutaneous immune response in mice. Drug Dev Res 53:29–32.

Wang C, Wang Z, Peng A, Hou J, Xin W. 1996. Interaction between fulvic acids of different origins and active oxygen radicals. Sci China C Life Sci 39(B):267–275.

Display from CAplus

Find documents citing this reference [$2.00]

CAS indexed 1 chemical substances from this document. [$2.00]

Title

Ointment containing conjugated oleic acid zinc salt and **humic** acid sodium salt

Inventor Name

Dong, Xin; Wumanjiang, Aili; Wen, Bin; Zhang, Yagang; Fan, Li; Ma, Li

Patent Assignee

Xinjiang Institute of Chemistry, Chinese Academy of Sciences, Peop. Rep. China

Publication Source

Faming Zhuanli Shenqing Gongkai Shuomingshu, 12 pp.

Identifier-CODEN

CNXXEV

Patent Information

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
CN 1372926	A	20021009	CN 2002-108604	20020328 <--

Priority Application Information

CN 2002-108604	20020328

Abstract

The ointment is composed of conjugated oleic acid Zn salt (such as 9Z,11E-, 9E,11Z-, 9E,11E-, 9Z,11Z-, 10E,12Z-, 10Z,12E-, 10Z,12Z-, 10E,12E-, 8Z,10E-, 8E,10Z-, 8Z,10Z-, 8E,10E-, 11E,13Z-, 11Z,13E-, 11E,13E-, and/or 11Z,13Z-oleic acid Zn salt) 10-40, **humic** acid Na salt 10-40, medical white oil 20-70, water 5-45, surfactant (such as Span, castor oil polyethylene glycol ether, sucrose polyester, glycerol monostearate, glycol distearate, propanediol tallate, xylitol tallate, and/or sorbitol stearate) 0.1-2, and antioxidant (such as Et pyrogallate, tert-Bu hydroquinone, tert-Bu 4-hydroxyanisole, or butylated hydroxytoluene) 0.01-0.3 part. The ointment may be used to treat skin diseases (such as eczema, psoriasis, dermatitis, and skin neoplasm).

International Patent Classification

International Patent Classification, Main

A61K031-201

International Patent Classification, Secondary

A61K045-00; A61K009-06; A61P017-02

Document Type

Patent

Language

Chinese

Accession Number

2003:652749 CAPLUS

Document Number

139:202477

United States Patent **6,946,144**
Jordan **September 20, 2005**

Transdermal delivery system

Abstract

The present invention relates to the discovery of a transdermal delivery system that can deliver high molecular weight pharmaceuticals and cosmetic agents to skin cells. A novel transdermal delivery system with therapeutic and cosmetic application and methods of use of the foregoing is disclosed.

Inventors: ***Jordan*; Frederick L.** (Santa Ana, CA)
Assignee: **ORYXE** (Anaheim, CA)
Appl. No.: **09/350,043**
Filed: **July 8, 1999**

Current U.S. Class: **424/449** ; 424/401
Field of Search: 424/401,45,195.1,78.02,78.05,78.07,725,744,449 514/937,947,887,944

References Cited [Referenced By]

U.S. Patent Documents

5318960	June 1994	Toppo
5431924	July 1995	Ghosh et al.
5472713	December 1995	Fein et al.
5571671	November 1996	Potter
5614212	March 1997	D'Angelo et al.
5665378	September 1997	Davis et al.
5708038	January 1998	Davis
5716625	February 1998	Hahn et al.
5744368	April 1998	Goldgaber et al.
5837853	November 1998	Takashima et al.
5840746	November 1998	Ducharme et al.
5849334	December 1998	Rivlin
5861268	January 1999	Tang et al.
5885597	March 1999	Botknecht et al.
5891651	April 1999	Roche et al.
5891857	April 1999	Holt et al.
5958384	September 1999	Holick
6103246	August 2000	Tisdale et al.

Foreign Patent Documents

WO 92/08470	May., 1992	WO
WO 97/09992	Mar., 1997	WO
WO 98/03163	Jan., 1998	WO
WO 98/33474	Aug., 1998	WO
98/34629	Aug., 1998	WO

Other References

O'Malley, P. et al., "Emu Products, Increasing Production and Profitability," Rural Industries Research & Development Corporation, pp. i-110, Dec. 1999. .
Biomedical Information Services Ltd., Inspection Criteria, General Standard for Testing Purity of Aloe Vera 7 pages, Copyright 1996. .
Chattem Inc., Packaging--Flexall QuickGel, Copyright 1999. .
Downing et al., Dermatology in General Medicine, Fitzpatrick, et al., eds., pp. 210-221 (1993). .
Ponec, M., Epidermal lipids in vivo, The Keratinocyte Handbook, Leigh, et al., eds., pp. 351-363 (1994)). .
Woodin, L., Cutting postop pain, RN, Aug.: 26-33 (1993). .
Amadio et al., Nonsteroidal anti-inflammatory drugs, Postgraduate Medicine, 93(4):73-97 (1993). .
Cohen, et al. in Wound Healing/Biochemical and Clinical Aspects, 1st ed. WB Saunders, Philadelphia (1992), page # ?. .
Grindlay and Reynolds, The Aloe Vera Phenomenon: A Review of the Properties and Modern Uses of the Leaf Parenchyma Gel, (1986) J. of Ethnopharmacology 16:117-151. .
Hart, et al., Two Functionally and Chemically Distinct Immunomodulatory Compounds in the Gel of Aloe Vera, (1988) J. of Ethnopharmacology 23:61-71. .
Hirata, et al., Biologically Active Constituents of Leaves and Roots of Aloe Arborescens var. natalensis, (1977) Z. Naturforsch, 32c:731-734. .
Bronaugh and Collier, In vitro Percutaneous absorption studies:Principle, Fundementals, and Applications, eds. Bronaugh and Maibach, Boca Raton, Fl, CRC Press, pp237-241 (1991). .
Nelson et al., Mid-Infrared Laser Ablation of Stratum Corneum Enhances inVitro Percutaneous Transport of Drugs, The Society for Investigative Dermatology, Inc.. 874-879 (1991). .
Collier et al., Maintenance of Skin Viability during in Vitro Percutaneous Absorption/Metabolism Studies, Toxicology and Applied Pharmacology, 99:522-533 (1989). .
Barel and Clarys, Study of the Stratum corneum Barrier Function by Transepidermal Water Loss Measurements: Comparison between Two Commercial Instruments, Skin Pharmacol. 8:186-195 (1995)..

Primary Examiner: Dodson; Shelley A.
Assistant Examiner: George; Konata M.
Attorney, Agent or Firm: Knobbe, Martens Olson & Bear, LLP.

Parent Case Text

This application claims priority under 35 U.S.C. .sctn. 119(e) to U.S. Provisional Application No. 60/092,061 entitled EFFECTIVE PAIN RELIEF SOLUTION, filed on Jul. 8, 1998 (now abandoned).

Claims

What is claimed is:

1. A transdermal delivery system comprising: an ethoxylated oil selected from the group consisting of ethoxylated castor oil, ethoxylated jojoba oil, ethoxylated corn oil, and ethoxylated emu oil; an alcohol mixed with the ethoxylated oil so as to form a penetration enhancer; an aqueous adjuvant mixed with the penetration enhancer, wherein the aqueous adjuvant comprises Aloe Vera; and a delivered agent mixed with the aqueous adjuvant and the penetration enhancer.

2. The transdermal delivery system of claim 1, wherein the ethoxylated oil has at least 20 ethoxylations per molecule.

3. The transdermal delivery system of claim 1, wherein about 0.1% to 40.0% by weight or volume is ethoxylated oil.

4. The transdermal delivery system of claim 1, wherein about 0.1% to 15% by weight or volume is alcohol.

5. The transdermal delivery system of claim 1, wherein about 0.1% to 85% by weight or volume is Aloe Vera.

6. The transdermal delivery system of claim 1, wherein the ratio of ethoxylated oil:alcohol:aqueous adjuvant is selected from the group consisting of 1:1:4, 1:1:14, 3:4:3, and 1:10:25.

7. The transdermal delivery system of claim 1, wherein the delivered agent is a molecule having a molecular weight of less than 6,000 daltons.

8. The transdermal delivery system of claim 1, wherein the delivered agent is a molecule having a molecular weight of greater than 6,000 daltons.

9. The transdermal delivery system of claim 1, wherein the delivered agent is selected from the group consisting of capsaicin, Boswellin, non steroidal anti-inflammatory drug, and collagen.

10. An apparatus comprising a vessel joined to an applicator and the transdermal delivery system of claim 1 incorporated in the vessel.

11. The apparatus of claim 10, wherein the applicator is a roll-on or a sprayer.

12. A transdermal delivery system comprising: an ethoxylated oil selected from the group consisting of ethoxylated castor oil, ethoxylated jojoba oil, ethoxylated corn oil, and ethoxylated emu oil; an alcohol mixed with the ethoxylated oil so as to form a penetration enhancer; Aloe Vera mixed with the penetration enhancer; and a therapeutically effective amount of capsaicin or NSAID or both mixed with the penetration enhancer and Aloe Vera.

13. The transdermal delivery system of claim 12, wherein the therapeutically effective amount of capsaicin is by weight or volume 0.01% to 13%.

14. The transdermal delivery system of claim 12, further comprising by weight or volume 0.1% to 10% Boswellin.

15. An apparatus comprising a vessel joined to an applicator and the transdermal delivery system of claim 12 incorporated in the vessel.

16. The apparatus of claim 15, wherein the applicator is a roll-on or a sprayer.

17. A transdermal delivery system comprising: an ethoxylated oil selected from the group consisting of ethoxylated castor oil, ethoxylated jojoba oil, ethoxylated corn oil, and ethoxylated emu oil; an alcohol mixed with the ethoxylated oil so as to form a penetration enhancer; an aqueous adjuvant mixed with the penetration enhancer, wherein the aqueous adjuvant comprises Aloe Vera; and a therapeutically effective amount of a delivered agent of less than or equal to 2,000,000 daltons mixed with the penetration enhancer and aqueous adjuvant.

18. The transdermal delivery system of claim 17, wherein the delivered agent is a collagen or fragment thereof.

19. The transdermal delivery system of claim 18, wherein the collagen has an approximate average molecular weight from about 2,000 daltons to about 500,000 daltons.

20. The transdermal delivery system of claim 18, wherein the therapeutically effective amount of collagen by weight or volume is 0.1% to 50.0%.

21. The transdermal delivery system of claim 18, wherein the collagen has an approximate average molecular weight of about 2,000 daltons and the therapeutically effective amount by weight or volume is 0.1% to 50.0%.

22. The transdermal delivery system of claim 18, wherein the collagen has an approximate average molecular weight of about 300,000 daltons and the therapeutically effective amount is 0.1% to 2.0%.

23. The transdermal delivery system of claim 18, wherein the collagen has an approximate average molecular weight of about 500,000 daltons and the therapeutically effective amount by weight or volume is 0.1% to 4.0%.

24. An apparatus comprising a vessel joined to an applicator and the transdermal delivery system of claim 17 incorporated in the vessel.

25. The apparatus of claim 24, wherein the applicator is a roll-on or a sprayer.

26. A method of making a transdermal delivery system comprising: providing an ethoxylated oil selected from the group consisting of ethoxylated castor oil, ethoxylated jojoba oil, ethoxylated corn oil, and ethoxylated emu oil; mixing the ethoxylated oil with an alcohol, nonionic solubilizer, or emulsifier so as to form a penetration enhancer; mixing the penetration enhancer with an aqueous adjuvant, wherein the aqueous adjuvant comprises Aloe Vera; and mixing the penetration enhancer and aqueous adjuvant with a delivered agent and thereby making the transdermal delivery system.

27. The method of claim 26, wherein the delivered agent is selected from the group consisting of capsaicin, Boswellin, non steroidal anti-inflammatory drug, and collagen.

28. The method of claim 26, wherein the delivered agent is less than or equal to 2,000,000 daltons.

29. The method of claim 26, further comprising incorporating the transdermal delivery system into an application device.

30. The method of claim 29, wherein the application device has a roll-on applicator or a sprayer.

31. The transdermal delivery system of claim 1, wherein the delivered agent is a non steroidal anti-inflammatory drug.

32. The transdermal delivery system of claim 31, wherein the non steroidal anti-inflammatory drug is selected from the group consisting of naproxin, ibuprofen, and aspirin.

33. The transdermal delivery system of claim 31, wherein the non steroidal anti-inflammatory drug is aspirin.

34. The transdermal delivery system of claim 1, wherein the ethoxylated oil is ethoxylated castor oil.

35. The transdermal delivery system of claim 1, wherein the ethoxylated oil is ethoxylated jojoba oil.

36. The transdermal delivery system of claim 1, wherein the ethoxylated oil is ethoxylated corn oil.

37. The transdermal delivery system of claim 1, wherein the ethoxylated oil is ethoxylated emu oil.

38. The method of claim 26, wherein the ethoxylated oil is ethoxylated castor oil.

39. The method of claim 26, wherein the ethoxylated oil is ethoxylated jojoba oil.

40. The method of claim 26, wherein the ethoxylated oil is ethoxylated corn oil.

41. The method of claim 26, wherein the ethoxylated oil is ethoxylated emu oil.

42. The composition of claim 1, wherein the delivered agent is less than or equal to 2,000,000 daltons.

43. The composition of claim 1, wherein the delivered agent is less than or equal to 1,500,000 daltons.

44. The composition of claim 1, wherein the delivered agent is less than or equal to 1,000,000 daltons.

45. The composition of claim 1, wherein the delivered agent is less than or equal to 700,000 daltons.

46. The composition of claim 1, wherein the delivered agent is less than or equal to 500,000 daltons.

47. The composition of claim 1, wherein the delivered agent is less than or equal to 300,000 daltons.

48. The composition of claim 1, wherein the delivered agent is less than or equal to 200,000 daltons.

49. The composition of claim 1, wherein the delivered agent is less than or equal to 100,000 daltons.

50. The composition of claim 1, wherein the delivered agent is less than or equal to 75,000 daltons.

51. The composition of claim 1, wherein the delivered agent is less than or equal to 50,000 daltons.

52. The composition of claim 1, wherein the delivered agent is less than or equal to 25,000 daltons.

53. The composition of claim 1, wherein the delivered agent is less than or equal to 20,000 daltons.

54. The composition of claim 1, wherein the delivered agent is less than or equal to 15,000 daltons.

55. The composition of claim 1, wherein the delivered agent is less than or equal to 10,000 daltons.

56. The composition of claim 1, wherein the delivered agent is less than or equal to 5,000 daltons.

57. The composition of claim 1, wherein the delivered agent is less than or equal to 2,500 daltons.

58. The composition of claim 1, wherein the delivered agent is less than or equal to 1,000 daltons.

59. The composition of claim 17, wherein the delivered agent is less than or equal to 1,500,000 daltons.

60. The composition of claim 17, wherein the delivered agent is less than or equal to 1,000,000 daltons.

61. The composition of claim 17, wherein the delivered agent is less than or equal to 700,000 daltons.

62. The composition of claim 17, wherein the delivered agent is less than or equal to 500,000 daltons.

63. The composition of claim 17, wherein the delivered agent is less than or equal to 300,000 daltons.

64. The composition of claim 17, wherein the delivered agent is less than or equal to 200,000 daltons.

65. The composition of claim 17, wherein the delivered agent is less than or equal to 100,000 daltons.

66. The composition of claim 17, wherein the delivered agent is less than or equal to 75,000 daltons.

67. The composition of claim 17, wherein the delivered agent is less than or equal to 50,000 daltons.

68. The composition of claim 17, wherein the delivered agent is less than or equal to 25,000 daltons.

69. The composition of claim 17, wherein the delivered agent is less than or equal to0,000 daltons.

70. The composition of claim 17, wherein the delivered agent is less than or equal to 15,000 daltons.

71. The composition of claim 17, wherein the delivered agent is less than or equal to 10,000 daltons.

72. The composition of claim 17, wherein the delivered agent is less than or equal to 5,000 daltons.

73. The composition of claim 17, wherein the delivered agent is less than or equal to 2,500 daltons.

74. The composition of claim 17, wherein the delivered agent is less than or equal to 1,000 daltons.

75. The method of claim 26, wherein the delivered agent is less than or equal to 1,500,000 daltons.

76. The method of claim 26, wherein the delivered agent is less than or equal to 1,000,000 daltons.

77. The method of claim 26, wherein the delivered agent is less than or equal to 700,000 daltons.

78. The method of claims 26, wherein the delivered agent is less than or equal to 500,000 daltons.

79. The method of claim 26, wherein the delivered agent is less than or equal to 300,000 daltons.

80. The method of claim 26, wherein the delivered agent is less than or equal to 200,000 daltons.

81. The method of claim 26, wherein the delivered agent is less than or equal to 100,000 daltons.

82. The method of claim 26, wherein the delivered agent is less than or equal to 75,000 daltons.

83. The method of claim 26, wherein the delivered agent is less than or equal to 50,000 daltons.

84. The method of claim 26, wherein the delivered agent is less than or equal to 25,000 daltons.

85. The method of claim 26, wherein the delivered agent is less than or equal to 20,000 daltons.

86. The method of claim 26, wherein the delivered agent is less than or equal to 15,000 daltons.

87. The method of claim 26, wherein the delivered agent is less than or equal to 10,000 daltons.

88. The method of claim 26, wherein the delivered agent is less than or equal to 5,000 daltons.

89. The method of claim 26, wherein the delivered agent is less than or equal to 2,500 daltons.

90. The method of claim 26, wherein the delivered agent is less than or equal to 1,000 daltons.

Description

FIELD OF THE INVENTION

The present invention relates to the discovery of a transdermal delivery system that can deliver high molecular weight pharmaceuticals and cosmetic agents to skin cells. A novel transdermal delivery system with therapeutic and cosmetic application and methods of use of the foregoing is disclosed.

BACKGROUND OF THE INVENTION

The skin provides a protective barrier against foreign materials and infection. In mammals this is accomplished by forming a highly insoluble protein and lipid structure on the surface of the corneocytes termed the cornified envelope (CE). (Downing et al., Dermatology in General Medicine, Fitzpatrick, et al., eds., pp. 210-221 (1993), Ponec, M., The Keratinocyte Handbook, Leigh, et al., eds., pp. 351-363 (1994)). The CE is composed of polar lipids, such as ceramides, sterols, and fatty acids, and a complicated network of cross-linked proteins; however, the cytoplasm of stratum comeum cells remains polar and aqueous. The CE is extremely thin (10 microns) but provides a substantial barrier. Because of

the accessibility and large area of the skin, it has long been considered a promising route for the administration of drugs, whether dermal, regional, or systemic effects are desired.

A topical route of drug administration is sometimes desirable because the risks and inconvenience of parenteral treatment can be avoided; the variable absorption and metabolism associated with oral treatment can be circumvented; drug administration can be continuous, thereby permitting the use of pharmacologically active agents with short biological half-lives; the gastrointestinal irritation associated with many compounds can be avoided; and cutaneous manifestations of diseases can be treated more effectively than by systemic approaches. Most transdermal delivery systems achieve epidermal penetration by using a skin penetration enhancing vehicle. Such compounds or mixtures of compounds are known in the art as "penetration enhancers" or "skin enhancers", While many of the skin enhancers in the literature enhance transdermal absorption, several possess certain drawbacks in that (i) some are regarded as toxic; (ii) some irritate the skin; (iii) some on prolonged use have a thinning effect on the skin; (iv) some change the intactness of the skin structure, resulting in a change in the diffusability of the drug; and (v) all are incapable of delivering high molecular weight pharmaceuticals and cosmetic agents. Clearly there remains a need for safe and effective transdermal delivery systems that can administer a wide-range of pharmaceuticals and cosmetic agents to skin cells.

BRIEF SUMMARY OF THE INVENTION

In aspects of the invention described below, transdermal delivery systems are provided that can be used to administer pharmaceuticals and cosmetic agents of various molecular weights. In several embodiments, the transdermal delivery system comprises a novel formulation of penetration enhancer and aqueous adjuvant that enables the delivery of a wide range of pharmaceuticals and cosmetic agents having molecular weights of less than 100 daltons to greater than 500,000 daltons. For example, embodiments of the transdermal delivery system described herein can be used to deliver a therapeutically effective amount of a non-steroidal anti-inflammatory drug (NSAID), a capsaicin or Boswellin containing pain-relief solution, or several different collagen preparations. Methods of making and using the transdermal delivery devices of the invention for the treatment and prevention of human disease and cosmetic condition are also provided.

Accordingly, in one embodiment a transdermal delivery system is provided that comprises an ethoxylated lipid, an alcohol mixed with the ethoxylated lipid so as to form a penetration enhancer, an aqueous adjuvant mixed with the penetration enhancer, wherein the aqueous adjuvant is a plant extract from the family of Liliaceae Liliaceae, and a delivered agent mixed with the aqueous adjuvant and the penetration enhancer. In some aspects of this embodiment, the ethoxylated lipid is a vegetable or animal oil having at least 20 ethoxylations per molecule. In other aspects of this embodiment, about 0.1% to 40.0% by weight or volume is ethoxylated lipid. Other embodiments of the invention include the transdermal delivery system described above wherein about 0.1% to 15% by weight or volume is alcohol or wherein about 0.1% to 85% by weight or volume is Aloe Vera. Still more embodiments of the invention have a ratio of ethoxylated lipid:alcohol:aqueous adjuvant selected from the group consisting of 1:1:4, 1:1:14, 3:4:3, and 1:10:25.

Desirably, the transdermal delivery systems described above have delivered agents that are molecules having a molecular weight of less than 6,000 daltons. In some embodiments, the transdermal delivery system described above has a delivered agent that is one or more of the compounds selected from the group consisting of capsaicin, Boswellin, non steroidal anti-inflammatory drug, and collagen. Preferably, however, the delivered agent is a molecule having a molecular weight of greater than 6,000 daltons. Additional embodiments include an apparatus comprising a vessel joined to an applicator and the transdermal delivery system described above incorporated in the vessel. Applicators in embodiments of the invention can be a roll-on or a sprayer.

In another aspect, a transdermal delivery system is provided which comprises an ethoxylated oil, an alcohol mixed with the ethoxylated oil so as to form a penetration enhancer, an Aloe extract mixed with the penetration enhancer, and a therapeutically effective amount of capsaicin or NSAID or both mixed with the penetration enhancer and Aloe extract. In some embodiments of this aspect, the therapeutically effective amount of capsaicin is by weight or volume 0.01% to 5.0% capsaicin or 1.0% to 13% oleoresin capsicum. In other embodiments of this aspect, the transdermal delivery system further includes by weight or volume 0.1% to 10% Boswellin. As above, an apparatus having a vessel joined to an applicator that houses the transdermal delivery system is also an- embodiment and preferred applicators include roll-on or a sprayer.

Several methods are also within the scope of aspects of the invention. For example, one approach involves a method of reducing pain or inflammation comprising the step of administering the transdermal delivery system described above to a subject in need and monitoring the reduction in pain or inflammation. Additional methods of the invention include approaches to treat cancer and Alzheimer's disease. For example, a method of treating or preventing cancer and Alzheimer's disease can comprise the step of identifying a subject in need of a COX enzyme inhibitor and administering the transdermal delivery system described above to the subject.

In addition to the delivery of low and medium molecular weight delivered agents, several compositions that have high molecular weight delivered agents (e.g., collagens) and methods of use of such compositions are embodiments of the invention. For example, one embodiment concerns a transdermal delivery system comprising an ethoxylated oil, an alcohol mixed with the ethoxylated oil so as to form a penetration enhancer, an Aloe extract mixed with the penetration enhancer, and a therapeutically effective amount of collagen mixed with the penetration enhancer and Aloe extract.

In different embodiments of this transdermal delivery system, the collagen has a molecular weight less than 6,000 daltons or greater than 6,000 daltons. Thus, in certain embodiments, the collagen can have an approximate molecular weight as low as 2,000 daltons or lower. In a certain embodiment, the molecular weight is from about 300,000 daltons to about 500,000 daltons. Further, these transdermal delivery systems can have a therapeutically effective amount of collagen by weight or volume that is 0.1% to 50.0% and the collagen can be Hydrocoll EN-55 when the therapeutically effective amount by weight or volume is 0.1% to 50.0%; the collagen can be Solu-Coll when the therapeutically effective amount is 0.1% to 2.0%; and the collagen can be Plantsol when the therapeutically effective amount by weight or volume is 0.1% to 4.0%. As above, an apparatus having a vessel joined to an applicator that houses the transdermal delivery system is also an embodiment and preferred applicators include roll-on or a sprayer.

Methods of reducing wrinkles and delivery of high molecular weight molecules are also embodiments of the invention. For example, by one approach, a method of reducing wrinkles in the skin comprises identifying a subject in need of skin tone restoration, administering the transdermal delivery system, such as is described above, to the subject and monitoring the restoration of skin tone. Further, methods of making a transdermal delivery system are within the scope of the invention. Accordingly, a method of making a transdermal delivery system can involve providing an ethoxylated oil, mixing the ethoxylated oil with an alcohol, nonionic solubilizer, or emulsifier so as to form a penetration enhancer, mixing the penetration enhancer with an aqueous adjuvant, wherein the aqueous adjuvant is an extract from a plant of the Liliaceae family, and mixing the penetration enhancer and aqueous adjuvant with a delivered agent and thereby making the transdermal delivery system.

In some aspects of this method, the delivered agent is selected from the group consisting of capsaicin, Boswellin, non steroidal anti-inflammatory drug, and collagen. In another embodiment of this method, the delivered agent has a molecular weight greater than 6,000 daltons. As above, an apparatus having a vessel joined to an applicator that houses the transdermal delivery system is also an embodiment and preferred applicators include a roll-on or a sprayer.

DETAILED DESCRIPTION OF THE INVENTION

In the following disclosure, several transdermal delivery systems are described that can administer an effective amount of a pharmaceutical or cosmetic agent to the human body. Although embodiments of the invention can be used to administer low or high (or both low and high) molecular weight pharmaceuticals and cosmetic agents, preferable embodiments include transdermal delivery systems that can administer compounds having molecular weights greater than 6,000 daltons. One embodiment, for example, includes a transdermal delivery system that can administer a therapeutically effective amount of a non-steroidal anti-inflammatory drug (NSAID). Another embodiment concerns a transdermal delivery system having a novel pain-relief solution (e.g., a formulation comprising capsaicin or Boswellin or both). Another aspect of the invention involves a transdermal delivery system that can administer a collagen preparation (e.g., soluble collagens, hydrolyzed collagens, and plant collagens). These examples are provided to demonstrate that embodiments of the invention can be used to transdermally deliver both low and high molecular weight compounds and it should be understood that many other molecules can be effectively delivered to the body, using the embodiments described herein, in amounts that are therapeutically, prophylactically, or cosmetically beneficial.

A transdermal delivery system has three components, a delivered agent, a penetration enhancer, and an aqueous adjuvant. Accordingly, one component of the transdermal delivery system of the invention is a "delivered agent". A molecule or a mixture of molecules (e.g., a pharmaceutical or cosmetic agent) that are delivered to the body using an embodiment of a transdermal delivery system of the invention are termed "delivered agents". A delivered agent that can be administered to the body using an embodiment of the invention can include, for example, a protein, a sugar, a nucleic acid, a chemical, or a lipid. Desirable delivered agents include, but are not limited to, glycoproteins, enzymes, genes, drugs, and ceramides. Preferred delivered agents include collagens, NSAIDS, capsaicin, and Boswein. In some embodiments, a transdermal delivery system comprises a combination of the aforementioned delivered agents.

The second component of a transdermal delivery system is a penetration enhancer. Desirable penetration enhancers comprise both hydrophobic and hydrophilic components. The "hydrophobic component" includes one or more polyether compounds. One preferred polyether compound is an ethoxylated lipid. Although an ethoxylated lipid can be created in many ways, a preferred approach involves the reaction of ethylene oxide with a vegetable or animal oil. The "hydrophilic component" can be, for example, an alcohol, a nonionic solubilizer or an emulsifier. Suitable hydrophilic components include, but are not limited to, ethylene glycol, propylene glycol,. dimethyl sulfoxide (DMSO), dimethyl

polysiloxane (DMPX), oleic acid, caprylic acid, isopropyl alcohol, 1-octanol, ethanol (denatured or anhydrous), and other pharamceutical grade or absolute alcohols with the exception of methanol.

Embodiments of the invention can also comprise a third component termed an "aqueous adjuvant". Aqueous adjuvants include, but are not limited to, water (distilled, deionized, filtered, or otherwise prepared), Aloe Vera juice, and other plant extracts. Thus, several embodiments of the invention have a penetration enhancer that includes a hydrophobic component comprising an ethoxylated oil (e.g., castor oil, glycerol, corn oil, jojoba oil, or emu oil) and a hydrophilic component comprising an alcohol, a nonionic solubilizer, or an emulsifier (e.g., isopropyl alcohol) and an aqueous adjuvant such as Aloe Vera extract. Other materials can also be components of a transdermal delivery system of the invention including fragrance, creams, ointments, colorings, and other compounds so long as the added component does not deleteriously affect transdermal delivery of the delivered agent. Unexpectedly, it has been found that compositions using extracts of plants of the Liliaceae family, such as Aloe Vera, provide superior benefits in transdermal delivery of high molecular weight delivered agents, including collagen having an average molecular weight greater than 6,000 daltons.

In addition to the aforementioned compositions, methods of making and using the embodiments of the invention are provided. In general, an embodiment of the invention is prepared by mixing a hydrophilic component with a hydrophobic component and an aqueous adjuvant. Depending on the solubility of the delivered agent, the delivered agent can be solubilized in either the hydrophobic, hydrophilic, or aqueous adjuvant components prior to mixing. In addition to physical mixing techniques (e.g., magnetic stirring or rocker stirring) heat can be applied to help coalesce the mixture. Desirably, the temperature is not raised above 40.degree. C.

Several formulations of transdermal delivery system are within the scope of aspects of the invention. One formulation comprises a ratio of hydrophilic component:hydrophobic component:aqueous adjuvant of 3:4:3. The amount of delivered agent that is incorporated into the penetration enhancer depends on the compound, desired dosage, and application. The amount of delivered agent in a particular formulation can be expressed in terms of percentage by weight, percentage by volume, or concentration. Several specific formulations of delivery systems are provided in the Examples described herein.

Methods of treatment and prevention of pain, inflammation, and human disease are also provided. In some embodiments, a transdermal delivery system comprising an NSAID, capsaicin, Boswellin or any combination thereof is provided to a patient in need of treatment, such as for relief of pain and/or inflammation. The use of transdermal delivery systems described herein which contain extracts of the Liliaecae family, such as Aloe Vera extract, are particularly beneficial in the delivery of these delivered agents. A patient can be contacted with the transdermal delivery system and treatment continued for a time sufficient to reduce pain or inflammation or inhibit the progress of disease.

Additionally, a method of reducing wrinkles and increasing skin tightness and flexibility is provided. By this approach, a transdermal delivery system comprising a collagen delivered agents is provided to a patient in need, the patient is contacted with the transdermal delivery system, and treatment is continued for a time sufficient to restore a desired skin tone (e.g., reduce wrinkles or restore skin tightness and flexibility). The transdermal delivery system described herein provides unexpectedly superior results in the delivery of collagen of all molecular weights.

In the disclosure below, there is provided a description of several of the delivered agents that can be incorporated into the transdermal delivery devices of the present invention.

Delivered Agents

Many different delivered agents can be incorporated into the various transdermal delivery systems of the invention and a non-exhaustive description of embodiments is provided in this section. While the transdermal delivery of molecules having a molecular weight in the vicinity of 6000 daltons has been reported, it has not been possible, until the present invention, to administer molecules of greater size transdermally. (U.S. Pat. No. 5,614,212 to D'Angelo et al.).

The described embodiments can be organized according to their ability to deliver a low or high molecular weight delivered agent. Low molecular weight molecules (e.g., a molecule having a molecular weight less than 6,000 daltons) can be effectively delivered using an embodiment of the invention and high molecular weight molecules (e.g., a molecule having a molecular weight greater than 6,000 daltons) can be effectively delivered using an embodiment of the invention. Desirably a delivery system of the invention can administer a therapeutically or cosmetically beneficial amount of a delivered agent having a molecular weight of 50 daltons to less than 6,000 daltons. Preferably, however, a delivery system of the invention can administer a therapeutically or cosmetically beneficial amount of a delivered agent having a molecular weight of 50 daltons to 2,000,000 daltons or less. That is, a preferred delivery system of the invention can administer a delivered agent having a molecular weight of less than or equal to 50, 100, 200, 500, 1,000, 1,500, 2,000,

2,500, 3,000, 3,500, 4,000, 4,500, 5,000, 5,500, 6,000, 7,000, 8,000, 9,000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, 20,000, 21,000, 22,000, 23,000, 24,000, 25,000, 26,000, 27,000, 28,000, 29,000, 30,000, 31,000, 32,000, 33,000, 34,000, 35,000, 36,000, 37,000, 38,000, 39,000, 40,000, 41,000, 42,000, 43,000, 44,000, 45,000, 46,000, 47,000, 48,000, 49,000, 50,000, 51,000, 52,000, 53,000, 54,000, 55,000, 56,000, 57,000, 58,000, 59,000, 60,000, 61,000, 62,000, 63,000, 64,000, 65,000, 66,000, 67,000, 68,000, 69,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, 100,000, 125,000, 150,000, 175,000, 200,000, 225,000, 250,000, 275,000, 300,000, 350,000, 400,000, 450,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1,000,000, 1,500,000, 1,750,000, and 2,000,000 daltons.

In one aspect, a low molecular weight compound (e.g., a pain relieving substance or mixture of pain relieving substances) is transdermally delivered to cells of the body using an embodiment of a transdermal delivery system of the invention. The delivered agent can be, for example, any one or more of a number of compounds, including non-steroidal anti-inflammatory drugs (NSAIDs) that are frequently administered systemically. These include ibuprofen (2-(isobutylphenyl)-propionic acid); methotrexate (N-[4-(2,4 diamino 6-pteridinyl-methyl]methylamino]benzoyl)-L-glutamic acid); aspirin (acetylsalicylic acid); salicylic acid; diphenhydramine (2-diphenylmethoxy)-NN-dimethylethylamine hydrochloride); naproxen (2-naphthaleneacetic acid, 6-methoxy-9-methyl-, sodium salt, (-)); phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione); sulindac-(2)-5-fuoro-2-methyl-1-[[p-(methylsulfinyl)phenyl]methylene-]-1H- indene-3-acetic acid; diflunisal (2',4', -difluoro-4-hydroxy-3-biphenylcarboxylic acid; piroxicam (4hydroxy-2-methyl-N-2-pyridinyl-2H-1,2-benzothiazine-2-carboxamide 1,1-dioxide, an oxicam; indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-H-indole-3-acetic acid); meclofenamate sodium (N-(2,6-dichloro-m-tolyl) anthranilic acid, sodium salt, monohydrate); ketoprofen (2-(3-benzoylphenyl)-propionic acid; tolmetin sodium (sodium 1-methyl-5-(4-methylbenzoyl-1H-pyrrole-2-acetate dihydrate); diclofenac sodium (2-[(2,6-dichlorophenyl)amino]benzeneatic acid, monosodium salt); hydroxychloroquine sulphate (2-{[4[(7-chloro-4-quinolyl)amino]pentyl]ethylamino}ethanol sulfate (1:1); penicillamine (3-mercapto-D-valine); flurbiprofen ([1,1-biphenyl]-4-acetic acid, 2-fluoro-alphamethyl-, (+-.)); cetodolac (1-8-diethyl-13,4,9, tetra hydropyrano-[3-4-13]indole-1-acetic acid; mefenamic acid (N-(2,3-xylyl)anthranilic acid; and diphenhydramine hydrochloride (2-diphenyl methoxy-N, N-di-methyletthmine hydrochloride).

The delivery systems of the invention having NSAIDs desirably comprise an amount of the compound that is therapeutically beneficial for the treatment or prevention of disease or inflammation. Several studies have determined an appropriate dose of an NSAID for a given treatment or condition. (See e.g., Woodin, RN, August: 26-33 (1993) and Amadio et al., Postgrduate Medicine, 93(4):73-97 (1993)). The maximum recommended daily dose for several NSAIDs is listed in Table 1. The amount of NSAID recommended in the literature and shown in Table 1 can be incorporated into a delivery system of the invention. Because the transdermal delivery system of the invention can administer a delivered agent in a site-specific manner, it is believed that a lower total dose of therapeutic agent, as compared to the amounts provided systemically, will provide therapeutic benefit. Additionally, greater therapeutic benefit can be gained by using a transdermal delivery system of the invention because a high dose of therapeutic agent (e.g., an NSAID) can be applied to the particular site of inflammation. That is, in contrast to systemic administration, which applies the same concentration of therapeutic to all regions of the body, a transdermal delivery system of the invention can site-specifically administer a therapeutic and, thereby, provides a much greater regional concentration of the agent than if the same amount of therapeutic were administered systemically.

TABLE 1 Agent Maximum Recommended Daily Dose Indomethacin 100 mg Ibuprofen 3200 mg Naproxen 1250 mg Fenoprofen 3200 mg Tolmetin 2000 mg Sulindac 400 mg Meclofenamate 400 mg Ketoprofen 300 mg Proxicam 10 mg Flurbiprofen 300 mg Diclofenac 200 mg

Additionally, desirable embodiments include a delivery system that can administer a pain relieving mixture comprising capsaicin (e.g., oleoresin capsicum) or Boswellin or both. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the pungent component of paprika and peppers, is a potent analgesic. (See U.S. Pat. No. 5,318,960 to Toppo, U.S. Pat. No. 5,885,597 to Botknecht et al., and U.S. Pat No. 5,665,378 to Davis et al.). Capsaicin produces a level of analgesia comparable to morphine, yet it is not antagonized by classical narcotic antagonists such as naloxone. Further, it effectively prevents the development of cutaneous hyperalgesia, but appears to have minimal effects on normal pain responses at moderate doses. At high doses capsaicin also exerts analgesic activity in classical models of deep pain, elevating the pain threshold above the normal value. Capsaicin can be readily obtained by the ethanol extraction of the fruit of capsicum frutescens or capsicum annum. Capsaicin and analogs of capsaicin are available commercially from a variety of suppliers, and can also be prepared synthetically by published methods. Aspects of the invention encompass the use of synthetic and natural capsaicin, capsaicin derivatives, and capsaicin analogs.

A form of capsaicin used in several desirable embodiments is oleoresin capsicum. Oleoresin capsicum contains primarily capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. The term "capsaicin" collectivelly refers to all forms of capsaicin, capsicum, and derivatives or modifications thereof. The pungency of these five compounds, expressed in Scoville units, are provided in Table 2.

TABLE 2 Compound Pungency .times. 100,000 SU Capsaicin 160 Dihydrocapsaicin 160 Nordihydrocapsaicin 91 Homocapsaicin 86 Homodihydrocapsaicin 86

The delivery systems of the invention having capsaicin desirably comprise by weight or volume 0.01% to 1.0% capsaicin or 1.0% to 10% oleoresin capsicum. Preferred amounts of this delivered agent include by weight or volume 0.02% to 0.75% capsaicin or 2.0% to 7.0% oleoresin capsicum. For example, the delivery systems of the invention having capsaicin can comprise by weight or volume less than or equal to 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.075%, 0.08%, 0.085%, 0.09%, 0.095%, 0.1%, 0.15%, 0.175%, 0.2%, 0.225%, 0.25%, 0.275%, 0.3%, 0.325%, 0.35%, 0.375%, 0.4%, 0.425%, 0.45%, 0.475%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, and 1.0% capsaicin. Although not a desirable embodiment, the delivery systems of the invention having capsaicin can comprise an amount of capsaicin by weight or volume that is greater than 1.0%, such as 1.2%, 1.5%, 1.8%, 2.0%, 2.2%, 2.5%, 2.8%, 3.0%, 3.5%, 4.0%, 4.5%, and 5.0%. Similarly, the delivery systems of the invention having oleoresin capsicum can comprise an amount of oleoresin capsicum less than 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5%, 10.0%, 11.0%, 12.0%, and 13.

Boswellin, also known as Frankincense, is an herbal extract of a tree of the Boswellia family. Boswellin can be obtained, for example, from Boswellia thurifera, Boswellia carteri, Boswellia sacra, and Boswellia serrata. There are many ways to extract Boswellin and Boswellin gum resin and boswellic acids are obtainable from several commercial suppliers (a 65% solution of Boswellic acid is obtainable from Nature's Plus). Some suppliers also provide creams and pills having Boswellin with and without capsaicin and other ingredients. Embodiments of the invention comprise Boswellin and the term "Boswellin" collectively refers to Frankincense, an extract from one or more members of the Boswellia family, Boswellic acid, synthetic Boswellin, or modified or derivatized Boswellin.

The delivery systems of the invention having Boswellin desirably comprise 0.1% to 10% Boswellin by weight or volume. Preferred amounts of this delivered agent include 1.0% to 5.0% Boswellin by weight. For example, the delivery systems of the invention having Boswellin can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 1.9%, 1.95%, and 2.0%, 2.1%, 2.15%, 2.2%, 2.25%, 2.3%, 2.35%, 2.4%, 2.45%, 2.5%, 2.55%, 2.6%, 2.65%, 2.7%, 2.75%, 2.8%, 2.85%, 2.9%, 2.95%, 3.0%, 3.1%, 3.15%, 3.2%, 3.25%, 3.3%, 3.35%, 3.4%, 3.45%, 3.5%, 3.55%, 3.6%, 3.65%, 3.7%, 3.75%, 3.8%, 3.85%, 3.9%, 3.95%, 4.0%,. 4.1%, 4.15%, 4.2%, 4.25%, 4.3%, 4.35%, 4.4%, 4.45%, 4.4%, 4.45%, 4.5%, 4.55%, 4.6%, 4.65%, 4.7%, 4.75%, 4.85%, 4.9%, 4.95%, and 5.0% Boswellin. Although not a desirable embodiment, the delivery systems of the invention having Boswellin can comprise amounts of Boswellin by weight that are greater than 5.0%, such as 5.5%, 5.7%, 6.0%, 6.5%%, 6.7%, 7.0%, 7.5%, 7.7%, 8.0%, 8.5%, 8.7%, 9.0%, 9.5%, 9.7%, and 10.0% or greater. Additionally, Boswellin from different sources can be combined to compose the Boswellin component of an embodiment. For example, in one embodiment an extract from Boswellia thunifera is combined with an extract from Boswellia serrata.

Additional embodiments of the invention comprise a transdermal delivery system that can administer a pain relieving solution comprising two or more members selected from the group consisting of NSAIDs, capsacin, and Boswellin. The delivery systems of the invention that include two or more members selected from the group consisting of NSAIDs, capsacin, and Boswellin desirably comprise an amount of delivered agent that can be included in a delivered agent having an NSAID, capsaicin, or Boswellin by itself. For example, if the delivered agent comprises an NSAID, the amount of NSAID that can be used can be an amount recommended in the literature (See e.g., Woodin, R N, August: 26-33 (1993) and Amadio, et al., Postgrduate Medicine, 93(4):73-97 (1993)), or an amount listed in Table 1. Similarly, if capsaicin is a component of the delivered agents then the delivery system can comprise by weight or volume less than or equal to 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.075%, 0.08%, 0.085%, 0.09%, 0.095%, 0.1%, 0.15%, 0.175%, 0.2%, 0.225%, 0.25%, 0.275%, 0.3%, 0.325%, 0.35%, 0.375%, 0.4%, 0.425%, 0.45% 0.475%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, and 1.0% capsaicin or less than 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5%, 10.0%, 11.0%, 12.0%, 13.0%, oleoresin capsicum. Further, if Boswellin is a component of the delivered agents, then the delivery system can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 9%, 1.95%, 2.0%, 2.1%, 2.15%, 2.2%, 2.25%, 2.3%, 2.35%, 2.4%, 2.45%, 2.5%, 2.55%, 2.6%, 2.65%, 2.7%, 2.75%, 2.8%, 2.85%, 2.9%, 2.95%, 3.0%, 3.1%, 3.15%, 3.2%, 3.25%, 3.3%, 3.35%, 3.4%, 3.45%, 3.5%, 3.55%, 3.6%, 3.65%, 3.7%, 3.75%, 3.8%, 3.85%, 3.9%, 3.95%, 4.0%,. 4.1%, 4.15%, 4.2%, 4.25%, 4.3%, 4.35%, 4.4%, 4.45%, 4.4%, 4.45%, 4.5%, 4.55%, 4.6%, 4.65%, 4.7%, 4.75%, 4.8%, 4.85%, 4.9%, 4.95%, 5.0%, 5.5%, 5.7%, 6.0%, 6.5%%, 6.7%, 7.0%, 7.5%, 7.7%, 8.0%, 8.5%, 8.7%, 9.0%, 9.5%, 9.7%, and 10.0% Boswellin.

In addition to low molecular weight delivered agents, many medium molecular weight delivery delivered agents (eg., humates) can be delivered to cells in the body by using an embodiment of the transdermal delivery system. Synthetic humates ("Hepsyls") are medium molecular weight compounds (1,000 to 100,000 daltons), which are known to be strong antiviral and antimicrobial medicaments (International Application Publication No. WO 9834629 to Laub). Hepsyls are generally characterzed as polymeric phenolic materials comprised of conjugated aromatic systems to which are attached hydroxyl, carboxyl, and other covalently bound functional groups. A delivery system that can administer Hepsyls to cells of the body has several pharmaceutical uses, including but not limited to, treatment of topical bacterial and viral infections.

Accordingly, in another aspect of the invention, a transdermal. delivery system that can administer a medium molecular weight compound (e.g., a form of Hepsyl) to cells of the body is provided. As described above, many different medium molecular weight compounds can be administered by using an embodiment of a transdermal delivery system of the invention and the use of a medium molecular weight Hepsyl as a delivered agent is intended to demonstrate that embodiments of the invention can deliver many medium molecular weight compounds to calls of the body.

In addition to low molecular weight delivered agents and medium molecular weight delivered agents, several high molecular weight delivered agents (e.g., glycoproteins) can be delivered to cells in the body by using an embodiment of the transdermal delivery system. Glycoproteins are high molecular weight compounds, which are generally characterized as conjugated proteins containing one or more heterosaccharides as prosthetic groups. The heterosaccharides are usually branched but have a relatively low number of sugar residues, lack a serially repeating unit, and are covalently bound to a polypeptide chain. Several forms of glycoproteins are found in the body. For example, many membrane bound proteins are glycoproteins, the substances that fill the intercellular spaces (e.g., extracellular matrix proteins) are glycoproteins, and the compounds that compose collagens, proteoglycans, mucopolysaccharides, glycosaminoglycans, and ground substance are glycoproteins. A delivery system that can administer glycoproteins to cells of the body has several pharmaceutical and cosmetic uses, including but not limited to, the restoration of skin elasticity and firmness (e.g., the removal of wrinkles by transdermal delivery of collagen) and the restoration of flexible and strong joints (e.g., water retention in joints can be increased by transdermal delivery of proteoglycans).

Accordingly, in another aspect of the invention, a transdermal delivery system that can administer a high molecular weight compound (e.g., a form of collagen) to cells of the body is provided. As described above, many different high molecular weight compounds can be administered by using an embodiment of a transdermal delivery system of the invention and the use of a high molecular weight collagen as a delivered agent is intended to demonstrate that embodiments of the invention can deliver many high molecular weight compounds to cells of the body.

Collagens exist in many forms and can be isolated from a number of sources. Additionally, several forms of collagen can be obtained commercially (e.g., Brooks Industries Inc., New Jersey). Many low molecular weight collagens can be made, for example, by hydrolysis. Several transdermal delivery systems of the invention can deliver collagens having molecular weights below 6,000 daltons. Additionally, several high molecular weight collagens exist. Some are isolated from animal or plant sources and some are synthesized or produced through techniques common in molecular biology. Several transdermal delivery systems of the invention can deliver collagens having molecular weights of 1,000 daltons to greater than 2,000,000 daltons. That is, embodiments of the transdermal delivery systems can deliver collagens having molecular weights of less than or equal to 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, 5,000, 5,500, 6,000, 7,000, 8,000, 9,000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, 20,000, 21,000, 22,000, 23,000, 24,000, 25,000, 26,000, 27,000, 28,000, 29,000, 30,000, 31,000, 32,000, 33,000, 34,000, 35,000, 36,000, 37,000, 38,000, 39,000, 40,000, 41,000, 42,000, 43,000, 44,000, 45,000, 46,000, 47,000, 48,000, 49,000, 50,000, 51,000, 52,000, 53,000, 54,000, 55,000, 56,000, 57,000, 58,000, 59,000, 60,000, 61,000, 62,000, 63,000, 64,000, 65,000, 66,000, 67,000, 68,000, 69,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, 100,000, 125,000, 150,000, 175,000, 200,000, 225,000, 250,000, 275,000, 300,000, 350,000, 400,000, 450,000, 500,000, 600,000, 700,000, 800,000, 900,000, 1,000,000, 1,500,000, 1,750,000, and 2,000,000 daltons.

In some embodiments, the commercially available collagen "Hydrocoll EN-55" was provided as the delivered agent and was delivered to cells of a test subject. This form of collagen is hydrolyzed collagen and has a molecular weight of 2,000 daltons. In another embodiment, the commercially available collagen "Solu-Coll" was provided as the delivered agent and was delivered to cells of a test subject. This form of collagen is a soluble collagen having a molecular weight of 300,000 daltons. An additional embodiment includes the commercially available collagen "Plantsol", which is obtained from yeast and has a molecular weight of 500,000 daltons. This collagen was also provided as a delivered agent and was delivered to cells of a test subject.

The delivery systems of the invention having a form of collagen as a delivered agent desirably comprise by weight or volume between 0.1% to 50.0% collagen depending on the type of collagen, its solubility, and the intended application.

That is, some transdermal delivery systems of the invention comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 04%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%,. 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.25%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0% 8.25%, 8.5%, 8.75%, 9.0%, 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%, 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.0%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, 40.0%, 41.0%, 42.0%, 43.0%, 44.0%, 45.0%, 46.0%, 47.0%, 48.0%, 49.0%, or 50.0% collagen.

For example, embodiments having Hydrocoll-EN55 can comprise by weight or volume less than or equal to 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%, 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.25%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0% 8.25%, 8.5%, 8.75%, 9.0%, 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%, 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.0%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, 40.0%, 41.0%, 42.0%, 43.0%, 44.0%, 45.0%, 46.0%, 47.0%, 48.0%, 49.0%, or 50.0%, Hydrocoll-EN-55. Further, delivery systems of the invention having Solu-Coll can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 1.9%, 1.95%, or 2.0% Solu-Coll. Additionally, delivery systems of the invention having Plantsol can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 1.9%, 1.95%, 2.0%, 2.1%, 2.15%, 2.2%, 2.25%, 2.3%, 2.35%, 2.4%, 2.45%, 2.5%, 2.55%, 2.6%, 2.65%, 2.7%, 2.75%, 2.8%, 2.85%, 2.9%, 2.95%, 3.0%, 3.1%, 3.15%, 3.2%, 3.25%, 3.3%, 3.35%, 3.4%, 3.45%, 3.5%, 3.55%, 3.6%, 3.65%, 3.7%, 3.75%, 3.8%, 3.85%, 3.9%, 3.95%, or 4.0% Plantsol.

In other embodiments of the invention, a transdermal delivery system that can administer a collagen solution comprising two or more forms of collagen (e.g., Hydro-Coll EN-55, Solu-coll, or Plantsol) is provided. The delivery systems of the invention that include two or more forms of collagen desirably comprise an amount of delivered agent that can be included in a delivered agent having the specific type of collagen by itself. For example, if the mixture of delivered agents comprises Hydro-Coll EN55, the amount of Hydro-Coll EN55 in the transdermal delivery system can comprise by weight or volume less than or equal to 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%, 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.250%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0%, 8.25%, 8.5%, 8.75%, 9.0%, 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%,. 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.0%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, 40.0%, 41.0%, 42.0%, 43.0%, 44.0%, 45.0%, 46.0%, 47.0%, 48.0%, 49.0%, or 50.0% Hydrocoll-EN-55. Similarly if the mixture of delivered agents have Solu-coll, then the amount of Solu-coll in the delivery device can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.1%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 1.9%, 1.95%, or 2.0% or Solu-Coll. Further, if the mixture of delivered agents have Plantsol, then the amount of Plantsol in the delivery system can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.1%, 1.15%, 1.2%, 1.25%, 1.3%, 1.35%, 1.4%, 1.45%, 1.5%, 1.55%, 1.6%, 1.65%, 1.7%, 1.75%, 1.8%, 1.85%, 1.9%, 1.95%, 2.0%, 2.1%, 2.15%, 2.2%, 2.25%, 2.3%, 2.35%, 2.4%, 2.45%, 2.5%, 2.55%, 2.6%, 2.65%, 2.7%, 2.75%, 2.8%, 2.85%, 2.9%, 2.95%, 3.0%, 3.1%, 3.15%, 3.2%, 3.25%, 3.3%, 3.35%, 3.4%, 3.45%, 3.5%, 3.55%, 3.6%, 3.65%, 3.7%, 3.75%, 3.8%, 3.85%, 3.9%, 3.95%, or 4.0% Plantsol.

In the section below, there is a description of the manufacture and use of a penetration enhancer that enables the delivery of both low and high molecular weight molecules to the skin cells of the body.

Penetration Enhancers

A penetration enhancer included in many embodiments of the invention is comprised of two components--a

hydrophobic component and a hydrophilic component. Desirably, the hydrophobic component comprises a polyether compound, such as an ethoxylated vegetable or animal oil, that has the ability to reduce the surface tension of materials that are dissolved into it. Preferable ethoxylated oils can be obtained or created from, for example, castor oil, jojoba oil, corn oil, and emu oil. Desirably, the ethoxylated compound comprises at least 20-25 ethoxylations per molecule and preferably the ethoxylated compound comprises at least 30-35 ethoxylations per molecule. Thus, in a preferred embodiment, an ethoxylated oil comprises a molar ratio of ethylene oxide:oil of 35:1. A 99% pure ethylene oxide/castor oil having such characteristics can be obtained commercially (BASF) or such an ethoxylated compound can be synthesized using conventional techniques. Desirable compounds often found in ethoxylated oils that are beneficial for some embodiments and methods of the invention are glycerol-polyethylene glycol ricinoleate, the fatty esters of polyethylene glycol, polyethylene glycol, and ethoxylated glycerol. Some of these desirable compounds exhibit hydrophilic properties and the hydrophilic-lipophilic balance (HLB) is preferably maintained between 10 and 18. In some embodiments, more than one ethoxylated compound is added or another hydrophobic compound is added (e.g., Y-Ling-Y-Lang oil; Young Living Essential Oils, Lehl, Utah)) to balance or enhance the penetration enhancer.

Depending on the type of delivered agent and the intended application, the amount of ethoxylated lipid(s) in the delivery system can vary. For example, delivery systems of the invention can comprise between 0.1% and 40% by weight or volume ethoxylated compound(s). That is, embodiments of the invention can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%,. 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.25%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0% 8.25%, 8.5%, 8.75%, 9.0%, 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%,. 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.0%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, and 40.0% ethoxylated lipid(s).

The hydrophilic component of the penetration enhancers of the invention desirably comprise an alcohol, a non-ionic solubilizer, or an emulsifier. Compounds such as ethylene glycol, propylene glycol, dimethyl sulfoxide (DMSO), dimethyl polysiloxane (DMPX), oleic acid, caprylic acid, isopropyl alcohol, 1-octanol, ethanol (denatured or anhydrous), and other pharmaceutical grade or absolute alcohols with the exception of methanol can be used. Preferred embodiments comprise an alcohol (e.g., absolute isopropyl alcohol), which is commercially available. As above, the amount of hydrophilic component in the penetration enhancer depends on the type of the delivered agent and the intended application. The hydrophilic component of a penetration enhancer of the invention can comprise between 0.1% and 50% by weight or volume. That is, a delivery system of the invention can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%,. 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.25%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0% 8.25%, 8.5%, 8.75%, 9.0%, 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%, 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.00%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5%, 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, 40.0%, 41.0%, 42.0%, 43.0%, 44.0%, 45.0%, 46.0%, 47.0%, 48.0%, 49.0%, or 50.0% hydrophilic component.

In addition to the delivered agent and penetration enhancer, desirable transdermal delivery devices comprise a third component--an aqueous adjuvant. In the section below, there is described the manufacture and use of a preferred aqueous adjuvant, Aloe Vera, that enhances the delivery of both low and high molecular weight molecules to the skin cells of the body.

Aqueous Adjuvants

The term "Aloe" refers to the genus of South African plants of the Liliaceaefamily, of which the Aloe barbadensis plant is a species. Aloe is an intricate plant, which contains many biologically active substances. (Cohen, et al. in Wound Heating/Biochemical and Clinical Aspects, 1st ed. W B Saunders, Philadelphia (1992)). Over 300 species of Aloe are known, most of which are indigenous to Africa. Studies have shown that the biologically active substances are located in three separate sections of the Aloe leaf--a clear gel fillet located in the center of the leaf, in the leaf rind or cortex of the leaf and in a yellow fluid contained in the pericyclic cells of the vascular bundles, located between the leaf rind and the internal gel fillet, referred to as the latex. Historically, Aloe products have been used in dermatological applications for the treatment of bums, sores and other wounds. These uses have stimulated a great deal of research in identifying compounds from Aloe plants that have clinical activity, especially anti-inflammatory activity. (See, e.g., Grindlay and

Reynolds (1986) J. of Ethnopharmacology 16:117-151; Hart, et al. (1988) J. of Ethnopharmacology 23:61-71). As a result of these studies there have been numerous reports of Aloe compounds having diverse biological activities, including anti-tumor activity, anti-gastric ulcer, anti-diabetic, anti-tyrosinase activity, (See e.g., Yagi, et al. (1977) Z. Naturforsch. 32c:731-734), and antioxidant activity (International Application Serial No. PCT/US95/07404). Recent research has also shown that Aloe Vera, a term used to describe the extract obtained from processing the entire leaf, isolated from the Aloe Vera species of Aloe, can be used as a vehicle for delivering hydrocortisone, .E-backward.-estradiol, and testosterone propionate. (Davis, et al, JAPMA 81:1 (1991) and U.S. Pat. No. 5,708,038 to Davis)). As set forth in Davis (U.S. Pat. No. 5,708,308), one embodiment of "Aloe Vera" can be prepared by "whole-leaf processing" of the whole leaf of the. Aloe barbadensis plant. Briefly, whole leaves obtained from the Aloe barbadensis plant are ground, filtered, treated with cellulase (optional) and activated carbon and lyophilized. The lyophilized powder is then reconstituted with water prior to use.

In some embodiments, Aloe Vera is commercially available and obtained through Aloe Laboratories. In some embodiments, the Aloe Vera is manufactured by manually harvesting the Aloe leaves. Next, the leaves are washed with water and the thorns on both ends are cut. The leaves are then hand-filleted so as to extract the inner part of the leaf. The inner gel is passed through a grinder and separator to remove fiber from the gel. Next, the gel is put into a pasturizing tank where L-Ascorbic Acid (Vitamin C) and preservatives are added. The gel is paturized at 85.degree. C. for 30 minutes. After pasturization, the gell is put into a holding tank for about one or two days, after which the gel is sent through a 1/2 micron filter. Finally, the gel is cooled down through a heat exchanger and stored in a steamed, sanitized and clean 55 gallon drum.

The above described sources and manufacturing methods of Aloe Vera are given as examples and not intended to limit the scope of the invention. One of ordinary skill in the art will recognize that Aloe Vera is a well known term of art, and that Aloe Vera is available from various sources and manufactured according to various methods.

Several embodiments of the invention are comprised of aqueous adjuvants such as Aloe Vera juice or water or both. Absolute Aloe Vera (100% pure) can be obtained from commercial suppliers (Lily of the Desert, Irving, Texas). Aloe Vera juice, prepared from gel fillet, has an approximate molecular weight of 200,000 to 1,400,000 daltons. Whole leaf Aloe Vera gel has a molecular weight of 200,000 to 3,000,000 depending on the purity of the preparation. Although, preferably, the embodiments of the invention having Aloe Vera comprise Aloe Vera juice, other extracts from a member of the Liliaceae family can be used (e.g., an extract from another Aloe species).

Transdermal delivery systems of the invention having Aloe Vera can comprise between 0.1% to 85.0% by weight or volume Aloe Vera. That is, embodiments of the invention can comprise by weight or volume less than or equal to 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%, 0.9%, 0.95%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.25%, 2.5%, 2.75%, 3.0%, 3.25%, 3.5%, 3.75%, 4.0%,. 4.25%, 4.5%, 4.75%, 5.0%, 5.25%, 5.5%, 5.75%, 6.0%, 6.25%, 6.5%, 6.75%, 7.0%, 7.25%, 7.5%, 7.75%, 8.0% 8.25%, 8.5%, 8.75%, 9.0% 9.25%, 9.5%, 9.75%, 10.0%, 10.25%, 10.5%, 10.75%, 11.0%,. 11.25%, 11.5%, 11.75%, 12.0%, 12.25%, 12.5%, 12.75%, 13.0%, 13.25%, 13.5%, 13.75%, 14.0%, 14.25%, 14.5%, 14.75%, 15.0%, 15.5%, 16.0%, 16.5%, 17.0%, 17.5%, 18.0%, 18.5%, 19.0%, 19.5%, 20.0%, 20.5%, 21.0%, 21.5%, 22.0%, 22.5%, 23.0%, 23.5%, 24.0%, 24.5%, 25.0%, 25.5%, 26.0%, 26.5%, 27.0%, 27.5%, 28.0%, 28.5%, 29.0%, 29.5% 30.0%, 30.5%, 31.0%, 31.5%, 32.0%, 32.5%, 33.0%, 33.5%, 34.0%, 34.5%, 35.0%, 35.5%, 36.0%, 36.5%, 37.0%, 37.5%, 38.0%, 38.5%, 39.0%, 39.5%, 40.0%, 40.25% 40.5%, 40.75%, 41.0%, 41.25%, 41.5%, 41.75%, 42.0%, 42.25%, 42.5%, 42.75%, 43.0%,. 43.25%, 43.5%, 43.75%, 44.0%, 44.25%, 44.5%, 44.75%, 45.0%, 45.25%, 45.5%, 45.75%, 46.0%, 46.25%, 46.5%, 46.75%, 47.0% 47.25%, 47.5%, 47.75%, 48.0%, 48.25%, 48.5%, 48.75%, 49.0%, 49.25%, 49.5%, 49.75%, 50.0%, 50.25%, 50.5%, 50.75%, 51.0%, 51.25%, 51.5%, 51.75%, 52.0%, 52.25%, 52.5%, 52.75%, 53.0%, 53.25%, 53.5%, 53.75%, 54.0%, 54.5%, 54.0%, 54.5%, 55.0%, 55.5%, 56.0%, 56.5%, 57.0%, 57.5%, 58.0%, 58.5%, 59.0%, 59.5%, 60.0%, 60.5%, 61.0%, 61.5%, 62.0%, 62.5%, 63.0%, 63.5%, 64.0%, 64.5%, 65.0%, 65.5%, 66.0%, 66.5%, 67.0%, 67.5%, 68.0%, 68.5%, 69.0%, 69.5%, 70.0%, 70.5%, 71.0%, 71.5%, 72.0%, 72.5%, 73.0%, 73.5%, 74.0%, 74.5%, 75.0%, 75.5%, 76.0%, 76.5%, 77.0%, 77.5%, 78.0%, 78.5%, 79.0%, 79.5%, 80.0%, 80.5%, 81%, 81.5%, 82%, 82.5%, 83%, 83.5%, 84%, 84.5%, and 85% Aloe Vera.

The amount of water in the delivery system generally depends on the amount of other reagents (e.g., delivered agent, penetration enhancer, and other aqueous adjuvants or fillers). Although water is used as the sole aqueous adjuvant in some embodiments, preferred embodiments use enough water to make the total volume of a particular preparation of a delivery system such that the desired concentrations of reagents in the penetration enhancer, aqueous adjuvant, and delivered agent are achieved. Suitable forms of water are deionized, distilled, filtered or otherwise purified. Clearly, however, any form of water can be used as an aqueous adjuvant.

In addition to the aforementioned compositions, methods of making and using the transdermal delivery systems of the invention are provided below.

Preparing Transdermal Delivery Systems

In general, an embodiment of the invention is prepared by combining a penetration enhancer with an aqueous adjuvant and a delivered agent. Depending on the solubility of the delivered agent, the delivered agent can be solubilized in either the hydrophobic or hydrophilic components of the penetration enhancer. Additionally, some delivered agents can be solubilized in the aqueous adjuvant prior to mixing with the penetration enhancer. Desirably, the pH of the mixture is maintained between 3 and 11 and preferably between 5 and 9. That is, during preparation and after preparation the pH of the solution is desirably maintained at less than or equal to 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5, 9.75, 10.0, 10.25, 10.5, 10.75, or 11.0. Several physical mixing techniques can be employed to help the delivery system coalesce. For example, a magnetic stir plate and bar can be used, however, the speed of stirring is preferably minimized so as not to drive air into the mixture. Additionally, a rocker can be used to bring components of the delivery system together. Heat can also be applied to help coalesce the mixture but desirably, the temperature is not raised above 40.degree. C. so that labile aqueous adjuvants or labile delivered agents are not degraded. Preferably, once the delivery system has coalesced, other components such as fragrances and colors are added or the delivery system is incorporated into a cream or ointment or a device for applying the delivery system.

Several formulations of delivery system are within the scope of aspects of the invention. Desirably, the ratio of hydrophilic component:hydrophobic component:aqueous adjuvant is 3:4:3, but preferred formulations comprise 1:1:4, 1:1:14, and 1:10:25. As described above, a sufficient amount of delivered agent to suit the intended purpose is incorporated into the delivery system. The amount of delivered agent that is incorporated into the penetration enhancer depends on the compound, desired dosage, and application.

Typically, a preferable transdermal delivery system is made by providing an ethoxylated oil, mixing the ethoxylated oil with an alcohol, non-ionic solubilizer, or emulsifier so as to form a penetration enhancer, mixing the penetration enhancer with an aqueous adjuvant (e.g., an extract from a plant of the Liliaeacae family), and mixing the penetration enhancer and aqueous adjuvant with a delivered agent and thereby making the transdermal delivery system. For example, an embodiment of a transdermal delivery system comprising a pain relief solution is manufactured as follows. A solution of 2.0% to 7.0% oleoresin capsicum, 2.5 grams of Boswellin, and 1.5 mls of a 65% solution of Frankensence is mixed with 400 ml of absolute carpilic alcohol or isopropyl alcohol, 300 ml of ethylene oxide admixed and reacted with castor oil, and 300 ml of a 100% solution of Aloe Vera. This transdermal delivery system has been observed to alleviate pain when rubbed on a targeted area.

The delivery systems of the invention having a form of Hepsyl as a delivered agent desirably are comprised by weight or volume of between 0.005% to 12.0% Hepsyl, depending on the type of Hepsyl, its solubility, and the intended application. For example, embodiments having Hepsyl CA 1501C. Hepsyl CGA 1501K., and Hepsyl RA 150K can be comprised by weight or volume of 0.01-2 grams of Hepsyl delivered agent, 0-50 mL of hydrophobic penetration enhancers (e.g., ethoxylated castor oil, jojoba oil, etc.), 0-50 mL of hydrophilic penetration enhancers, nomonic solubilizers, or emulsifiers (e.g., isopropyl. alcohol, DMSO, etc.), and 0-50 mL of aqueous adjuvant (e.g., water, Aloe Vera extract, etc.). A particularly desirable embodiment of the invention is comprised of 0.1-0.5 gram of Hepsyl, 5-10 mL of ethoxylated castor oil, 5-10 mL of isopropyl alcohol, and 5-10 mL of Aloe Vera extract.

By using these formulations, other delivered agents can incorporated into a transdermal delivery system. Formulations of transdermal delivery systems having collagens are described in Example 2.

In the disclosure below, several therapeutic, prophylactic and cosmetic applications are provided.

Therapeutic, Prophylactic, and Cosmetic Applications

Many embodiments are suitable for treatment of subjects either as a preventive measure (e.g., to avoid pain or skin disorders) or as a therapeutic to treat subjects already afflicted with skin disorders or who are suffering pain. In one embodiment, a method of treatment or prevention of inflammation, pain, or human diseases, such as cancer, arthritis, and Alzheimer's disease, comprises using a trnsdennal delivery system of the invention. Because delivered agents such as NSAIDs, capsaicin, and Boswellin interfere and/or inhibit cyclooxygenase enzymes (COX and COX-2), they will provide a therapeutically beneficial treatment for cancer and Alzheimer's disease when administered by a transdermal delivery system of the invention. (U.S. Pat. No. 5,840,746 to Ducharmne et al., and U.S. Pat. No. 5,861,268 to Tang et al.).

By one approach, a taansdermal delivery system comprising a delivered agent that is effective at reducing pain or inflammation (e.g., NSAIDS, capsaicin, Boswellin, or any combination thereof) is administered to a subject in need and the reduction in pain or inflammation is monitored. An additional approach involves identifying a subject in need of a

COX enzyme inhibitor (e.g., a subject suffering from cancer or Alzheimer's disease) and administering a transdermal delivery system comprising a delivered agent that inhibits a COX enzyme (e.g., NSAIDS, capsaicin, Boswellin, or any combination thereof). Although many individuals can be at risk for contracting cancer or Alzheimer's disease, those with a family history or a genetic marker associated with these maladies are preferably identified. Several diagnostic approaches to identify persons at risk of developing these diseases have been reported. (See e.g., U.S. Pat. Nos., 5,891,857; 5,744,368; 5,891,651; 5,837,853; and 5,571,671). The transdermal delivery system is preferably applied to the skin at a region of inflammation or an area associated with pain or the particular condition and treatment is continued for a sufficient time to reduce inflammation, pain, or inhibit the progress of the disease. Typically, pain and inflammation will be reduced in 5-20 minutes after application. Cancer and Alzheimer's disease can be inhibited or prevented with prolonged use.

In another method of the invention, an approach to reduce wrinkles and increase skin tightness and flexibility (collectively referred to as "restoring skin tone") is provided. Accordingly, a transdermal delivery system comprising a form of collagen as a delivered agent is provided and contacted with the skin of a subject in need of treatment. By one approach, a subject in need of skin tone restoration is identified, a transdermal delivery system comprising collagen is administered to the subject, and the restoration of the skin tone is monitored. Identification of a person in need of skin restoration can be based solely on visible inspection and the desire to have tight, smooth, and flexible skin. Treatment with the delivery system is continued until a desired skin tone is achieved. Typically a change in skin tone will be visibly apparent in 15 days but prolonged use may be required to retain skin tightness and flexibility. The form of collagen in the delivered agent can be from various sources and can have many different molecular weights, as detailed above. Preferably, high molecular weight collagens are used.

The transdermal delivery systems of this invention can be processed in accordance with conventional pharmacological and cosmetological methods to produce medicinal agents and cosmetics for administration to patients, e.g., mammals including humans. The transdermal delivery systems described herein can be incorporated into a pharmaceutical or cosmetic product with or without modification. The compositions of the invention can be employed in admixture with conventional excipients, e.g., pharmaceutically acceptable organic or inorganic carrier substances suitable for topical application that do not deleteriously react with the molecules that assemble the delivery system. The preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, coloring, aromatic substances and the like that do not deleteriously react with the active compounds. They can also be combined where desired with other active agents.

The effective dose and method of administration of a carrier system formulation can vary based on the individual patient and the stage of the disease, as well as other factors known to those of skill in the art. Although several doses of delivered agents have been indicated above, the therapeutic efficacy and toxicity of such compounds in a delivery system of the invention can be determined by standard pharmaceutical or cosmetological procedures with experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical and cosmetological compositions that exhibit large therapeutic indices are preferred. The data obtained from animal studies is used in formulating a range of dosages for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors that may be taken into account include the severity of the disease state, age, weight and gender of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Short acting compositions are administered daily whereas long acting pharmaceutical compositions are administered every 2, 3 to 4 days, every week, or once every two weeks. Depending on half-life and clearance rate of the particular formulation, the pharmaceutical compositions of the invention are administered once, twice, three, four, five, six, seven, eight, nine, ten or more times per day.

Routes of administration of the delivery systems of the invention are primarily topical, although it is desired to administer some embodiments to cells that reside in deep skin layers. Topical administration is accomplished via a topically applied cream, gel, rinse, etc. containing a delivery system of the invention. Compositions of delivery system-containing compounds suitable for topical application include, but are not limited to, physiologically acceptable ointments, creams, rinses, and gels.

In some embodiments, the mixture of penetration enhancer, aqueous adjuvant, and delivered agent is incorporated into a device that facilitates application. These apparatus generally have a vessel joined to an applicator, wherein a transdermal

delivery system of the invention is incorporated in the vessel. Some devices, for example, facilitate delivery by encouraging vaporization of the mixture. These apparatus have a transdermal delivery system of the invention incorporated in a vessel that is joined to an applicator such as a sprayer (e.g., a pump-driven sprayer). These embodiments can also comprise a propellant for driving the incorporated transdermal delivery system out of the vessel. Other apparatus can be designed to allow for a more foccused application. A device that facilitates a foccused application of a transdermal delivery system of the invention can have a roll-on or swab-like applicator joined to the vessel that houses the transdermal delivery system. Several devices that facilitate the administration of a delivery system of the invention have a wide range of cosmetic or therapeutic applications.

In the example below a clinical study is described that was performed to evaluate the efficacy of a transdermal delivery system that administered a low molecular weight pain relief solution comprising capsaicin.

EXAMPLE 1

In this example, evidence is provided that a transdermal delivery system of the invention can administer a therapeutically effective amount of a low molecular weight delivered agent (e.g., 0.225% oleoresin capsicum). A clinical study was performed to evaluate the effectiveness of a transdermal delivery system of the invention comprising 0.225% capsaicin ("EPRS") as compared to a commercially available cream comprising Boswellin, 10% methyl salicylate, and 0.25% capsaicin. (Nature's Herbs). The two pain relief preparations were tested on six subjects who suffer from degenerative arthritis, debilitating back pain, and/ or bursitis. For the first five days of the study, the subjects applied the commercially available cream three times a day. On day six, application of the commercially available cream was stopped and subjects applied the EPRS transdermal delivery system. The EPRS pain relief solution was also applied for five days, three times a day. Daily analysis of the efficacy of the particular pain relief formulations was taken by the subjects and observations such as the time of administration, odor, and therapeutic benefit were recorded after each administration.

The five day use of the commercially available cream was found to provide only minimal therapeutic benefit. The cream was reported to irritate the skin, have a noxious smell, and provide little decrease in pain or increase in flexibility or range of motion. In contrast, the five day use of EPRS was reported to provide significant pain relief, relative to the relief obtained from the oral consumption of NSAIDs. Further, EPRS was reported to increase flexibility and range of motion within five to twenty minutes after application. Additionally, EPRS did not present a significant odor nor did it cause skin irritation. The results of this study demonstrate that a delivery system comprising a low molecular weight compound, capsaicin, can effectively administer the delivered agent to cells of the body where it provides therapeutic benefit.

In the example below, a clinical study is described that was performed to evaluate the efficacy of a transdermal delivery system that administered low and high molecular weight collagens.

EXAMPLE 2

In this example, evidence is provided that a transdermal delivery system of the invention can administer a therapeutically effective amount of a low and high molecular weight delivered agent (e.g., a low and high molecular weight collagen). A clinical study was performed to evaluate the effectiveness of several transdermal delivery systems of the invention comprising various penetration enhancers, aqueous adjuvents, and collagen delivered agents. The various transdermal delivery systems that were evaluated are provided in Table 3. Of the formulations that were originally screened, three were extensively evaluated by ten subjects (three men and seven women) in a single blind study. The formulations analyzed in the single blind study are indicated in Table 3 by a dagger. That is, the three different formulations ("P1", "P2", and "F4") were evaluated.

The P1 formulation comprised approximately 0.73% to 1.46% Solu-Coll, a soluble collagen having a molecular weight of 300,000 daltons. The P2 formulation comprised approximately 1.43% to 2.86% Plantsol, a plant collagen obtained from yeast having a molecular weight of 500,000 daltons. The F4 formulation comprised approximately 11.0% of HydroColl EN-55, a hydrolyzed collagen having a molecular weight of 2,000 daltons. The evaluation of the P1, P2, and F4 formulations was as follows. Left, right, and center mug-shot photographs were taken with a Pentax camera having a zoom 60.times. lens and Kodak-Gold 100 film before beginning the study. Shortly after, each subject was given a bottle having a formulation of transdermal delivery system and was instructed to apply the solution to the right side of the face and neck, leaving the left side untreated, twice daily for 15 days. The F4 formulation was tested first and the application was carried out after showering or washing and before application of any other product to the treated area of the face. After the 15 day period, three mug-shot photographs were again taken, the subjects recorded their observations on the effectiveness of the formulation in a questionaire, and a 7 day period without application of a collagen product provided. The questionaire requested the subject to assign a score (e.g., a numerical value that represents effectiveness) on characteristics of the transdermal delivery system formulation. Characteristics that were

evaluated included tackiness, odor, marketability, and overall effectiveness of the formulation, as well as, whether the formulation tightened the skin, decreased lines, conditioned or softened the skin, and had any negative side-effects. The scale for the scoring was 1-10, with 1 being the worst rating and 10 being the best rating.

Following the test of F4, the evaluation detailed above was conducted on the P1 formulation. Again, photographs were taken before and after the second 15 day protocol, a questionaire evaluating the efficacy of the particular formulation was completed, and a 7 day period without application of a collagen product was provided. Further, after the test of P1, the same evaluation was conducted on the P2 formulation, photographs were taken before and after the trial, and a questionaire evaluating the efficacy of the particular formulation was completed.

The data from the three evaluation questionaires were pooled, analyzed using a "t-table" and standard deviation calculations were made. See Table 4. An overall rating for each particular formulation was assigned. A perfect score by this system was a 7.875 overall rating. P1 was found to have a 4.25 overall rating (approximately 54% effective), P2 was found to have a 4.625 overall rating (approximately 59% effective), and F4 was found to have a 5.625 overall rating (approximately 71% effective).

The before and after treatment photographs also revealed that the three tested transdermal delivery systems provided therapeutic benefit. A decrease in wrinkles was observed and an increase in skin tightness and firmness can be seen. That is, P1, P2, and F4 all provided therapeutic and/or cosmetic benefit in that they restored skin tone in the subjects tested. The results presented above also demonstrate that transdermal delivery systems of the invention can be used to administer high molecular weight delivered agents.

TABLE 3 ECO Aloe IPA Plantsol EN-55 Solu-coll DMPX YYO Score ID 29.7%* 50.0%* 5.0%* 0* 8.3%* 0* 0* 0* 2 F-1 10.4% 79.0% 5.3% 0 8.7% 0 0 0 3 F-2 5.2% 63.0% 5.3% 0 17.4% 0 0 0 3 F-3 5.0% 70.0% 5.0% 0 11.0% 0 0 0 3+ F-4 .dagger. 4.5% 18.2% 4.6% 0 0 0.7% to 0 0 3+ P-1 .dagger. 1.5% 8.3% 8.3% 8.3% 0.7% to 4.6% 0.3% to 0 0 2 Y-500 1.4% 0.7% 0.7% 22.2% 11.1% 1.3% to 0 0 0 0 3+ P-501 2.7% 0.4% 35.7% 3.6% 1.1% to 0 0 0 0 2 P-502 2.1% 0.9% 8.7% 0 0 0 2.3% to 0 0 1 SC-1 4.6% 1.8% 18.5% 0 0 44.8% 0 0 0 3+ SC-2 1.8% 17.9% 7.1% 0 43.2% 0 0 0 3 SC-3 0.9% 9.4% 4.7% 0 34.3% 0.3% to 0 0 1 PSCE 0.6% N 1.8% 31.3% 6.3% 1.3% to 0 0 0 0 3+ P-1A 2.5% 0.8% 19.2% 3.8% 1.5% to 0 0 7.7% 0.3% 5 P-1C 3.1% 0.7% 17.9% 7.1% 1.4% to 0 0 1.1% 0.3% 5 P-2 2.9% .dagger. 0.7% 22.2% 11.1% 1.3% to 0 0 0 0 3+ P-501 2.7% Abbreviations: ECO--ethoxylated castor oil (BASF) Aloe--Aloe Vera (Aloe Labs; (800)-258-5380) IPA--Absolute isopropyl alcohol (Orange County Chemical, Santa Ana, California) Plantsol--Yeast extract collagen (Brooks Industries Inc., Code No. 06485) EN-55--hydrolyzed bovine collagen (Brooks Industries Inc., Code No. 01000) SoluColl--soluble collagen (Brooks Industries Inc., Code No. 01029) DMPX--dimethyl polysiloxane (5 centistokes) (Sigma) YYU--Y-ling-Y-lang oil (Young Living Essential Oils, Lehl, Utah) ID--Identification number *The percentages reflect volume to volume. .dagger. Sample used in the 45 day clinical trial.

TABLE 4 Collagen T-Table standard Formulations P1 P2 F4 deviation Tackiness 5 3 10 2.94 Skin tightness 7 5 8 1.25 Odor 2 8 8 2.83 Decrease lines 2 2 1 0.47 Soften skin 8 7 4 1.7 Total skin 5 5 6 0.47 restoration Market Buying 5 7 8 1.25 Power Side effects 0 0 0 0 Total Score 4.25 4.63 5.63 1.36 (Average)

Several in vitro techniques are now widely used to assess the percutaneous absorption of delivered agents. (See e.g., Bronaugh and Collier in In vitro Percutaneous absorption studies: Principle, Fundementals, and Applications, eds. Bronaugh and Maibach, Boca Raton, Fla., CRC Press, pp237-241 (1991) and Nelson et al., J. Invest. Dermatol. 874-879 (1991), herein incorporated by reference). Absorption rates, and skin metabolism can be studied in viable skin without the interference from systemic metabolic processess. In the example below, several approaches are described that can be used to evaluate the administration of a delivered agent by using a transdermal delivery system of the invention.

EXAMPLE 3

Skin barrier function can be analyzed by examining the diffusion of fluorescent and colored proteins and dextrans of various molecular weights ("markers") across the spin of nude mice or swine. Swine skin is preferred for many studies because it is inexpensive, can be maintained at -20.degree. C., and responds similarly to human skin. Prior to use, frozen swine skin is thawed, hair is removed, and subcutaneous adipose tissue is dissected away. Preferably, a thickness of skin that resembles the thickness-of human skin is obtained (e.g., several millimeters) so as to prepare a membrane that accurately reflects the thickness of the barrier layer. A dermatome can be pushed across the surface of the skin so as to remove any residual dermis and prepare a skin preparation that accurately reflects human skin. Elevation of temperature can also be used to loosen the bond between the dermis and the epidermis of hairless skin. Accordingly, the excised skin is placed on a hot plate or in heated water for 2 minutes at a temperature of approximately 50.degree. C.-60.degree. C. and the dermis is removed by blunt dissection. Chemical approaches (e.g., 2M salt solutions) have also been used to separate the dermis from the epidermis of young rodents.

Many different buffers or receptor fluids can be used to study the transdermal delivery of delivered agents across excised skin prepared as described above. Preferably, the buffer is isotonic, for example a normal saline solution or an isotonic buffered solution. More physiological buffers, which contain reagents that can be metabolized by the skin, can also be used. (See e.g., Collier et al., Toxicol. Appl. Pharmacol. 99:522-533 (1989)).

Several different markers with molecular weight from 1,000 daltons to 2,000,000 daltons are commercially available and can be used to analyze the transdermal delivery systems of the invention. For example, different colored protein markers having a wide range of molecular weights (6,500 to 205,000 daltons) and FITC conjugated protein markers (e.g., FITC conjugated markers from 6,500 to 205,000 daltons) are available from Sigma (C3437, M0163, G7279, A2065, A2190, C1311, T9416, L8151, and A2315). Further, high molecular weight FITC conjugated dextrans (e.g., 250,000, 500,000, and 2,000,000 daltons) are obtainable from Sigma. (FD250S, FD500S, and FD2000S).

Accordingly, in one approach, swine skin preparations, obtained as described above, are treated with a delivery system lacking a delivered agent and control swine skin preparations are treated with water. Subsequently, the skin is contacted with a 1 mM solution of a marker with a known molecular weight suspended in Ringer's solution (pH 7.4) at 37.degree. C. After one hour, the skin is frozen and sliced at a thickness of 5:m. The sections are counter stained with 5:g/ml propidium iodide and, if the marker is FITC conjugated, the sections are analyzed by fluoresence microscopy. If the marker is a colored marker, diffusion of the marker can be determined by light microscope. The marker will be retained in the upper layers of the stratum corneum in the untreated mice but the delivery system treated mice will be found to have the dye distributed throughout the stratum corneum and any dermal layer that remains.

Additionally, modifications of the experiments described above can be performed by using a delivery system comprising various molecular weight markers. Accordingly, skin preparations are treated with the delivery system comprising one or more markers and control skin preparations are treated with water. After one hour, the skin is frozen and sliced at a thickness of 5:m. The sections can be counter stained with 5:g/ml propidium iodide and can be analyzed by fluoresence microscopy (e.g., when a fluorescent marker is used) or alternatively, the sections are analyzed under a light microscope. The various markers will be retained in the upper layers of the straum comeum in the untreated mice but the delivery system treated mice will be found to have the marker distributed throughout the stratum corneum and any dermal layer that remains.

In another method, the transdermal water loss (TEWL) of penetration enhancer-treated skin preparations can be compared to that of untreated skin preparations. Accordingly, skin preparations are obtained, as described above, and are treated with a delivery system of the invention lacking a delivered agent (e.g., a penetration enhancer). Control skin preparations are untreated. To assess TEWL, an evaporimeter is used to analyze the skin preparation. The Courage and Khazaka Tewameter TM210, an open chamber system with two humidity and temperature sensors, can be used to measure the water evaporation gradient at the surface of the skin. The parameters for calibrating the instrument and use of the instrument is described in Barel and Clarys Skin Pharmacol. 8: 186-195 (1995) and the manufacturer's instructions. In the controls, TEWL will be low. In contrast, TEWL in penetration enhancer-treated skin preparations will be significantly greater.

Further, skin barrier function can be analyzed by examining the percutaneous absorption of labeled markers (e.g., radiolabeled, fluorescently labeled, or colored) across skin preparations in a diffusion chamber. Delivery systems of the invention having various molecular weight markers, for example, the proteins and dextrans described above, are administered to swine skin preparations. Swine skin preparations are mounted in side-by-side diffusion chambers and are allowed to stabilize at 37.degree. C. with various formulations of penetration enhancer. Donor and receiver fluid volumes are 1.5 ml. After 1 hour of incubation, a labeled marker is added to the epidermal donor fluid to yield a final concentration that reflects an amount that would be applied to the skin in an embodiment of the invention. Five hundred:1 of receiver fluid is removed at various time points, an equal volume of penetration enhancer is added to the system. The aliquot of receiver fluid removed is then analyzed for the presence of the labeled marker (e.g., fluorescent detection, spectroscopy, or scintillation counting). Control swin skin preparations are equilibrated in Ringer's solution (pH 7.4) at 37.degree. C.; the same concentration of labeled marker as used in the experimental group is applied to the donor fluid after one hour of equilibration; and 500:1 of receiver fluid is analyzed for the presence of the marker. In the experimental group, the steady-state flux of labeled marker in the skin will be significantly greater than that of the control group.

By using these approaches, several transdermal delivery systems can be evaluated for their ability to transport low and high molecular weight delivered agents across the skin.

Although the invention has been described with reference to embodiments and examples, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All references cited herein are hereby expressly incorporated by reference.

* * * * *


Images
View Cart
Add to Cart
Hit List
Next
Top
Home
Quick
Advanced
Pat Num
Help

Dermatol. Monatsschr. 165 (1979) 505—509

Aus der Hautklinik (Direktor: Prof. Dr. sc. med. H. SCHUBERT)
und dem Institut für Medizinische Mikrobiologie (Direktor: Prof. Dr. sc. med. M. SPRÖSSIG)
der Medizinischen Akademie Erfurt

Ergebnisse einer orientierenden klinischen Prüfung von Ammoniumhumat zur Lokalbehandlung von Herpesvirus-hominis-(HVH)-Infektionen

Von F. SCHILLER, RENATE KLÖCKING, P. WUTZLER und INGE FÄRBER

Zusammenfassung: Es wird über eine erfolgreiche Lokaltherapie von Herpesvirus-hominis-(Herpes-simplex-Virus)-Erkrankungen mit 1%iger Ammoniumhumatlösung berichtet. Bei 70 von insgesamt 78 Fällen wurde der Krankheitsverlauf günstig beeinflußt. Damit konnte die in vitro nachgewiesene antivirale Wirksamkeit von Ammoniumhumat gegenüber Herpes-simplex-Infektionen auch am Menschen bestätigt werden. Die Fortsetzung der klinischen Prüfung von Ammoniumhumat wird als gerechtfertigt angesehen.

Schlüsselwörter: Herpes-simplex-Virus-Infektion – Virustatika – Ammoniumhumat.

Topical ammoniumhumat treatment of human HSV infections – Preliminary clinical results

Summary: Successful local treatment of herpesvirus hominis (herpes simplex virus) infections with a solution of 1% ammonium humate is reported. In 70 out of 78 cases ammonium humate showed a beneficial action on the course of the disease. The antiviral effectiveness of this substance proved in vitro against herpesvirus hominis infections could be confirmed in human beings too. Therefore, continuation of clinical trials with ammonium humate is considered to be justified.

Key-words: Herpes simplex virus infections – Ammoniumhumate – Antiviral treatment.

Herpesvirus-hominis-(Herpes-simplex-Virus)-Infektionen der Haut sind zum Teil außerordentlich unangenehm und schmerzhaft. Sie beeinflussen viele Patienten auch unter ästhetischen Gesichtspunkten schwer. Obwohl die Hauteffloreszenzen normalerweise keine Narben hinterlassen, können häufige Rezidive in loco zur entzündlich bedingten Verlegung der Lymphwege mit Bildung eines stabilen Ödems und einer irreversiblen elephantiasisartigen Schwellung der Weichteile führen [6].

Eine gezielte kausale Therapie rezidivierender HVH-Infektionen ist bislang nicht möglich. Die Immunisierung mit Herpesvirus-Impfstoffen, wie sie von BIBERSTEIN und JESSNER [1], SÖLTZ-SZÖTZ [20], DEGOS und TOURAINE [2], KOINIS und WÜTHRICH [13] sowie von NASEMANN [18] beschrieben wurde, führt nur bei einem Teil der Patienten zur Heilung bzw. zum verlängerten rezidivfreien Intervall, so daß der Lokalbehandlung auch weiterhin Bedeutung zukommt. Neben zahlreichen austrocknend wirkenden Lösungen und Pasten, denen antimikrobiell wirkende Substanzen und Steroide zugesetzt sind [17], werden vor allem 5-Jod-2'-desoxyuridin (IDU) enthaltende Präparate verwendet. Über Erfolge mit IDU berichteten zuerst KAUFMANN [4] und KAUFMANN und MALONEY [5], später unter anderen THIEL und WACKER [23] sowie NASEMANN und BRAUN-FALCO [19]. Da IDU eine ganze Reihe von Nebenwirkungen an der menschlichen Zelle, speziell an Histiozyten und Lymphozyten (Chromosomenbrüche) entfaltet [26] und infolge seines Eingriffs in die DNS-Synthese als potentielles Kanzerogen und Teratogen anzusehen ist, kommt es für eine standardmäßige interne Therapie nicht in Betracht. Nach WOLLENSAK und KYPKE [27] wird IDU auch in Korneazellen eingebaut und kann zu fibrinoider

Nekrose und granulärer Degeneration des Hornhautparenchyms mit nachfolgender Perforation der Kornea führen. Über gute Erfahrungen mit fotodynamischer Farbstofftherapie berichten MEFFERT und EL BASHIR [15] und über den therapeutischen Einsatz von Dipyridamol (Curantyl®) GÜNTHER und Mitarb. [3].

Obwohl in letzter Zeit die Anwendung von Phosphonoessigsäure im Tierexperiment [7] gewisse Hoffnungen auf die Verhinderung von Rezidiven bei HVH-induzierten Hautläsionen geweckt hat, ist ein optimales Lokaltherapeutikum für HVH-Infektionen bisher nicht bekannt. Die Einschätzung NASEMANNs [16], daß jede Verbesserung antiherpetischer Externa dem Therapeuten willkommen sein wird, hat auch heute noch ihre volle Gültigkeit und veranlaßte uns, die Wirksamkeit von Ammoniumhumat bei der Lokalbehandlung von HVH-Infektionen zu untersuchen.

Bei der verwendeten Substanz handelt es sich um ein Phenolkörperpolymerisat natürlicher Herkunft, das, wie auch die durch enzymatische Oxydation von o-Diphenol-Verbindungen hergestellten synthetischen Phenolkörperpolymerisate, im Konzentrationsbereich von 10 bis 1 µg/ml gegen HVH Typ 1 und HVH Typ 2 in vitro antiviral wirksam ist [10, 21]. Auch in vivo konnte an der mit HVH Typ 1 infizierten Kaninchenkornea die Wirksamkeit eines Huminsäurepräparates, das im Frühstadium der Infektion lokal appliziert wurde, nachgewiesen werden [11].

Ammoniumhumat besitzt außer seiner antiviralen auch eine antiphlogistische Wirkkomponente [8]. Nachdem die Prüfung der Substanz auf Hautverträglichkeit im Konjunktival-, Epikutan- und Gebrauchstest beim Menschen sowie die Prüfung der Sensibilisierungspotenz am Meerschweinchen keinerlei Hinweise auf Unverträglichkeitserscheinungen ergeben hatte, wurde mit der vorliegenden Studie begonnen.

Material und Methoden

Auswahl der Probanden: Wir behandelten insgesamt 78 Patienten (56 ♀, 22 ♂) mit rezidivierenden HVH-Infektionen. Davon hatten 12 erstmalig Hauterscheinungen, 66 kamen nach einem oder mehreren Rezidiven zur Behandlung. 50 Patienten wurden im Blasen-, 28 im Blasen- und Krustenstadium behandelt. Vor der Behandlung wurde versucht, eine Virusisolierung aus den Hautefloreszenzen sowie eine Antikörperbestimmung gegen HVH durchzuführen.

Ammoniumhumat: Behandelt wurde mit einer 1%igen wässrigen Ammoniumhumatlösung durch mehrmaliges tägliches Abtupfen der betroffenen Hautregion. Das wasserlösliche Ammoniumsalz von Moorwasserhuminsäuren wurde nach einem bereits früher beschriebenen Verfahren [9] hergestellt. Das Präparat ist diskelektrophoretisch einheitlich und enthält keine freien Nichthuminstoffe. Seine chemischen und physiko-chemischen Eigenschaften wurden von WITTHAUER und Mitarb. [25] beschrieben.

Virusisolierung und -typisierung: Bläscheninhalt, Krustenmaterial und Abstriche von Hautefloreszenzen wurden sofort nach Abnahme in Viruserhaltungsmedium gebracht und bei −20°C eingefroren. Nach dem Auftauen erfolgte die Verimpfung der Suspension auf humane Lungenfibroblasten- und Strumazellkulturen. Traten innerhalb von 8 Tagen keine sichtbaren zytopathischen Veränderungen auf, wurden zwei weitere Zellpassagen angeschlossen. Die Typisierung isolierter zytopathogener Agenzien erfolgte mit entsprechenden Hyperimmunseren von Kaninchen oder Meerschweinchen im Neutralisationstest.

Serologische Untersuchungen: Zum Nachweis von Antikörpern gegen HVH in Patientenseren wurden der Fluoreszenzantikörpertest und die Komplementbindungsreaktion herangezogen. Beim Fluoreszenzantikörpertest dienten mit HVH Typ 1 infizierte humane Strumazellen nach Azetonfixierung als Antigen. Die Komplementbindungsreaktion wurde als Mikromethode mit einem Rohantigen aus HVH Typ 1 infizierten humanen Lungenfibroblastenzellen bei Verwendung von 2 Antigen- und 2 Komplementeinheiten durchgeführt.

Ergebnisse

Lokalbehandlung mit Ammoniumhumat: Bei der Beurteilung gingen wir wie WEITGASSER [24] davon aus, daß die durchschnittliche Dauer des Krankheitsverlaufes einer HVH-Infektion der Haut mit 10 Tagen anzunehmen ist. Der Therapieerfolg wurde daher wie in Tabelle I dargestellt beurteilt:

Tabelle I. Ergebnis der Behandlung von Herpesvirus-hominis-Dermatosen mit 1%igem Ammoniumhumat

Lokalisation der Herpeseruptionen	Anzahl d. Pat.	Beurteilung des Therapieerfolges		
		++	+	∅
Herpes labialis	42	34	5	3
Herpes facialis (außer Herpes labialis)	25	18	5	2
Herpes integumentalis	11	8	–	3
Gesamtzahl	78	60	10	8

++ Abklingen der Erscheinungen innerhalb von 5 Tagen (sehr guter bis guter Erfolg)
+ Abklingen der Erscheinungen innerhalb von 8 Tagen (guter bis befriedigender Effekt)
∅ Abklingen der Erscheinungen nach mehr als 8 Tagen (kein Effekt)

Virologische und serologische Untersuchungen: Bei 63 von 78 Patienten mit klinischen Zeichen einer HVH-Infektion konnte aus den Effloreszenzen Herpesvirus hominis isoliert werden. In 15 Fällen gelang der Virusnachweis bei einmaliger Materialabnahme nicht. Es handelte sich dabei zumeist um Patienten, denen erst 3 bis 4 Tage nach Beginn der Erkrankung bzw. im Krustenstadium Material zur Anzüchtung entnommen wurde. Einmal war keine sichere Aussage möglich, da nach beginnenden typischen zytopathischen Veränderungen der Gewebekulturansatz stark bakteriell verunreinigt war. Bei 2 Patienten mit negativem Virusisolierungsversuch konnten zudem auch keine Antikörper gegen HVH nachgewiesen werden, so daß das Vorliegen einer HVH-Infektion wenig wahrscheinlich ist. Ein weiterer Patient dieser Gruppe zeigte einen mehr als vierfachen Anstieg komplementbindender Antikörper gegen Adenovirus mit entsprechender klinischer Symptomatik.

Die Antikörpertiter gegen HVH Typ 1 betrugen bei den Patienten in der Komplementbindungsreaktion 1:8 bis 1:64 und im Fluoreszenzantikörpertest 1:8 bis 1:256.

Diskussion

Von 78 getesteten Patienten mit dem klinischen Bild einer HVH-Infektion sprachen 70 auf die Therapie mit 1%iger Ammoniumhumatlösung an. Bei 60 Patienten konnte der Therapieeffekt als sehr gut bis gut eingeschätzt werden. Nur bei 8 Patienten wurde die Erkrankung durch die Behandlung nicht beeinflußt. Davon gelang bei 2 Erkrankten die Virusisolierung nicht. Bei einem weiteren Patienten wiesen die serologischen Befunde eine akute Adenovirusinfektion aus, die möglicherweise das klinische Erscheinungsbild der HVH-Infektion ausgelöst und in seinem Verlauf beeinflußt hat.

Im Ergebnis unserer Untersuchungen können wir die Erfahrungen anderer Autoren [14, 19, 20], die für eine gute Wirksamkeit von Lokaltherapeutika ihre frühzeitige Anwendung als entscheidend ansehen, bestätigen. Es ist sicher am günstigsten, wenn die Behandlung bereits im Prodromalstadium begonnen wird, das die Patienten mit rezidivierenden HVH-Infektionen infolge plötzlichen Juckreizes und schmerzhaften Spannungsgefühls im betreffenden Hautareal bereits Stunden vor dem Aufschießen der Hauteffloreszenzen bemerken. Bei frühzeitigem Einsatz der Therapie mit Ammoniumhumat in einer Konzentration von 1% ist eine deutliche Verkürzung der Krankheitsdauer zu verzeichnen; es kann sogar zur vollständigen Unterdrückung des

Blasenschubes kommen. Diese Feststellung entspricht auch der von In-vitro-Untersuchungen bekannten Tatsache, daß Virustatika vom Typ der Phenolkörperpolymerisate in der Frühphase der Virus-Zell-Interaktion ihre Wirkung entfalten.

Nebenwirkungen durch lokale Applikation von Ammoniumhumat sind uns weder bei den bisherigen Behandlungsversuchen noch bei der klinischen und pharmakologischen Vortestung der Präparate bekannt geworden. Durch den relativ hohen Prozentsatz von Patienten mit gehäuften rezidivierenden HVH-Infektionen in unseren Untersuchungen ergibt sich die Möglichkeit, bei Wiederauftreten der Infektion die Behandlungsergebnisse durch Plazebo-Präparate weiter abzusichern. Entsprechende Untersuchungen sind vorgesehen.

Schrifttum

1. Biberstein, H., und Jessner, M.: Experiences with Herpin in recurrent herpes simplex together with a review and analysis of the literature on the use of CNS-substance as a virus antigencarrier. Dermatologica 117 (1958) 267–287.
2. Degos, R., und Touraine, R.: Traitment de l'herpès récidivant par un vaccin spécifique. Bull Soc. franç. Dermatol. & Syphiligr. T. 71, A. 74 (1964) 161–166.
3. Günther, W., Waschke, S. R., Meffert, H., El Bashir, M. H., Diezel, W., und Sönnichsen, N.: Untersuchungen zum therapeutischen Einsatz von Dipyridamol. Dt. Gesundh. Wesen 32 (1977) 1955–1959.
4. Kaufmann, H. E.: Clinical cure of herpes simplex keratitis by 5-Iodo-2'-deoxyuridine. Proc Soc. exper. Biol. & Med. 109 (1962) 251–259.
5. Kaufmann, H. E., und Maloney, E. D.: IDU and hydrocortisone in experimental herpe simplex keratitis. Arch. Ophthalmol. 68 (1962) 396–398.
6. Keining, E., und Braun-Falco, O.: Dermatologie und Venerologie. 2., völl. überarb. u erw. Aufl. München: Lehmann 1969, S. 34.
7. Klein, R. J., und Buimovici-Klein, E.: Latent infection after topical antiviral treatmen of herpes simplex virus infection in mice. 10th Intern. Congr. Chemother. Zürich (Switzerland 1977, Abstr. No. 487.
8. Klöcking, R., Hofmann, R., und Mücke, D.: Tierexperimentelle Untersuchungen zur ent zündungshemmenden Wirkung von Humaten. Arzneimittelforschung 18 (1966) 941–942.
9. Klöcking, R., und Mücke, D.: Isolierung wasserlöslicher Huminsäuren (Fulvosäuren aus ihren Blei(II)-Chelatverbindungen. Z. Chemie 9 (1969) 453–454.
10. Klöcking, R., und Sprössig, M.: Wirkung von Ammoniumhumat auf einige Virus-Zell Systeme. Z. allg. Mikrobiol. 15 (1975) 25–30.
11. Klöcking, R., Thiel, K.-D., und Sprössig, M.: Antiviral activity of humic acids from pea water. Proc. 5th Internat. Peat Congr. Poznan, VR Polen, 1 (1976) 446–455.
12. Klöcking, R., Töpke, H., Graumann, G., Blumöhr, T., Eichhorn, U., und Drabke, P. Antiviral effect of sodium and ammonium humate in experimental herpes infection of rabbi cornea. 2nd Internat. Sympos. Socialist. Countr. on Antiviral Substances, Reinhardsbrun (GDR), April 6–9, 1976 Abstr. (1976), S. 18.
13. Koinis, G., und Wüthrich, B.: Bericht über eine Herpes simplex-Vakzine (Lupidon H und G für die Behandlung des rezidivierenden Herpes simplex. Schweiz. Rdsch. Med., Praxis 6 (1975) 1176–1179.
14. Luger, A.: Verlauf und Behandlung der Infektionen mit dem Herpesvirus hominis. I. Therapie. Z. Haut- u. Geschlechtskr. 46 (1971) 399–408.
15. Meffert, H., und El Bashir, M. H.: Fotodynamische Therapie von Herpes-simplex-Er krankungen. Dermatol. Monatsschr. 163 (1977) 563–564.
16. Nasemann, Th.: Schlußwort zu [18]. Arch. klin. u. exper. Dermatol. 237 (1970) 261.
17. Nasemann, Th.: Die Infektionen durch das Herpes-simplex-Virus. Jena: Fischer 196 S. 20. (Infektionskrankheiten u. ihre Erreger. 2.)
18. Nasemann, Th.: Neuere Behandlungsmethoden unterschiedlicher Herpes simplex-Infek tionen. Arch. klin. u. exper. Dermatol. 237 (1970) 234–237.
19. Nasemann, Th., und Braun-Falco, O.: Virustatische Substanzen in der Lokalbehandlun herpetischer Dermatosen. Ther. Gegenwart 109 (1970) 222–236.
20. Söltz-Szötz, I.: Neue Methoden einer spezifischen Vaccination bei rezidivierendem Herpe simplex. Hautarzt 11 (1960) 465–467.
21. Thiel, K.-D., Klöcking, R., und Helbig, B.: In vitro-Untersuchungen zur antivirale Aktivität enzymatisch oxydierter o-Diphenolverbindungen gegenüber Herpes simplex-Viru Typ 1 und Typ 2. Zbl. Bakteriol., Parasitenkd., Infekt.-Kr. u. Hyg., Abt. I, Orig. A 23 (1976) 159–169.

22. THIEL, K.-D., KLÖCKING, R., SCHWEIZER, H., und SPRÖSSIG, M.: Untersuchungen in vitro zur antiviralen Aktivität von Ammoniumhumat gegenüber Herpes simplex-Virus Typ 1 und Typ 2. Zbl. Bakteriol., Parasitenkd., Infekt.-Kr. u. Hyg., Abt. I, Orig. A 239 (1977) 304–321.
23. THIEL, R., und WACKER, A.: Die Behandlung der Keratitis herpetica mit Thymin-analogen Verbindungen. Klin. Monatsbl. Augenheilkd. u. augenärztl. Fortb. 141 (1962) 94–99.
24. WEITGASSER, H.: Neue Behandlungsmöglichkeiten des Herpes simplex in der dermatologischen Praxis. Hautarzt 24 (1973) 298–301.
25. WITTHAUER, J., KLÖCKING, R., HELBIG, B., und DRABKE, P.: Chemical and physicochemical chracterization of antivirally active humic acids. Proc. 5th Internat. Peat Congr. Poznan (VR Polen) 1 (1976) 456–466.
26. WOLLENSAK, J.: Antimetaboliten zur Therapie von Viruserkrankungen am Auge. In: 3. International Congress of Chemotherapy, 1963, Stuttgart. Hrsg. Kuemmerle, H. P., und Preziosi, P., Verhandlungen, Vol. 1, S. 222–228. Stuttgart: Thieme 1964.
27. WOLLENSAK, J., und KYPKE, W.: Hemmung von Epithelregeneration und kornealer Wundheilung bei Kaninchen durch Antimetaboliten. Albrecht v. Graefes Arch. Ophthalmol. 168 (1965) 102–115.

Eingegangen am 11. 7. 1978

Anschr. d. Verf.: Doz. Dr. sc. med. FRIEDRICH SCHILLER,
Hautklinik der Medizinischen Akademie,
DDR-5010 Erfurt, Klement-Gottwald-Str. 34

Interaction of Humic Acids and Humic-Acid-Like Polymers with Herpes Simplex Virus Type 1

Renate Klöcking and Björn Helbig
Institute of Medical Microbiology
Medical Academy Erfurt, DDR-5010 Erfurt, GDR

Abstract

The study was performed in order to compare the antiviral activity against herpes simplex virus type 1 (HSV-1) of synthetic humic-acid-like polymers to that of their low-molecular-weight basic compounds and naturally occurring humic acids (HA) in vitro. HA from peat water showed a moderate antiviral activity at a minimum effective concentration (MEC) of 20 µg/ml. HA-like polymers, i.e. the oxidation products of caffeic acid (KOP), hydrocaffeic acid (HYKOP), chlorogenic acid (CHOP), 3,4-dihydroxyphenylacetic acid (3,4-DHPOP), nordihydroguaretic acid (NOROP), gentisinic acid (GENOP), pyrogallol (PYROP) and gallic acid (GALOP), generally inhibit virus multiplication, although with different potency and selectivity. Of the substances tested, GENOP, KOP, 3,4-DHPOP and HYKOP with MEC values in the range of 2 to 10 µg/ml, proved to be the most potent HSV-1 inhibitors. Despite its lower antiviral potency (MEC 40 µg/ml), CHOP has a remarkable selectivity due to the high concentration of this polymer that is tolerated by the host cells (>640 µg/ml). As a rule, the antiviral activity of the synthetic compounds was restricted to the polymers and was not preformed in the low-molecular-weight basic compounds. This finding speaks in favour of the formation of antivirally active structures during the oxidative polymerization of phenolic compounds and, indirectly, of corresponding structural parts in different HA-type substances.

Introduction - Historical Background

Naturally occurring humic acids (HA) and synthetic HA-like polymers derived from phenolic compounds are polyanionic substances of the humic acid type (HA-type substances). They may be characterized as negatively charged, yellow to dark brown polymers which are precipitable by heavy metals and partially degradable to low-molecular phenolic degradation products by reduction with sodium amalgam or by alkaline pressure hydrolysis.

Despite their different origins, HA and HA-like polymers have a great number o biological activities in common. For example, they exert antiviral activity against various DNA as well as RNA viruses.

The possible interaction of naturally occurring humic acids (HA) with viruses was first discussed by Schultz [1] in 1962 when he successfully used HA-containing peat dust litter for combating an outbreak of foot-and-mouth disease in pigs. He supposed the virus inactivation to be due to the protein-denaturing effect of HA.

In the past two decades, more detailed studies with isolated HA showed that: 1) HA also affect viruses that are pathogenic for man, RNA as well as DNA viruses; 2) HA work selectively at an antiviral concentration range without impairing the viability of host cells; and 3) HA, like other polyanionic compounds, such as heparin and pentosan polysulfate, mainly interfere with an early step of virus-cell interaction [2-6]. The antiviral spectrum of HA includes herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), influenza A, coxsackie A9 and rhinovirus 1 B [5]. Vaccinia virus and adenovirus type 2 are not affected [3]. The reason for the different sensitivity of viruses to HA is still unknown. Sensitivity seems to be independent of the nucleic acid type and is not influenced by the presence or absence of viral envelope.

After discovering the antiviral properties of HA the question arose which structural regions of the HA molecule are responsible for this activity. In this context, the contribution of Hampton and Fulton [7] deserved great interest. These authors found, in 1961, that enzymatically oxidized phenols inactivate phytopathogenic viruses. In 1970, Fulton succeeded in isolating an oxidation product of chlorogenic acid from virus-infected tobacco leaves. Additionally, he was able to synthesize a protein-quinone complex by enzymatic oxidation of chlorogenic acid in the presence of albumin [8].

Modifying Fulton's procedure by omitting albumin, we started synthesizing about 30 phenolic polymers in the mid seventies; first by enzymatic and later by chemical oxidation of various o- and p-diphenolic compounds. The resulting polymers proved to be negatively charged, brown substances sharing the following properties with naturally occurring HA [9,10] (Table 1):

Table 1 Common properties of humic acids and humic-acid-like polymers (according to [10]).

Solubility in alkaline media.
Precipitation by heavy metals.
Anodic migration in an electric field.
Formation of complexes with cationic dyes.
High content of stable free radicals.
Partial degradation to low-molecular phenolic compounds by reductive cleavage with sodium amalgam or by alkaline pressure hydrolysis.

Because of the existence of more than 30 synthetic HA-like polymers produced from well-known, low-molecular compounds, it was possible, for the first time, to

determine the biological activities dependent on the structure of the starting material. Furthermore, it is now possible to compare the activities of synthetic polymers to those of natural HA, as well as to those of low-molecular phenolic compounds. The antiherpetic activity served as the starting point for the present study, which attempted to answer the question whether the biological activity investigated is already present in the low molecular starting material or if it appears during oxidative polymerization.

Materials and Methods

HA were isolated from peat water by utilizing their lead(II) chelate compounds, as described previously [11]. Phenolic compounds were supplied from SERVA Feinbiochemica Heidelberg, FRG (caffeic acid, chlorogenic acid, gallic acid), FLUKA AG, Buchs SG, Switzerland (hydrocaffeic acid, gentisinic acid, nordihydroguaretic acid = 4,4'-(2,3-dimethyltetramethylene)-dicatechol), FERAK, Berlin-West (pyrogallol) and Calbiochem, San Diego, California, USA (3,4-dihydroxyphenylacetic acid).

To synthesize phenolic polymers, 10 mmoles of the phenolic compounds were dissolved in distilled water, adjusted with NaOH to pH 8.5 and oxidized with 2.5 mmoles sodium periodate. After heating a short time at 50°C, the solution was left overnight at room temperature. The next day, HA-like polymers were isolated by lead precipitation, as mentioned above for HA [11].

Monolayer cell cultures of human embryo lung fibroblasts (HELF) were used to determine the antiviral activity and cytotoxicity of the test substances. Culturing was carried out in 96-well microtest plates (MLW Polyplast Halberstadt, GDR) with 0.2 ml Eagle's minimum essential medium per well. For antiviral screening, HELF were infected with 2 $TCID_{50}$/cell HSV-1 strain Kupka. Virus was allowed to adsorb for 1 h at 37°C. The infectious medium was then removed and replaced by virus-free maintaining medium. The antiviral activity of the substances was tested by means of different test designs where the cells were exposed to the test substances either 1 h before, 1 h during, or 24 h after virus adsorption. Additionally, we studied the effect of a 24-h-exposure during the entire virus replication cycle. Dependent on the experimental design, test substances were added in two-fold dilution series before, at the time of or after virus adsorption. After incubation for 24 h at 37°C, monolayers were checked microscopically for virus-induced cell alterations using a score system (0=0%, 1=up to 25%, 2=up to 50%, 3=up to 75%, 4=more than 75% of the cells are altered or destroyed). The substance concentrations reducing the cytopathogenic effect (CPE) by more than 50% were within the antiviral concentration range. The substance concentration necessary for complete suppression of virus-induced CPE was defined as minimum effective concentration (MEC).

Cytotoxicity was detected using the same microtest plate containing uninfected HELF. The maximum tolerated concentration (MTC) corresponds to the highest substance concentration not inducing any morphological cell alterations.

Each experiment has been repeatedly performed. The values given in the tabl are the geometric means of 3 to 5 single experiments.

Results and Discussion

Table 2 illustrates the antiviral and cytotoxic activities of GENOP and KOl respectively. If the substances were present during the whole replication cycle (0...2 h p.i.), the antiviral concentration range was between 1 and 32 µg/ml for GENO and 0.2 and 32 µg/ml for KOP. Higher concentrations were not tolerated witho morphological alterations of cells. For GENOP or KOP a concentration of 2 µg/n was also sufficient for complete suppression of CPE if the substances were prese for only 1 h during the adsorption phase. The effect could not be enhanced b prolonging the time of influence. No (KOP) or only weak (GENOP) effects we found when the substances were present before or after viral adsorption. The resul confirm the high sensitivity of the early phase of virus-cell interaction, as has als been found for other polyanionic compounds [12-14]. Therefore, test substances liste in Table 3 were exclusively screened at the adsorption phase.

Table 2 Antiviral activity of GENOP and KOP dependent on the time of influence. Virus-cell system: HSV Kupka/HELF. Concentration range tested: 0.25-1024 µg/ml.

Test substance	Time of influence h p.i.	Antivirally active concentration µg/ml	MEC[1] µg/ml	MTC[2] µg/ml
GENOP	0.....24	1.....32	2	32
	1.....0	32.....64	64	64
	0.....1	0.5.....128	2	64
	1.....24	16.....32	n a	32
KOP	0.....24	0.2.....32	2	16
	1.....0			64
	0.....1	1.....128	2	64
	1.....24			8

[1]Minimum effective concentration; n.a. = not achieved
[2]Maximum tolerated concentration

Table 3 demonstrates the results for 8 of 22 tested pairs of substances (HA-like polymers and corresponding low-molecular starting compounds) as well as results fo sodium humate. The polymers more or less strongly inhibit the virus adsorption whereas the low-molecular-weight compounds failed to do so. The only exception i 4,4'-(2,3-dimethyltetramethylene)-dicatechol (nordihydroguaretic acid), which turne brown shortly after addition to the cells. Probably, the substance reacted to th corresponding polymer and thus affected the virus-cell interaction. Both the antivira activity and the cytotoxicity of the substances display remarkable, structure-dependen differences. GENOP, KOP, 3,4-DHPOP and HYKOP having MEC values between

and 10 µg/ml, proved to be the most potent antiviral substances. In spite of its lower antiviral potency CHOP shows a remarkable selectivity, since it is best tolerated by the cells (MTC > 640 µg/ml). The low-molecular phenolic compounds themselves are not antivirally active. This means that the antiviral activity is restricted to the HA-like polymers and must be initiated by fundamental alterations of the chemical structure during the oxidative polymerization.

Table 3 Screening of humic-acid-like polymers and their low-molecular-weight starting compounds for antiviral activity in vitro. Virus-cell system: Herpes simplex virus type 1/ human embryo lung fibroblasts. Concentration range tested: 0.6-640 µg/ml. Time of influence: 0-1 h post infection.

Test substance	Antiviral concentration range µg/ml	MEC[1] µg/ml	MTC[2] µg/ml
Caffeic acid			160
KOP	1...160[3]	5	80[3]
Hydrocaffeic acid			320
HYKOP	5....320	10	160
Chlorogenic acid			640
CHOP	5....640	40	>640
3,4-Dihydroxyphenyl-acetic acid			160
3,4-DHPOP	2....160	5	40
4,4'-(2,3-Dimethyltetra-methylene)-dicatechol	20....80	n.a	5
NOROP	20....80	n.a	40
Gentisinic acid			340
GENOP	1....160	2	80
Pyrogaltol			2
PYROP	40....80	n.a	20
Gallic acid			640
GALOP	10...320	20	40
Sodium humate	20...320	80	80

[1] Minimum effective concentration; n.a = not achieved
[2] Maximum tolerated concentration
[3] In virus-infected cells, the cytotoxicity was often shifted to higher substance concentrations.

Moreover, we would like to suggest that during these reactions new structural features are formed, which are essential for antiviral activity as well as for HA structure, and which are more or less similar in different HA-type substances. Following up this idea, a common basic structure of HA-type substances might be assumed.

The interaction of HA-type substances with viruses implies practical aspects, too. Hence it was found that adsorption to and recovery of enteroviruses from water filters are influenced by HA [15,16]. Furthermore, HA-virus interactions might be of great importance for limiting virus spread in plants, surface waters and soils and also offer new prospects for the development of antiviral drugs.

References

1. Schultz, H. Deutsche Tierärztliche Wochenschrift 69:613 (1962).
2. Klöcking, R. and M. Sprössig. Experientia 28:607 (1972).
3. Klöcking, R. and M. Sprössig. Zeitschrift für Allgemeine Mikrobiologie 15:25 (1975).
4. Thiel, K.-D., R. Klöcking, H. Schweizer and M. Sprössig. Zentralblatt für Bakteriologie I. Abteilung 239:304 (1977).
5. Klöcking, R., M. Sprössig, P. Wutzler, K.-D. Thiel and B. Helbig. Zeitschrift für Physiotherapie 33:95 (1983).
6. Klöcking, R., K.-D. Thiel and M. Sprössig. In: Peat and Peatlands in the Natural Environment Protection Vol. 1, pp. 446-455. Proc. 5th Int. Peat Congr. (Warszawa: WCIN, 1976).
7. Hampton, R.E. and R.W. Fulton. Virology 13:44 (1961).
8. Hampton, R.E. Phytopathology 60:1677 (1970).
9. Helbig, B. and R. Klöcking. Zeitschrift für Physiotherapie 33:31 (1983).
10. Hänninen, K.I., R. Klöcking and B. Helbig. Sci. Tot. Environ. 62:201 (1987).
11. Klöcking, R., B. Helbig, and P. Drabke. Pharmazie 32:297 (1977).
12. Vahery, A. and K. Cantell. Virology 21:661 (1963).
13. Voss, H., H. Sensch and P. Panse. Zentralblatt für Bakteriologie I. Abteilung 192:137 (1964).
14. Baba, M., R. Snoeck, R. Pauwels and E. De Clercq. Antimicrobial Agents and Chemotherapy 32:1742 (1988).
15. Guttman-Bass, N. and J. Catalano-Sherman. Appl. Environ. Microbiol. 49:1260 (1985).
16. Sobsey M.D. and A.R. Hickey. Appl. Environ. Microbiol. 49:259 (1985).

Antiviral Chemistry & Chemotherapy 13:241–249

Anti-HSV-1 activity of synthetic humic acid-like polymers derived from *p*-diphenolic starting compounds

R Klöcking*, B Helbig, G Schötz, M Schacke and P Wutzler

Institute for Antiviral Chemotherapy, Friedrich-Schiller-University Jena, Jena, Germany

*Corresponding author: Tel: +49 361 741 1482; Fax: +49 361 741 1166; E-mail: rkloeck@zmkh.ef.uni-jena.de

A panel of ten humic-acid-like polymers was synthesized by oxidation of *p*-diphenolic compounds and characterized by relative molecular weights, FT-IR spectra and functional group analysis. Using the XTT-based tetrazolium reduction assay EZ4U, both the low-molecular starting compounds and the synthesized polymers were examined for antiviral and cytotoxic activities in HSV-1-infected Vero cells. With the exception of hydroquinone, 2,5-dihydroxytoluene and 2,5-dihydroxybenzoquinone, the starting compounds failed to inhibit herpesvirus replication. The polymeric oxidation products, however, developed anti-HSV-1 activity with EC_{50} values in the range of 0.65 (2,5-DHPOP) and 322 µg/ml (2,5-DHBQOP). The CC_{50} values of the polymers varied among 32.0 (TMHYDROP) and >512 µg/ml (2,5-DHBQOP, HYDSULFOP). The most effective polymers were found to be 2,5-DHPOP, 2,5-DHTOP and GENOP (EC_{50}: 0.65, 1.6 and 2.2 µg/ml, respectively, and SI: ≥400, ≥80 and ≥58, respectively). Functional group analysis revealed that increasing numbers of carboxyl groups together with a high content of hydroxyl groups tend to enhance the antiviral activity of polymers derived from *p*-diphenolic compounds.

Keywords: *p*-diphenolic compounds, humic acid-like polymers, FT-IR spectra, herpes simplex virus type 1, structure–activity relationship

Introduction

Humic acids (HA) are fractally organised polyanionic substances with a putative melanin-like chemical structure (Cataldo, 1998). They are widely distributed in decaying plant material, soils and surface waters. Peat, as the main source of naturally occurring HA, has been used effectively to prevent the spread of foot-and-mouth disease even 40 years ago (Schultz, 1962). In the 1970s, the antiviral activity of HA against enteroviruses, herpesviruses and influenza viruses was detected in cell cultures (Klöcking & Sprößig, 1972, 1975). Time-of-addition experiments in HSV-1-infected human lung fibroblasts revealed virus adsorption to be the most humate-sensitive step of virus replication (Klöcking & Sprößig, 1975).

A major hindrance for mechanistic investigations of HA is the fact that chemical components forming the HA molecule vary depending on where peat samples are collected. Therefore, we prepared origin-defined HA-like polymers by enzymatic (Thiel *et al.*, 1976) as well as by chemical synthesis (Helbig & Klöcking, 1983; Helbig *et al.*, 1994) starting from single low-molecular ortho- and paradiphenolic compounds. Like naturally occurring HA, the synthetic polymers were found to inhibit an early stage of HSV-1, HSV-2 as well as HCMV replication (Thiel *et al.*, 1976; Klöcking *et al.*, 1983; Thiel *et al.*, 1984, Neyts *et al.*, 1992; Helbig *et al.*, 1997; Meerbach *et al.*, 2001), probably by interaction with positively charged domains of viral envelope glycoproteins (Neyts *et al.*, 1992). In contrast to the natural model, the synthetic polymers enabled us to investigate structure–activity relationships referring to the starting compounds.

In a previous paper, we have shown the influence of aromatic ring substituents on the anti-HSV-1 activity and cytotoxicity of *o*-diphenol-derived polymers (Helbig *et al.*, 1997). In the present study, we introduce another panel of 10 HA-like polymers synthesized by oxidation of *p*-diphenolic starting compounds and characterize them by molecular weight determination, FT-IR spectra and functional group analysis. We report data for antiviral and cytotoxic activities of both the low-molecular starting compounds and the synthesized polymers and discuss the influence of special structural features of the starting compounds on the biological activity of the polymeric oxidation products.

Finally we consider the results with regard to the putative structure of HA-like polymers.

Materials and methods

Chemistry

Test substances. The following *p*-diphenolic starting compounds (even numbers) and corresponding HA-like polymers (odd numbers) were investigated: **1**, HYDROP; **2**, hydroquinone; **3**, 2,5-DHTOP; **4**, 2,5-dihydroxytoluene; **5**, GENOP; **6**, gentisinic acid; **7**, 2,5-DHPOP; **8**, 2,5-dihydroxyphenylacetic acid; **9**, 2,5-DHBAOP; **10**, 2,5-dihydroxybenzaldehyde; **11**, HYDSULFOP; **12**, hydroquinone sulfonic acid (K-salt); **13**, 2,3-DMHYDROP; **14**, 2,3-dimethylhydroquinone; **15**, TMHYDROP; **16**, trimethylhydroquinone; **17**, 2-MOHYDROP; **18**, 2-methoxyhydroquinone; **19**, 2,5-DHBQOP; **20**, 2,5-dihydroxybenzoquinone. All the compounds are listed in Table 1.

The nucleoside analogue acyclovir (Zovirax™) was obtained from GlaxoSmithKline (Munich, Germany). Pentosan polysulfate (SP54) was kindly supplied by bene-Arzneimittel GmbH (Munich, Germany).

Preparation of phenolic polymers. The synthesis of HA-like polymers has been described previously (Helbig *et al.*, 1997). Briefly, 10^{-2} M solutions of *p*-diphenolic starting compounds in de-ionized sterile water were oxidized with 2.5×10^{-3} M sodium metaperiodate at 55°C and pH 8–9 for 1 h and left at room temperature overnight. After that, the formed dark-brown polymers were precipitated and repeatedly washed with ethanol. The yields of polymers amounted to 50–80%.

Molecular weight determinations. The molecular weights of the synthetic polymers were determined by gel permeation chromatography using Controlled Pore Glass, (CPG, 120 Å, Electro Nucleonics Inc., Fairfield, Conn., USA) fillings in a 1×100 cm column. Polymers were eluted with 0.05 M Tris/HCl in 0.5 M NaCl, pH 8.0, at a rate of 120 ml/h. Sample concentration was 1% and 0.5 ml polymer sample solution was used. Absorbance at 254 nm was measured using the BioLogic-System UV monitor from BIO-RAD (Hercules, Calif., USA). The molecular weights given in Table 1 correspond to the mean peak elution volume of at least three different runs. To construct the calibration curve, water-soluble calibration proteins of known molecular weights (Combithek calibration kit, Boehringer, Mannheim, Germany) were used. The molecular weights determined by this method represent relative values described as protein equivalent molecular weights.

Determination of acid oxygen containing groups. To determine total acidity and carboxyl groups concentration, potentiometric titrations were performed using $Ba(OH)_2$ and $Ca(Ac)_2$ exchange, respectively, according to published

Table 1. Characterization of the synthesized HA-like-polymers and their *p*-diphenolic starting compounds by relative molecular weight (MW) and the content of carboxyl (COOH) and hydroxyl (OH) groups

Compound	Test substance		MW	COOH (eq/mol)	OH (eq/mol)
	HA-like polymer	*p*-Diphenolic compound			
1	HYDROP		5200	5	110
2		Hydroquinone	110	0	2
3	2,5-DHTOP		4700	5	78
4		2,5-Dihydroxytoluene	124	0	2
5	GENOP		5500	5	99
6		Gentisinic acid	154	1	2
7	2,5-DHPOP		5500	10	104
8		2,5-Dihydroxyphenylacetic acid	168	1	2
9	2,5-DHBAOP		6200	2	111
10		2,5-Dihydroxybenzaldehyde	138	0	2
11	HYDSULFOP		7800	5	99
12		Hydroquinone sulfonic acid	190	0	2
13	2,3-DMHYDROP		2400	4	38
14		2,3-Dimethylhydroquinone	138	0	2
15	TMHYDROP		2200	1	46
16		Trimethylhydroquinone	152	0	2
17	2-MOHYDROP		6300	2	111
18		2-Methoxyhydroquinone	140	0	2
19	2,5-DHBQOP		3400	1	30
20		2,5-Dihydroxybenzoquinone	140	0	2

HA, humic acid.

procedures (Schnitzer & Khan, 1972). Phenolic hydroxyl concentration was considered to be the difference between total acidity and carboxyl group concentration.

FT-IR spectroscopy. FT-IR spectroscopy was carried out in an Impact 410 spectroscope (Nicolet Instrument Corporation, Madison, Wis., USA) equipped with a DTSG detector. The sample pellet consisted out of 1.0 mg sample in 200 mg KBr. Four to 16 scans were measured. IR spectra were analysed with Omnic software (Nicolet Instrument Corporation).

Virology

Cells and virus strain. Vero cells (ATCC, CCL 81) were cultivated in modified Eagle medium (MEM) with L-glutamate (GIBCO BRL Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, Md., USA) and antibiotics.

HSV-1 strain Kupka isolated 1962 by Dr Benda (Prague) from a patient with herpes labialis (Benda *et al.*, 1972) was kindly provided by Dr J Rajcani, Bratislava. For the experiments, the virus was propagated in Vero cells. The $TCID_{50}$ of the cell-free virus suspension was 10^7–10^8/ml.

Antiviral screening assay. Antiviral screening was performed in 96-well flat-bottom microtitre plates using the XTT-based tetrazolium reduction assay EZ4U (Biozol, Eching, Germany). In this way, it is possible to determine the inhibition of viral cytopathogenicity as well as substance-induced cytotoxic effects. The assay has been described in detail by Klöcking *et al.* (1995). Briefly, 2×10^4 cells in 200 µl medium were applied to each of the 60 inner wells of the plate. To avoid marginal effects, the 36 external wells were filled with 200 µl MEM. Cells were incubated to confluence in a humid 1% CO_2-containing atmosphere at 37°C for 2 days. After removing growth medium, 200 µl of eight serial dilution steps of antiviral compounds dissolved in MEM were added to 8×6 wells. Three wells of each group received 2×10^4 $TCID_{50}$ of HSV-1 strain Kupka. The residual 12 wells served as cell and virus controls, respectively, and remained free of test substances. After further incubation at 37°C for 120 h, plates were centrifuged at 200×*g* for 10 min and the supernatant was replaced by fresh MEM without phenol red. Added to each well was 25 µl of the EZ4U preparation. After an incubation time of 3 h, optical density (OD) was measured at 450 nm (reference wavelength 620 nm) in a microplate reader (Ceres 900C, BIO-TEK, Winooski, Vt., USA). The percentage of antiviral and cytotoxic activities of the test compounds were calculated from the measured OD values according to Pauwels *et al.* (1988). Substance concentrations at 50% virus inhibition (EC_{50}) and 50% cytotoxicity (CC_{50}) were obtained by means of regression analysis. Average values and standard deviations of at least three independent experiments were calculated from $\log_{10}$ transformed EC_{50} and CC_{50} values, respectively. Back-transformation into linear scale results in mean EC_{50} and CC_{50} values with corresponding confidence limits.

Results

Preparation and chemical properties of HA-like polymers

HA-like polymers were synthesized by periodate oxidation of the low-molecular *p*-diphenolic compounds listed in Table 1. In this way, we obtained the oxidation products (OP) from hydroquinone (HYDROP), 2,5-dihydroxytoluene (2,5-DHTOP), gentisinic acid (GENOP), 2,5-dihydroxyphenylacetic acid (2,5-DHPOP), 2,5-dihydroxybenzaldehyde (2,5-DHBAOP), hydroquinone sulfonic acid (HYDSULFOP), 2,3-dimethylhydroquinone (2,3-DMHYDROP), trimethylhydroquinone (TMHYDROP), 2-methoxyhydroquinone (2-MOHYDROP) and 2,5-dihydroxybenzoquinone (2,5-DHBQOP).

The dark-brown HA-like polymers are stable at room temperature and soluble in neutral and diluted alkaline media. As determined by gel permeation chromatography on CPG (Table 1), the relative molecular weights of the polymers range between 2200 (TMHYDROP) and 7800 (HYDSULFOP) while the molecular weights of the starting compounds are between 110 (hydroquinone) and 190 (hydroquinone sulfonic acid). The COOH content of the polymers was found to be between 1 eq/Mol (TMHYDROP, 2,5-DHBQOP) and 10 eq/Mol (2,5 DHPOP). For comparison, the low-molecular starting compounds contain 1 eq/Mol COOH (gentisinic acid, 2,5-dihydroxyphenylacetic acid) or they are free from COOH (the remaining starting compounds). The content of phenolic OH was between 30 eq/Mol (2,5-DHBQOP) and 111 eq/Mol (2,5-DHBAOP, 2-MOHYDROP) in the polymers and amounted to 2 eq/Mol in the starting compounds.

IR spectra band assignment

The IR spectra band assignment for the polymers investigated is shown in Table 2. The broad O–H stretching maximum at ~3400 cm^{-1} demonstrates the presence of exchangeable protons typical for alcoholic, phenolic and carboxylic groups. The maximum was found between 3398 cm^{-1} (2,5-DHBQOP) and 3452 cm^{-1} (HYDSULFOP) indicating the presence of intermolecular H-bonds with a relatively low association degree (Wang & Wang, 1983). Aliphatic C–H valence vibrations were observed at ~2900 cm^{-1} (CH_2, CH_3) as one (2,5-DHTOP, 2,5-DHPOP, 2,5-

Table 2. Infrared spectra band assignment for the p-diphenol-derived HA-like polymers tested

IR band (cm^{-1})	Band assignment	HYDROP 1	DHTOP 3	GENOP 5	DHPOP 7	DHBAOP 9	HYDSULFOP 11	DMHYDOP 13	TMHYDOP 15	MOHYDOP 17	DHBQOP 19
3500–3100	O–H stretching	3419	3430	3419	3419	3403	3452	3419	3441	3419	3398
3100–2700	C–H stretching	2925	2946		2914	2919	2973	2925	2925	2941	2941
		2855								2844	2790
2400–2200	–C≡C– stretching										2446
1780–1680	C=O stretching bands nonconjugated	1710				1688			1691		
1650–1550	C=O stretching conjugated	1616	1596	1585	1582	1627	1616	1643	1647	1633	1636
	C=C Stretching conjugated		1582		1562	1609	1541			1605	1544
						1579					
1470–1420	C–H bending vibrations	1442				1465	1415	1435	1450	1438	
						1432				1420	
1400–1330	Symmetric COO^- stretching vibrations	1370	1394	1387	1380	1398	1377	1380	1363	1401	
			1335		1336		1363			1326	
1300–1000	C–O–C stretching vibrations in esters	1196		1237	1206	1127	1288	1285	1225	1220	1271
							1182			1103	
1060–600	Sulfonic acid groups						1049				
							618				
900–650	Polysubstituted aromatic compounds	871	871	669	874	840	799	809	871	847	792
		813	775		775	782	761	755	721	799	763
					669	698		738		775	724

DHBAOP, HYDSULFOP, 2,3-DMHYDROP, TMHYDROP) or two distinct maxima (HYDROP, 2-MOHYDROP, 2,5-DHBQOP). Only GENOP did not show any peak in this area. Peak maxima in the region of 1700 cm^{-1} (C=O of carbonyl from R_2CO or COOH) were observed in the spectra of HYDROP (1710 cm^{-1}), 2,5-DHBAOP (1688 cm^{-1}) and TMHYDROP (1691 cm^{-1}). Signals of C=C stretching (from C=C double bonds and from aromatic C=C bonds) and of conjugated C=O stretching vibrations were registered regularly between 1582 cm^{-1} (2,5-DHPOP) and 1647 cm^{-1} (TMHYDROP). Asymmetric and symmetric COO^- stretching vibrations were present in the regions of 1541–1582 cm^{-1} (2,5-DHTOP, 2,5-DHPOP, 2,5-DHBAOP, HYDSULFOP, 2,5-DHBQOP) and 1330–1400 cm^{-1} (all polymers except for DHBAOP). Aliphatic C–H bending vibrations around 1450 cm^{-1} were registered in the spectra of HYDROP, 2,5-DHBAOP, HYDSULFOP, 2,3-DMHYDROP, TMHYDROP and 2-MOHYDROP. Peak maxima at 1100–1300 cm^{-1} corresponding to C–O single bonds from esters, ethers or phenols could be observed regularly, except for 2,5-DHTOP. Absorption maxima in the region of 700–900 cm^{-1} indicate the presence of polysubstituted aromatic compounds and occurred in all the spectra measured. Typical signals of sulfonic groups were detectable only in HYDSULFOP (peaks at 1049 and 618 cm^{-1}, respectively).

Antiviral activity and cytotoxicity

The synthesized HA-like polymers as well as their low-molecular weight starting compounds were tested for antiviral activity in HSV-1-infected, and for cytotoxicity in uninfected, Vero cells. Dose–response relationships of the starting compounds (hydroquinone, 2,5-dihydroxytoluene, gentisinic acid, 2,5-dihydroxybenzaldehyde) and of the corresponding polymers (HYDROP; 2,5-DHTOP; GENOP; 2,5-DHBAOP) are given in Figure 1. The examples (Figure 1b, d, f and h) clearly show the dose-dependent antiviral activity of the polymers accompanied with minimal cytotoxicity only. This is in contrast to the dose-response curves of low-molecular starting compounds, which demonstrate a more differentiated picture. Gentisinic acid (Figure 1e) exerts neither antiviral activity nor cytotoxicity. The antiviral effect of hydroquinone (Figure 1a) and 2,5-dihydroxytoluene (Figure 1c) is restricted to a small non-toxic concentration range. 2,5-Dihydroxybenzaldehyde (Figure 1g) fails to inhibit virus replication but is cytotoxic to Vero cells at 32 µg/ml.

Table 3 summarizes the EC_{50}, CC_{50} and SI values of all the substances tested. The EC_{50} values of the *p*-diphenol-derived polymers ranged between 0.65 µg/ml (2,5-DHPOP) and 322 µg/ml (2.5-DHBQOP). The most effective polymers (EC_{50}<10 µg/ml, CC_{50}≥128 µg/ml, SI >10) were 2,5-DHPOP>2,5-DHTOP>GENOP>HYDROP>2,5-DHBAOP. The highest SI (≥400) was found for 2,5-DHPOP, followed by 2,5-DHTOP (≥80) and GENOP (≥58). Less anti-HSV-1 activity (EC_{50}≥10 µg/ml, CC_{50}<128 µg/ml, SI≥5) was detected for 2,3-DMHYDROP>HYDSULFOP>2-MOHYDROP. Only marginal antiviral activity was detectable for 2,5-DHBQOP (EC_{50}=322 µg/ml, SI≥1.6). Under the same experimental conditions, the EC_{50} values of the reference substances were 0.14 µg/ml for acyclovir and 7.4 µg/ml for pentosan polysulfate.

Among the low-molecular starting compounds, hydroquinone, 2,5-dihydroxytoluene and 2,5-dihydroxybenzoquinone developed detectable anti-HSV-1 activity, the SI, however, reached only values of 1.6, 1.7 and 3.5, respectively.

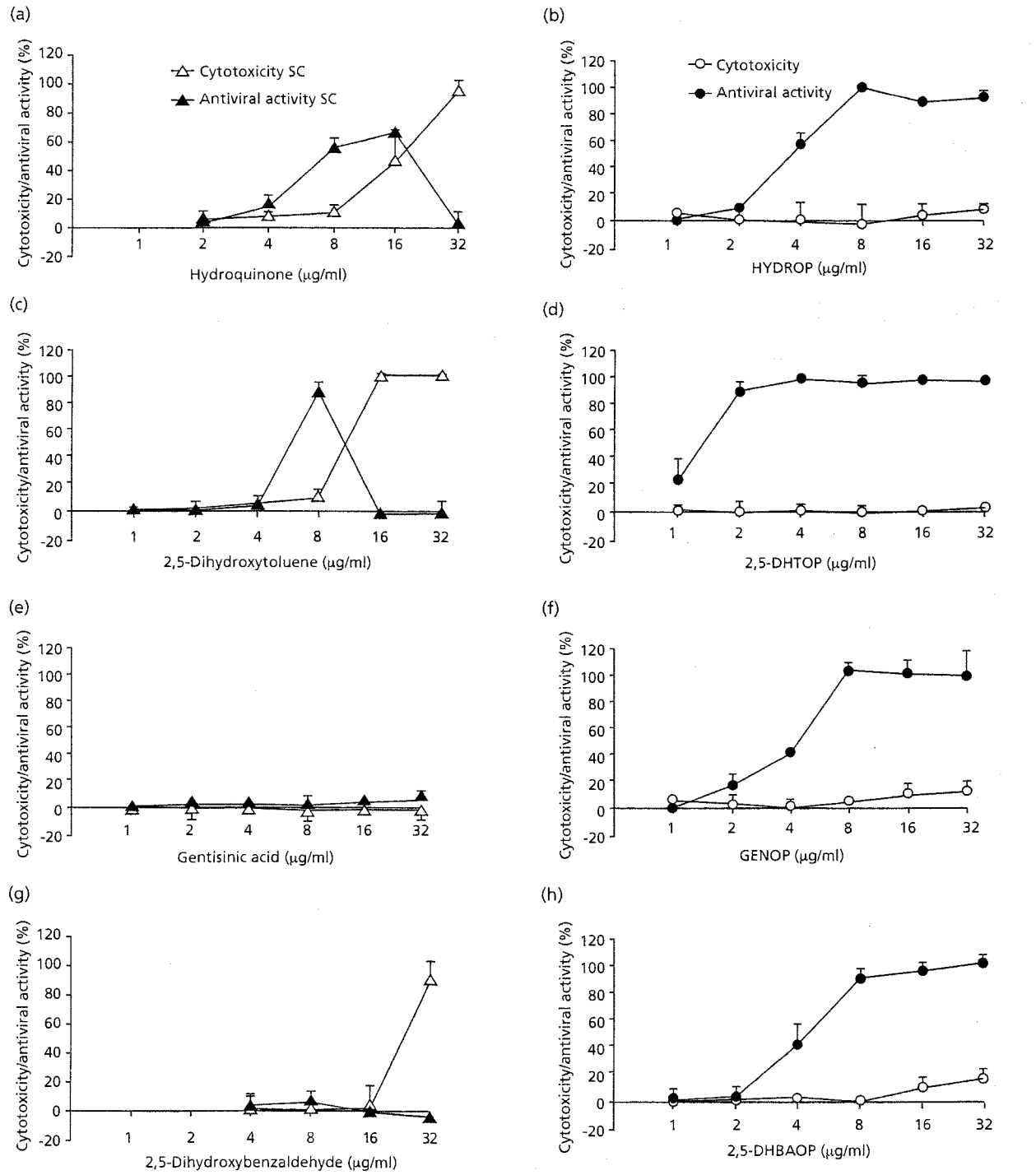
The CC_{50} values of the p-diphenol-derived polymers were between 32.1 µg/ml (TMHYDROP) and >512 µg/ml (HYDSULFOP, 2,5-DHBQOP). Further non-toxic polymers within the concentration range tested were 2,5-DHPOP, MOHYDROP, HYDROP, 2,5-DHTOP, GENOP and 2,5-DHBAOP as well as the reference substances acyclovir and pentosan polysulfate. 2,3-DMHYDROP (CC_{50}=83.7 µg/ml) and TMHYDROP (CC_{50}=32.1 µg/ml) were found to be the most cytotoxic polymers tested.

The low-molecular starting compounds were generally more toxic than the polymers synthesized from them. Only gentisinic acid, hydroquinone sulfonic acid and 2,5-dihydroxybenzoquinone proved to be non-toxic within the concentration range tested (CC_{50}>512 µg/ml). The highest cytotoxicity was observed for hydroquinone (CC_{50}=13.6 µg/ml) and its methylated derivatives 2,5-dihydroxytoluene (CC_{50}=9.9 µg/ml), 2,3-dimethylhydroquinone (CC_{50}=8.0 µg/ml), trimethylhydroquinone (CC_{50}=14.1 µg/ml) and 2-methoxyhydroquinone (CC_{50}=16,1 µg/ml).

Discussion

The diversity of chemical compounds contributing to the formation of HA in nature entails wide variation in the antiviral activity and toxicity of HA extracted or otherwise derived from natural sources. In contrast to this, HA-like polymers synthesized by oxidative polymerisation of individual phenolic starting compounds allow more precisely to identify chemical precursor structures of biologically active humic polymers. This concept has been described in detail for polymers derived from ortho-diphenolic compounds (Helbig *et al.*, 1997) and is extended in the present study to 10 other polymers obtained by periodate oxidation of *p*-diphenolic compounds. The low-molecular starting compounds comprised 2,5-dihydroxybenzene (hydroquinone) and nine of its derivatives, including three different methylation products and three carboxylic acids.

Figure 1. Antiviral and cytotoxic dose–response curves of four selected *p*-diphenolic starting compounds and their corresponding HA-like polymers



(a) Hydroquinone, (b) HYDROP, (c) 2,5-dihydroxytoluene, (d) 2,5-DHTOP, (e) gentisinic acid, (f) GENOP, (g) 2,5-dihydroxybenz-aldehyde, (h) 2,5-DHBAOP.
Data points correspond to the mean of at least three independent experiments.
HA, humic acid; SC, starting compounds.

Table 3. Anti-HSV-1 activity of *p*-diphenol derived HA-like polymers and of their *p*-diphenolic starting compounds*

Compound	Test substance	CC_{50}† (μg/ml)	EC_{50}‡ (μg/ml)	SI§
1	HYDROP	>128	3.7 (3.41–4.09)	>35
2	Hydroquinone	13.6 (10.8–17.1)	8.7(8.28–9.12)	1.6
3	2,5-DHTOP	>128	1.6 (1.31–1.98)	>80
4	2,5-Dihydroxytoluene	9.9 (8.87–11.0)	5.9 (5.64–6.15)	1.7
5	GENOP	>128	2.2 (0.54–4.02)	58
6	Gentisinic acid	>512	>512	NC
7	2,5-DHPOP	>256	0.65 (0.53–0.77)	>394
8	2,5-Dihydroxyphenylacetic acid	82.8 (65.9–104)	∅**	NC
9	2,5-DHBAOP	>128	3.9 (3.25–4.70)	>33
10	2,5-Dihydroxybenzaldehyde	24.2 (22.1–26.4)	∅	NC
11	HYDSULFOP	>512	29.1 (28.9–29.3)	>18
12	Hydroquinone sulfonic acid	>512	∅	NC
13	2,3-DMHYDROP	83.7 (74.2–94.3)	15.5 (13.1–18.3)	5.4
14	2,3-Dimethylhydroquinone	8.0 (7.55–8.55)	∅	NC
15	TMHYDROP	32.1 (22.6–45.7)	14.0 (12.7–15.3)	2.3
16	Trimethylhydroquinone	14.1 (10.0–19.8)	∅	NC
17	2-MOHYDROP	>256	31.6 (29.8–33.5)	>8.1
18	2-Methoxyhydroquinone	16.1 (15.3–17.0)	∅	NC
19	2,5-DHBQOP	>512	322(269–385)	>1.6
20	2,5-Dihydroxybenzoquinone	>512	147(138–157)	>3.5
Reference substances				
Acyclovir		>128	0.14 (0.14–0.15)	>914
Pentosan polysulfate		>128	7.4 (7.13–7.57)	>17

*Data represent the mean CC_{50} and EC_{50} values, respectively, of at least three independent experiments, confidence intervals in brackets.
†CC_{50}, concentration required to reduce XTT formazan production in Vero cells by 50%.
‡EC_{50}, concentration required to prevent Vero cells from virus-induced impairment of formazan production by 50%.
§SI, selectivity index=CC_{50}/EC_{50}.
**∅, EC_{50} is not achieved within the nontoxic concentration range of the test substance.
HA, humic acid; NC, not calculable.

The oxidative polymerisation of the *p*-diphenolic starting compounds resulted in HA-like polymers with relative molecular weights in the range of 2200 and 7800. Except for the oxidation product of chlorogenic acid (MW 14000), the molecular weights of *o*-diphenol-derived polymers (3800–9000) are of the same order of magnitude (Helbig *et al.*, 1997). This is also valid for the content of hydroxyls, which amounts to 30–111 eq/mol for para- and 11–112 for *o*-diphenol derived polymers. Greater differences exist between the COOH content of *para*- (1–10 eq/mol) and *o*-diphenol-derived polymers (4–28 eq/mol). The FT-IR spectra of *p*-diphenol-derived polymers (Table 2) reveal the similarity of HA-like polymers with naturally occurring HA. The broad peak at 3400 cm^{-1} corresponds to hydrogen bridge-bound hydroxyl groups (Bellamy, 1980). Peak maxima in the regions of 1500–1600 cm^{-1} and 700–900 cm^{-1}, respectively, can be allocated to aromatic ring vibrations of the polymer (Hänninen *et al.*, 1989). As known from nuclear magnetic resonance studies, the aromaticity of phenolic polymers is higher than that of naturally occurring HA (Hänninen *et al.*, 1987).

The proof for antiviral activity and cytotoxicity of the synthesized *p*-diphenol-derived polymers revealed significant antiviral activity against HSV-1 with clear differences between the individual polymers. According to EC_{50} values the anti-HSV-1 activity ranked as follows: 2,5-DHPOP>2,5-DHTOP>GENOP>HYDROP>2,5-DHBAOP>TMHYDROP>2,3-DMHYDROP>HYDSULFOP>2-MOHYDROP>>2,5-DHBQOP. The data of 2,5-DHPOP, 2,5-DHTOP and GENOP confirmed partly earlier results obtained with the KOS strain of HSV-1 (Neyts *et al.*, 1992), even though the Kupka strain seems to be more sensitive to the polymers (10-fold to 2,5-DHPOP, 75-fold to 2,5-DHTOP and 9-fold to GENOP) and the relative position of 2,5-DHTOP and GENOP in the ranking order has been changed.

The remaining 17 compounds (10 starting compounds and 7 polymers) are completely new and have not been tested before.

The antiviral potency of HA-like polymers depends, at least partially, on the content of acidic functional groups. This tendency is confirmed by the results of the present study demonstrating the increase of antiviral activity with the equivalents/Mol of carboxyl groups (Table 1).

Differences in the content of carboxyl groups can also be recognised from the infrared spectra interpreted according to Bellamy (1980), and Hadzija & Spoljar (1995). All the polymers tested possess distinctive peak maxima in the region of the symmetric (1370–1500 cm^{-1}) and asymmetric carboxylate anion vibrations (1600 cm^{-1}), even such polymers that were synthesized from phenolic starting compounds lacking carboxyl groups (except for GENOP and 2,5-DHPOP). Only HYDROP, 2,5-DHBEOP and TMHYDROP show peak maxima at 1700 cm^{-1} (C=O from COOH groups) too. The appearance of carboxyl vibrations in the case of polymers synthesized from carboxyl-free phenolic starting compounds prove the assumption that a few of aromatic rings are cleaved during the oxidative polymerisation. In this way, carboxyl groups and aliphatic side chains will be formed.

Concerning the structure and antiviral activity, the differences between the synthetic HA-like polymers and their low-molecular starting compounds are much higher than among the different polymers themselves. This is evidenced by a comparison of the EC_{50} values of HA-like polymers and of their starting compounds. Seven of 10 low-molecular starting compounds did not possess detectable antiviral activity. Only hydroquinone, 2,5-dihydroxytoluene and, more weakly, 2,5-dihydroxy benzoquinone, demonstrated anti-HSV-1 activity, probably due to spontaneously formed polymers during incubation time.

On the other hand, the CC_{50} values were generally found to be higher than the EC_{50} values of polymers and, except for 2,3-DMHYDROP and TMHYDROP, outside the concentration range tested. High cytotoxicities were found only for carboxyl-free phenolic compounds such as hydroquinone, 2,5-dihydroxy toluene, 2,5-dihydroxy benzaldehyde, 2,3-dimethyl hydroquinone, trimethyl hydroquinone and 2-methoxy hydroquinone. The highest SI values were reached by 2,5-DHPOP (≥400), 2,5-DHTOP (≥80) and GENOP (≥58).

Together with earlier findings, the results of the present study identify the consistent antiviral properties of naturally occurring HA and synthetic *o*- and *p*-diphenol-derived HA-like polymers suggesting a common fundamental principle of the underlying chemical structure. The tremendous number of naturally occurring and synthetically available phenolic compounds provide an opportunity to specifically increase the antiviral effectiveness and cell compatibility of these polymers. Further progress may be expected from the elucidation of the hitherto unknown backbone structure of HA-like polymers and from the knowledge on the steric relationships between intramolecular COOH and OH functional groups. Due to roughly the same potency of HA-like polymers to both types of HSV (Thiel *et al.*, 1976; Klöcking *et al.*, 1983; Neyts *et al.*, 1992; Helbig *et al.*, 1997; Meerbach *et al.*, 2001), the results make synthetic HA-like polymers interesting candidates for further studies as anti-HSV-1 as well as anti-HSV-2 agents.

Acknowledgement

The excellent technical help of Christa Rothmann is gratefully acknowledged.

References

Bellamy IJ (1980) *The Infrared Spectra of Complex Molecules*, 2nd edn; vol. 2. Advances in Infrared Group Frequencies. London, New York: Chapman & Hall.

Benda R, Petrovic S & Rychterova V (1972) Transmission of herpesvirus along the trigeminal nerve in rabbits. *Acta virologica* **16**:459–465.

Cataldo F (1998) On the structure of macromolecules obtained by oxidative polymerisation of polyhydroxyphenols and quinones. *Polymer International* **46**:263–268.

Hadzija O & Spoljar B (1995) Quantitative determination of carboxylate by infrared spectroscopy application to humic acids. *Fresenius' Journal of Analytical Chemistry* **351**:692–693.

Hänninen KI, Klöcking R & Helbig B (1987) Synthesis and characterization of humic acid-like polymers. *Science of the Total Environment* **62**:201–210.

Hänninen KI, Klöcking R & Helbig B (1989) Structural studies on phenolic polymers derived from pyrogallol oxidation. *Suomen Akatemian Julkaisuja* **5**:141–144.

Helbig B & Klöcking R (1983) Darstellung und Charakterisierung von Huminsäure-Modellsubstanzen. *Zeitschrift für Physiotherapie* **33**:31–37.

Helbig B, Klöcking R & Wutzler P (1997) Anti-herpes simplex virus type 1 activity of humic acid-like polymers and their *o*-diphenolic starting compounds. *Antiviral Chemistry & Chemotherapy* **8**:265–273.

Helbig B, Klöcking R & Hänninen KI (1994) Phenolkörperpolymerisate als Modellsubstanzen für natürliche Huminsäuren: Darstellung und vorläufige Charakterisierung. *TELMA* **24**:131–142.

Klöcking R, Schacke M & Wutzler P (1995) Primärscreening antiherpetischer Verbindungen mit EZ4U. *Chemotherapie Journal* **5**:141–147.

Klöcking R & Sprößig M (1972) Antiviral properties of humic acids. *Experientia* **28**:607.

Klöcking R & Sprößig M (1975) Wirkung von Ammoniumhumat auf einige Virus-Zell-Systeme. *Zeitschrift für Allgemeine Mikrobiologie* **15**:25–30.

Klöcking R, Sprößig M, Wutzler P, Thiel K-D & Helbig B (1983) Antiviral wirksame Huminsäuren und huminsäureähnliche Polymere. *Zeitschrift für Physiotherapie* **35**:95–101.

Meerbach A, Neyts J, Balzarini J, Helbig B, De Clerq E & Wutzler P (2001) In vitro activity of polyhydroxycarboxylates against herpesviruses and HIV. *Antiviral Chemistry & Chemotherapy* **12**:337–345.

Neyts J, Snoeck R, Wutzler P, Cushman M, Klöcking R, Helbig B, Wang P & De Clercq E (1992) Poly(hydroxy)carboxylates as selective inhibitors of cytomegalovirus and herpes simplex virus replication. *Antiviral Chemistry & Chemotherapy* **3**:215– 222.

Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J & De Clercq E (1988) Rapid and automated tetrazoli-

um-based colorimetric assay for the detection of anti-HIV compounds. *Journal of Virological Methods* **20:**309–321.

Schnitzer M & Khan SU (1972) *Humic Substances in the Environment.* New York: Marcel Dekker.

Schols D, Wutzler P, Klöcking R, Helbig B & De Clercq E (1991) Selective inhibitory activity of polyhydroxycarboxylates derived from phenolic compounds against human immunodeficiency virus replication. *Journal of Acquired Immune Deficiency Syndrome* **4:**677–685.

Schultz H (1962) Die viricide Wirkung der Huminsäuren im Torfmull auf das Virus der Maul- und Klauenseuche. *Deutsche Tierärztliche Wochenschrift* **69:**613–616.

Thiel KD, Wutzler P, Helbig B, Klöcking R, Sprößig M & Schweizer H (1984) Antivirale Wirksamkeit von enzymatisch und nichtenzymatisch oxidierter Kaffee- und Hydrokaffeesäure gegenüber Herpesvirus hominis Typ 1 und Typ 2 *in vitro*. *Pharmazie* **39:**781–782.

Thiel KD, Klöcking R & Helbig B (1976) *In vitro* studies of the antiviral activity of enzymatically oxidized *o*-diphenolic compounds against herpes simplex virus type 1 and type 2. *Zentralblatt für Bakteriologie und Hygiene Abteilung Originale A* **234:**159–169.

Wang TSC & Wang MC (1983) Catalytic synthesis of humic substances by using aluminas as catalysts. *Soil Science* **136:**226–230.

Received 8 May 2002; accepted 7 August 2002

Display from MEDLINE

Title

[In vitro studies of the antiviral activity of ammonium humate against **herpes** simplex virus type 1 and type 2 (author's transl)]. Untersuchungen in vitro zur antiviralen Aktivitat von Ammonium-humat gegenuber **Herpes** simplex-Virus Typ 1 und Typ 2.

Author

Thiel K D; Klocking R; Schweizer H; Sprossig M

Publication Source

Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Erste Abteilung Originale. Reihe A: Medizinische Mikrobiologie und Parasitologie, (1977 Nov) 239 (3) 304-21.
Journal code: 0331570. ISSN: 0300-9688.

Document Type

Journal; Article; (JOURNAL ARTICLE)

Language

German

Abstract

Ammonium humate, isolated from peat water, is a higher molecular polyphenolic compound with a strong antiviral activity against **herpes** simplex virus type 1 and type 2. Three cell lines were chosen for examining the cytotoxicity of ammonium humate: rabbit kidney primary cells, HEp-2- and FL-cells. As the light microscopic examination of both, the monolayers and the stained cover-glass cultures, revealed, concentrations of up to 5 microgram/ml ammonium humate can be used without hesitation for rabbit kidney primary cells (Fig. 1, 2) 200 microgram/ml for HEp-2-cells (Fig. 3, 4, 5), and 2000 microgram/ml for FL-cells. Fresh prepared rabbit kidney primary cells and suspended FL-cells (Tab. 1) are most sensitive against ammonium humate than monolayers. A remarcable plaque inhibition effect on the multiplication of **herpes** simplex virus type 1 and type 2 has been observed in all cell systems at concentrations of 0.5-20 microgram/ml ammonium humate (Fig. 6). The inhibition of virus multiplication at concentrations of greater than 10 microgram/ml ammonium humate was independent of the incubation temperature (Fig. 8). The adsorption of the virus to the cell surface has been found to be the most humate-sensitive phase of the herpesvirus multiplication cycle (Fig. 7, 9, 10). A prophylactic effect in vitro could be observed at concentrations greater than 10 microgram/ml ammonium humate (Fig. 11, 12).

Accession Number

78099292 MEDLINE

Display from CAplus

Title

The effect of sodium humate on metabolism and resistance of highly productive poultry

Author

Stepchenko, L. M.; Zhorina, L. V.; Kravtsova, L. V.

Organization

Dnepropetr. S-kh. Inst., Dnepropetrovsk, USSR

Publication Source

Biologicheskie Nauki (Moscow) (1991), (10), 90-5

Identifier-CODEN

BINKBT

ISSN

0470-4606

Abstract

Supplementation of the broiler chick diet with sodium humate at 250 mg/kg for 3 wk starting from age 20 days increased their body wt. by 7-10%, survival rates by 4-5%, and mproved resistance to **hepatitis**, diathesis, and hypoavitaminosis A, E, and D. The supplement increased blood alk. reserve (by 6-10%), lysozyme and bactericidal activities of blood serum (by 30-35%), erythrocyte Hb levels (by 6-10%), improved amino acid and protein metab., and affected lipid, mineral, and water metab.

Document Type

Journal

Language

Russian

Accession Number

1992:40267 CAPLUS

Document Number

116:40267

Display from MEDLINE

Title

[A comparative evaluation of the effect of different types of sapropel on dynamic liver function in intact rats and in the modelling of toxic **hepatitis**].
Sravnitel'naia otsenka vliianiia raznykh tipov sapropelia na dinamiku funktsional'nogo sostoianiia pecheni intaktnykh krys i pri modelirovanii toksicheskogo gepatita.

Author

Kuz'menko D I; Sidorenko G N; Levitskii E F; Laptev B I; Dzhuraeva E I

Publication Source

Voprosy kurortologii, fizioterapii, i lechebnoi fizicheskoi kultury, (1998 Mar-Apr) (2) 37-8.
Journal code: 2984868R. ISSN: 0042-8787.

Document Type

Journal; Article; (JOURNAL ARTICLE)

Language

Russian

Abstract

A course of silicic sapropel applications compared to calcareous sapropel induced a reversible fall of total lipid concentration in blood serum of intact rats. Sapropels of different kinds and of the same kind but obtained from different depths of the same deposit varied by their ability to correct hepatic function in rats with toxic **hepatitis**. The highest benefit was registered in application of carbonate sapropels taken from the depth of 1.5-2.5 m.

Accession Number

1998307117 MEDLINE

Biomed. Biochim. Acta 45 (1986) 9, 1173–1179

Hemmung ausgewählter Influenzavirusstämme der Typen A und B durch Phenolkörperpolymerisate

JUTTA HILS[1], ANNELIES MAY[1], M. SPERBER[1], RENATE KLÖCKING[2], B. HELBIG[1] und M. SPRÖSSIG[2]

[1] Institut für Angewandte Virologie, 1190 Berlin, Britzer Str. 1–3
[2] Institut für Mikrobiologie der Medizinischen Akademie Erfurt, 5010 Erfurt, Nordhäuser Str. 74

(Eingegangen am 1. Juli 1985; nach Revision am 4. März 1986)

Zusammenfassung

10 synthetische Phenolkörperpolymerisate (KOP, HYKOP, CHOP, 3,4-DHTOP, Na-ADROP, NH_4-ADROP, Na-NORADROP, NH_4-NORADROP, GENOP und 2,5-DHTOP) und 2 Phenolkörperpolymerisate natürlicher Herkunft (Na- und NH_4-humat) wurden im Primärscreening mit dem Testsystem Allantois-on-shell auf antivirale Wirksamkeit gegenüber verschiedenen Influenzavirusstämmen der Typen A und B untersucht. Die Kontrolle der Virusvermehrung erfolgte durch Bestimmung des Infektionstiters. Eine Hemmwirkung konnte bei allen Testsubstanzen gegenüber ausgewählten Influenzavirusstämmen des Typs A nachgewiesen werden. 3,4-DHTOP, Na- und NH_4-ADROP sowie GENOP wirkten außerdem hemmend auf Influenzavirus B/Singapore/222/79.

Die höchsten Titerreduktionen ($> 4.0 \log 10\ ID_{50}$) wurden mit KOP und HYKOP gegen Influenzavirus A/Brazil/11/78 und mit 3,4-DHTOP, KOP und HYKOP gegen Influenzavirus A/Hongkong/1/68 erreicht. Nur GENOP zeigte antivirale Wirksamkeit gegenüber allen eingesetzten Testviren.

Einleitung

Für die Bekämpfung der Grippe gewinnt die Chemoprophylaxe und -therapie als eine wirksame Ergänzung der Immunprophylaxe immer größere Bedeutung. Als bisher erfolgreichste Virostatika sind Amantadin und Rimantadin aus der Substanzklasse der Adamantane in ausgedehnten Feldversuchen getestet worden bzw. für eine Massenanwendung zum Einsatz gekommen [8, 13]. Ein Nachteil der genannten Substanzen besteht darin, daß sich ihre Hemmwirkung ausschließlich auf Influenzavirusstämme des Typ A erstreckt [4–6]. Die Suche nach Substanzen mit breiterem Wirkungsspektrum steht daher im Mittelpunkt der gegenwärtigen Virostatikaforschung.

Wie mit dem Plaque-Reduktionstest bereits gezeigt werden konnte, besitzen enzymatisch synthetisierte Phenolkörperpolymerisate (PKP) antivirale Aktivität gegenüber Influenzavirus A/Krasnodar/101/59 (H2N2). Ihre Wirkung erstreckt sich sowohl auf die Adsorptionsphase als auch auf postadsorptive Stadien der Virusvermehrung [11]. MICHEL et al. [12] konnten in vitro eine Hemmung der Influenzavirus-Transkriptase durch verschiedene PKP nachweisen. In der vorliegenden Arbeit wird geprüft, ob auch andere Influenzavirusstämme verschiedener Typen und Subtypen gegenüber

PKP empfindlich sind und ob die Hemmwirkung von der Struktur der Polymere abhängig ist. Dabei wurden zum ersten Mal vorzugsweise PKP eingesetzt, die durch chemische Oxydation, d. h. auf nichtenzymatischem Wege, synthetisiert wurden [3].

Material und Methoden

Virusstämme

Verwendet wurden die WHO-Referenzstämme des Influenzavirus A/Brazil/11/78 (H1N1), Pass.: En + 3, $EID_{50} = 10^{7,3}$; A/Krasnodar/101/59 (H2/N2), Pass.: En + 3, $EID_{50} = 10^{8,2}$; A/Hongkong/1/68 (H3N2), Pass.: En + 3, $EID_{50} = 10^{7,8}$; B/Singapore/222/79, Pass.: En + 2, $EID_{50} = 10^{7,9}$. Die Virusvermehrung erfolgte auf 11tägigen embryonierten Hühnereiern. Die infektiöse Allantois wurden 48 bzw. 72 h p.i. geerntet und das vereinigte Virusmaterial portioniert bei −70 °C bis zu Versuchsbeginn gelagert.

Testsystem

Als Grundlage des antiviralen Screenings diente das von ILJENKO [7] für Influenzaviren empfohlene Allantois-on-shell-System. Die bereits von FAZEKAS DE ST. GROTH und WHITE [2] beschriebene Methode wurde in Bezug auf das eingesetzte Medium (Hanks-Lösung gepuffert mit 0,2 M Tris-HCl) leicht modifiziert [4, 5]. Für die Versuche verwendeten wir 120 × 255 mm große Plastikplatten mit 12 × 6 Vertiefungen (Fassungsvermögen je 2 ml), die je 0,4 ml Medium enthielten. In diese wurden Eischalenstücke mit Chorioallantoismembran von etwa 5 × 5 mm Größe eingelegt. Die Konzentration aller Testsubstanzen betrug 100 µg/ml. Die Wirkstoffzugabe erfolgte 1 h vor Virusinokulation. Beimpft wurde mit 0,1 ml der jeweiligen Virusverdünnung (10^{-1} bis 10^{-10} EID_{50}/0,1 ml). Der Wirkstoff verblieb während der gesamten Inkubationszeit — 48 h bei Influenza A bzw. 72 h bei Influenzavirus B — im Medium. Die Inkubationstemperatur betrug 36 °C bei Influenza A bzw. 33 °C bei Influenza B. Nach Ablauf der Inkubationszeit wurde der Endpunkt der Virusvermehrung anhand der hämagglutinierenden und/oder der Neuraminidaseaktivität bestimmt. Bei einigen PKP konnte die Auswertung nicht durch Bestimmung der hämagglutinierenden Aktivität erfolgen, da schon die Polymerisate zur Agglutination führten. Die Neuraminidaseaktivität wurde durch die PKP nicht beeinflußt. Als Kontrolle für die Inhibitorwirkung der PKP dienten entsprechende Ansätze ohne Wirkstoff. Mit jeder Testsubstanz wurden pro Virusstamm 3 bis 5 Ansätze mit jeweils 6 Parallelen durchgeführt.

Bestimmung der hämagglutinierenden Aktivität

Nach Ablauf der Inkubationszeit und Entfernen der Eischalenstücke mit der Chorioallantoismembran aus den Proben wurden 25 µl einer 5%igen Hühnererythrozytensuspension je Probe zugegeben. Die Platten blieben 1 h bei Zimmertemperatur stehen. Anschließend wurde mit Hilfe des auf diesem Wege ermittelten Endpunktes der Virusvermehrung die ID_{50} nach SPERMAN und KAERBER bestimmt und daraus die Titerreduktion gegenüber der unbehandelten Kontrolle berechnet [10].

Bestimmung der Neuraminidaseaktivität

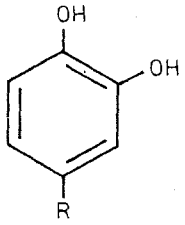
Für die Bestimmung der Neuraminidaseaktivität wurde der Neuramikrotiter TYP OX-604/6 (Labor MIM Budapest, VR Ungarn) eingesetzt. Die verwendeten Titerplatten hatten eine Abmessung von 130 × 90 × 15 mm. Jede der 10 × 6 Vertiefungen faßte ein Flüssigkeitsvolumen von 0,6 ml. Der Bestimmung lag die Methode von AYMARD-HENRY et al. [1] zugrunde, wobei Ovomukoid anstelle von Fetuin als Substrat verwendet wurde und das Ausschütteln des Farbkomplexes mit Butanol entfiel. Die erforderlichen Reagenzien wurden in folgenden Volumina eingesetzt: 50 µl virushaltiges Medium aus jeder Probe, 50 µl Substratlösung (50 mg/ml Ovomukoid), 25 µl Perjodat-, 50 µl Arsenit- und 250 µl Thiobarbitursäurereagenz. Die Substratlösung wurde 18 h bei 37 °C mit dem virushaltigen Medium inkubiert. Nach Zugabe der übrigen Reagenzien wurden die Proben mit Heizstäbchen ca. 6 min auf 90 bis 100 °C erhitzt. Der bei Anwesenheit von Neuraminidase sich entwickelnde rote Farbkomplex zeigt den Endpunkt der Virusvermehrung an. Danach wurden die ID_{50} sowie die Reduktion des Infektionstiters gegenüber der unbehandelten Kontrolle berechnet [10].

Testsubstanzen

Insgesamt wurden 8 in den Tabellen 1 und 2 näher bezeichnete synthetische PKP in Form ihrer Na-Salze, zwei davon (ADROP und NORADROP) zusätzlich auch in Form ihrer NH_4-Salze auf antivirale Aktivität gegenüber Influenzaviren geprüft. Ein Polymerisat (ADROP) lag sowohl in enzymatisch als auch in chemisch synthetisierter Form vor. Als Vertreter natürlich vorkommender PKP wurden wasserlösliche Huminsäuren (HS) in Form ihres Na- und NH_4-Salzes in die Testung einbezogen. Ihre Isolierung aus dem HS-reichen Wasser eines Küstenhochmoores der DDR erfolgte über die Blei(II)-Chelatver-

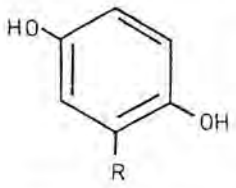
Tabelle 1

Verzeichnis der Testsubstanzen (Phenolkörperpolymerisate) und ihrer Ausgangsverbindungen: 3,4-Dihydroxyphenylderivate



Ausgangsverbindung	–R	Bezeichnung des Polymerisates	Molmasse
Kaffeesäure	$-CH=CH-COOH$	KOP	6500
Hydrokaffeesäure	$-CH_2-CH_2-COOH$	HYKOP	10200
Chlorogensäure	$-CH=CH-COOC_7H_{11}O_5$	CHOP	11000
3,4-Dihydroxytoluen	$-CH_3$	3,4-DHTOP	6000
Adrenalin	$-CHOH-CH_2NHCH_3$	ADROP	5400
Noradrenalin	$-CHOH-CH_2-NH_2$	NORADROP	7200

Tabelle 2
Verzeichnis der Testsubstanzen (Phenolkörperpolymerisate) und ihrer Ausgangsverbindungen: 2,5-Dihydroxyphenylderivate



Ausgangsverbindung	—R	Bezeichnung des Polymerisates	Molmasse
Gentisinsäure	—COOH	GENOP	5900
2,5-Dihydroxytoluen	$-CH_3$	2,5-DHTOP	10100

bindungen nach einem an anderer Stelle ausführlich beschriebenen Verfahren [9]. Zur Herstellung der synthetischen PKP wurden die o- und p-diphenolischen Ausgangsverbindungen mit einem milden chemischen Oxydationsmittel [3] oder enzymatisch mit Hilfe von Tyrosinase (E. C. 1.10.3.1) oxydiert [9]. Ihre Isolierung erfolgte in gleicher Weise wie die der HS.

Alle Testsubstanzen sind in wässrigen Medien bei *p*H 7,4 löslich. Ihre mittleren Molmassen, bestimmt durch Gelpermeationschromatographie an Controlled Pore Glass[18] der Porengröße 120 Å (Electro-Nucleonics, Inc., Fairfield, N.J., USA) liegen zwischen 5400 und 11000. Die Molmassen von Na- und NH_4-Humat betragen 7900 bzw. 9000. Da die Molmassen zu Beginn dieser Studie noch nicht bekannt waren, erfolgte die Dosierung einheitlich in µg/ml. Die Angaben in den Tabellen 1 und 2 ermöglichen eine rasche Orientierung über die eingesetzten molaren Konzentrationen für jede Testsubstanz.

Ergebnisse und Diskussion

Die für das antivirale Screening im Allantois-on-shell-System eingesetzte Substanzkonzentration von 100 µg ml wurde von der Chorioallantoismembran gut toleriert und führte in keinem Fall zu mikroskopisch sichtbaren Zellveränderungen. Untersuchungen an FL-Zellen, einer permanenten humanen Amnionepithelzellinie, erbrachten bis zu PKP-Konzentrationen um 1000 µg/ml keine Hinweise auf eine toxische Beeinflussung der Membranpermeabilität von Ruhezellen (Ergebnisse nicht dargestellt).

Die Ergebnisse zur antiviralen Wirksamkeit der untersuchten PKP sind in Abb. 1 und 2 dargestellt. Als Maß für die Hemmung der Virusvermehrung durch die Testsubstanzen wird die Reduktion des Infektionstiters gegenüber der unbehandelten Kontrolle angegeben. Dabei entspricht eine Erniedrigung des Infektionstiters um eine logarithmische Stufe einer 90%igen Hemmwirkung und stellt den Grenzwert für die Einstufung „empfindlich" bzw. „unempfindlich" dar.

Die Säulendiagramme zeigen, daß alle eingesetzten Substanzen bei mehreren Influenza-A-Virusstämmen eine Hemmung der Virusvermehrung von mindestens

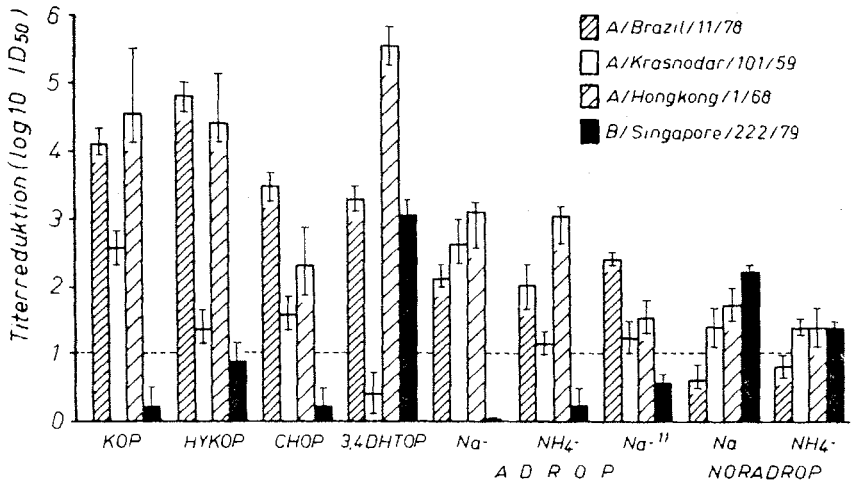


Abb. 1. Empfindlichkeit ausgewählter Influenzavirusstämme Typ A und B gegenüber Polymerisaten aus 3,4-Dihydroxyphenylverbindungen.
Konzentration der Testsubstanzen 100 µg/ml. [1] enzymatisch synthetisiert

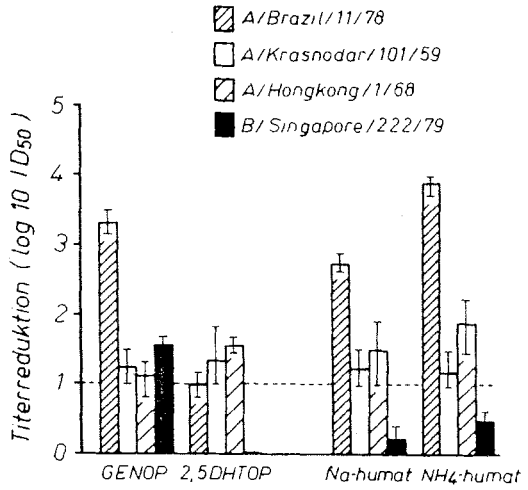


Abb. 2. Empfindlichkeit ausgewählter Influenzavirusstämme Typ A und B gegenüber Polymerisaten aus 2,5-Dihydroxyphenylverbindungen und gegenüber natürlich vorkommenden Phenolkörperpolymerisaten (Na- und NH_4-humat).
Konzentration der Testsubstanzen 100 µg/ml.

einer logarithmischen Stufe gegenüber der Kontrolle bewirken. Influenzavirus Typ B wird von 4 Testsubstanzen, nämlich 3,4-DHTOP, Na- und NH_4-NORADROP sowie GENOP in seiner Vermehrung so gehemmt, daß der o. g. Grenzwert für die Empfindlichkeit erreicht bzw. überschritten wird.

Unter den getesteten PKP verdient GENOP besonderes Augenmerk, da nur gegen diese Substanz alle ausgewählten Influenzavirusstämme „empfindlich" reagierten. Es gehört jedoch nicht zu den am stärksten wirksamen Inhibitoren, denn die höchsten Titerreduktionen (> 4.0 log 10) wurden mit KOP und HYKOP gegen Influenzavirus A/Brazil/11/78 und mit 3,4-DHTOP, KOP und HYKOP gegen A/Hongkong/1/68 erzielt.

Zwischen den Na- und NH_4-Salzen der einzelnen Verbindungen traten keine wesentlichen Unterschiede in der Hemmwirkung auf. Desgleichen zeigte auch das enzymatisch synthetisierte Na-ADROP keine grundlegenden Differenzen in seiner Hemmwirkung gegenüber dem chemisch synthetisierten Polymeren.

Die vorliegenden Testergebnisse bestätigen die von Mentel et al. [11] nachgewiesene Hemmung der PKP gegenüber Influenzavirus A/Krasnodar/101/59 (H2N2), wenn sich auch auf Grund der unterschiedlichen Screeningmethoden (Plaquetechnik auf Hühnerembryofibroblasten bzw. Reduktion des Infektionstiters im Allantois-on-shell-System) keine quantitativen Vergleiche anstellen lassen.

Auffällig ist, daß nur wenige Polymerisate zur Hemmung von Influenzavirus B in der Lage sind. Auch die natürlich vorkommenden PKP-Vertreter Na- und NH_4-Humat hemmen, wie die meisten synthetischen Polymerisate, nur die A-Stämme. Relativ kleine Unterschiede in der chemischen Struktur der phenolischen Ausgangsverbindungen bedingen dabei deutliche Unterschiede in der Wirksamkeit der Polymerisate (z.B. 2,5-DHTOP unwirksam — 3,4-DHTOP wirksam, ADROP unwirksam — NORADROP wirksam). Diese für die Aufklärung des antiviralen Wirkungsmechanismus der PKP interessierenden Befunde könnten auch für die gezielte Synthese von Influenza-B-Inhibitoren auf PKP Basis in Zukunft von Bedeutung sein. Dabei ist zu berücksichtigen, daß die in vitro gefundenen Ergebnisse nicht unmittelbar auf das Verhalten der Wirkstoffe in vivo schließen lassen. Hierüber kann nur das Tierexperiment Aufschluß geben.

Abschließend sei zu dem hier angewandten Testsystem Allantois-on-shell bemerkt, daß es sich im Hinblick auf seinen relativ geringen Material- und Zeitaufwand als Screening-System für antivirale Substanzen bewährt hat.

Literatur

[1] Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, R. G. Webster: Bull. WHO **48**, 199 (1973)

[2] Fazekas de St., S. Groth, D. O. White: J. Hyg. **56**, 151 (1958)

[3] Helbig, B., R. Klöcking, M. Sprössig, D. Bertram, H. Lettau: WP (DDR) Reg.-Nr. 2516977 (C 07 G) v. 3. Juni 1983

[4] Hils, J., A. May, H. Giese, G. Pohl, H. Scharfenorth: Dtsch. Gesundheitswes. **38**, 398—400 (1983)

[5] Hils, J., A. May, M. Sperber, G. Pohl, H. Scharfenorth: Z. Klin. Med. **40**, 279 bis 281 (1985)

[6] Hils, J., A. May, K. Lehmann, G. Pohl, H. Scharfenorth: Z. Klin. Med. **40**, 1297 bis 1299 (1985)

[7] Iljenko, V. J. in: Methoden zur Untersuchung und Bewertung der antiviralen Aktivität chemischer Verbindungen gegenüber Influenzaviren. Methodische Hinweise des Allunionsforschungsinstitutes für Grippe des Ministeriums für Gesundheitswesen der UdSSR, Leningrad 1977, S. 11—15

[8] Indulen, M. K., V. A. Kalninya in: Developments in Antiviral Therapy. L. H. Collier, J. Oxford (Eds.), Academic Press, London 1980

[9] Klöcking, R., B. Helbig, P. Drabke: Pharmazie **32**, 297 (1977)

[10] Mayr, A., P. A. Bachmann, B. Bibrack, G. Wittmann: Virologische Arbeitsmethoden. Bd. I und II. Fischer, Jena 1977

[11] MENTEL, R., B. HELBIG, R. KLÖCKING, L. DÖHNER, M. SPRÖSSIG: Biomed. Biochim. Acta **42**, 1353–1356 (1983)
[12] MICHEL, S., E. SCHREIER, B. SCHWEIGER, R. T. POHL, A. MÜLLER, P. SCHULZE: Isolierung und Charakterisierung der Ribonukleoprotein-Partikel durch In-vitro-Testung von Inhibitoren der Transkriptase. 2. Tagung der Sektion Virologie der Gesellschaft für Mikrobiologie und Epidemiologie der DDR, Gera, 9.–12. Mai 1983
[13] OXFORD, J. S., A. GALBRAITH: Pharmacol. Ther. **11**, 181–262 (1980)

Summary

J. HILS, A. MAY, M. SPERBER, R. KLÖCKING, B. HELBIG, and M. SPRÖSSIG: Inhibitory effect of phenolic polymers on several strains of influenza virus type A and B

Ten synthetic polymers of diphenolic compounds (KOP, HYKOP, CHOP, 3,4-DHTOP Na-ADROP, NH_4-ADROP, Na-NORADROP, NH_4-NORADROP, GENOP, and 2,5-DHTOP) as well as two phenolic polymers of natural origin (Na-humate, NH_4-humate) were tested for their effectiveness on several strains of influenza virus type A and B. The allantois-on-shell system was used for primary screening. Virus multiplication was assessed by means of the infectious titer. All substances tested were found to exert inhibitory effects on influenza virus type A, while 3,4-DHTOP, Na-ADROP, NH_4-ADROP as well as GENOP were effective against either type. The highest titer reductions ($>$ 4.0 log 10 ID_{50}) were obtained with KOP and HYKOP against influenzavirus A/Brasil/11/78 and with 3,4-DHTOP, KOP and HYKOP against influenzavirus A/Hongkong/1/68. Only GENOP showed antiviral activity against all virus strains tested so far.

Arch Virol (2002) 147: 273–284

Archives of Virology

In vitro anti-influenza virus activity of synthetic humate analogues derived from protocatechuic acid

F.-J. Lu[1], S.-N. Tseng[2], M.-L. Li[2], and S.-R. Shih[2,3]

[1]Department of Biochemistry, College of Medicine,
National Taiwan University, Taipei, Taiwan
[2]School of Medical Technology, Chang Gung University, Tao-Yuan, Taiwan
[3]Clinical Virology Center, Chang Gung Memorial Hospital, Tao-Yuan, Taiwan

Accepted October 15, 2001

Summary. Two humic-like substances, the oxidative polymer of protocatechuic acid (OP-PCA) and humic acid inhibit the in vitro replication of influenza virus A/WSN/33 (H1N1) in Madin-Darby canine kidney (MDCK) cells at concentrations of no cytotoxicity. The 50% inhibitory concentration (IC_{50}) for OP-PCA was 6.59 ± 1.02 μg/ml when the compound was added at the stage of viral adsorption. When OP-PCA was added after virus adsorption, the IC_{50} was 53.27 ± 8.12 μg/ml. The IC_{50} for humic acid was 48.61 ± 7.32 μg/ml and 55.27 ± 5.46 μg/ml respectively when the compound was added at the stage of viral adsorption or post-adsorption. In spite of structural resemblance of these two compounds, they exhibit different actions of anti-flu. The OP-PCA inhibits virus-induced hemagglutination and low pH-induced cell–cell fusion. Humic acid inhibits the endonuclease activity of viral RNA polymerase. The monomer of PCA shows no inhibition on influenza virus replication.

Introduction

Influenza viruses cause acute respiratory disease with high morbidity and mortality in human and animals. Amantadine and rimantadine (adamantane derivatives) have been proven to be clinically useful in the prevention and treatment of influenza A viruses. Zanamivir and oseltamivir, the influenza neuraminidase inhibitors, have recently been shown inhibiting both influenza A and influenza B viruses replication in vitro and in the ferret model [1–8]. However, we believe that development of other anti-flu agents is absolutely needed for more efficient combinatorial therapy.

Protocatechuic acid (PCA) is a simple phenolic compound capable of modulating certain cellular enzymes as well as having anti-oxidative and anti-mutagenic

activities [9]. This phenolic chemical has also been proven possessing potent chemopreventive activity [10]. Oxidation of PCA forms phenolic polymer that is a humic-like substance. Humic substances exist abundantly in plants, soil, well water and other sources. It is a polymeric compound of high molecular weight. Several phenolic complexes have been shown to have anti-influenza virus activity. For example, a phenolic complex isolated from plant [11] is shown to exhibit the selective inhibition of viral protein synthesis. Another phenolic complex, a plant flavonoid, is shown to prevent flu infection in experimental mice [12]. Tea polyphenols were shown to bind to the hemagglutinin of influenza virus, inhibit its adsorption to MDCK cells, and thus block its infectivity [13]. In this study, we test the anti-flu activity of the oxidative polymer of protocatechuic acid (OP-PCA). Meanwhile we use its monomer (PCA) as well as its analogue (humic acid) for comparison. We also investigate the mechanism of their anti-viral activities.

Materials and methods

Compounds

Protocatechuic acid (PCA; 3,4-dihydroxybenzoic acid, Fig. 1) was purchased from Wako (Chuo-Ku, Osaka, Japan). Oxidation of PCA to form polymeric compound was carried out as previously described [14]. Briefly, the polymer was prepared by the oxidation of PCA with sodium periodate in a 50 °C shaking bath for 24 h. After centrifugation, the supernatant was collected and acidified to pH 1.0 with 0.1 N HCl. The mixture was further centrifuged and the polymers in the precipitate was subsequently dissolved in a 0.1 N NaOH. The polymer of oxidative PCA solution was further purified by passing through cellulose ester membrane. The solution was concentrated to dry powder using a vacuum evaporator. Humic acid was purchased from Aldrich (Milwaukee, WI, USA). The monomers of humic acid include protocatechuic acid, vanillic acid, catechol, gallic acid, syringic acid, caffeic acid, and ferulic acid.

Viruses and cells

Influenza virus A/WSN/33 (H1N1) was grown and propagated in Madin-Darby canine kidney (MDCK) cells.

Plaque inhibition assay

Triplicate plates (6-well plates) containing monolayers of MDCK cells were washed and infected with equal volume of virus suspension (50–100 plaque-forming units). The plates were incubated at 4 °C for 1 h. A 0.3% agarose overlay containing different drug concentration was then added to each plate. Plates were further incubated at 37 °C for another 48 h. Plaques were stained with crystal violet and counted. The percentage of plaque inhibition

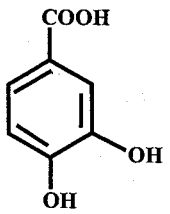


Fig. 1. Chemical structure of protocatechuic acid

relative to the infected control (no drug) plates was determined for each drug concentration, and the 50% inhibitory concentration (IC_{50}) was calculated.

Cellular toxicity

The effects of tested compounds on the viability of MDCK cells were determined in 96-well plastic microtiter plates by the tetrazolium-based colorimetric-MTT assay [15]. Approximately 10^4 cells/well were seeded and incubated with drug-containing medium for 48 h before assay. The percentage of cell viability was calculated as the ratio of the optical density values ($OD_{595, 650}$) between the drug-treated and non drug-treated cells.

Viral protein synthesis

The cell monolayers in duplicate wells of the 6-well plates were challenged with influenza virus (MOI = 50 PFU/cell) for 1 h at 4 °C in the presence or absence of tested anti-viral compounds. After incubation for 24 h at 35 °C the monolayers were labeled for 20 min with 50 μCi ^{35}S-methionine/well and lysed. The equal amount (10 μg) of proteins were then analyzed by electrophoresis on a 14% polyacrylamide gel.

Endonuclease inhibition assay

Inhibition of endonuclease activity of influenza virus by tested anti-viral compounds (100 μg/ml) was studied by the method described by [16]. Influenza A/WSN/33 was purified from MDCK cells by sucrose/D_2O cushion method and viral cores were isolated by velocity gradient method. The β-eliminated AIMV RNA4 (4.56 μg) was capped and methylated in vitro with guanylyltransferase in the presence of α-^{32}P-GTP. Cap-labeled mRNAs were gel-purified, extracted by phenol/chloroform, filtered through G-25 Sephadex spin columns, and then precipitated by ethanol. RNA substrate was incubated with purified virus core in the presence or absence of tested compounds for 30 min at 31 °C. Reaction was terminated with the addition of formamide-stop solution, and the mixture was analyzed on a 20% polyacrylamide-urea gel.

Hemagglutination inhibition test

25 μl (1000 PFU/μl) of virus suspension was mixed with tested compound of different concentrations at room temperature for 1 h. The mixture was two-fold serial diluted with PBS. 50 μl of 0.5% Guinea pig red blood cells (RBC) was added at 4 °C. The result was read after 2 h incubation.

Virus-induced cell–cell fusion assay

CV-1 cells were cultured in 6-well plates to reach confluence. Each well was infected with virus (1 m.o.i) for 9 h. The tested compounds in 2% FBS-DMEM of pH 5.0 or pH 7.2 were added into each well individually for 10 min at 37 °C. The medium was then refreshed with 2% FBS-DMEM of pH 7.2 without tested compounds. The result was read under microscopy after 2 h incubation.

Results

Cytotoxicity

We first evaluated the cytotoxicity of these tested compounds. Confluent MDCK monolayers treated for 48 h with Aldrich humic acid at concentrations of

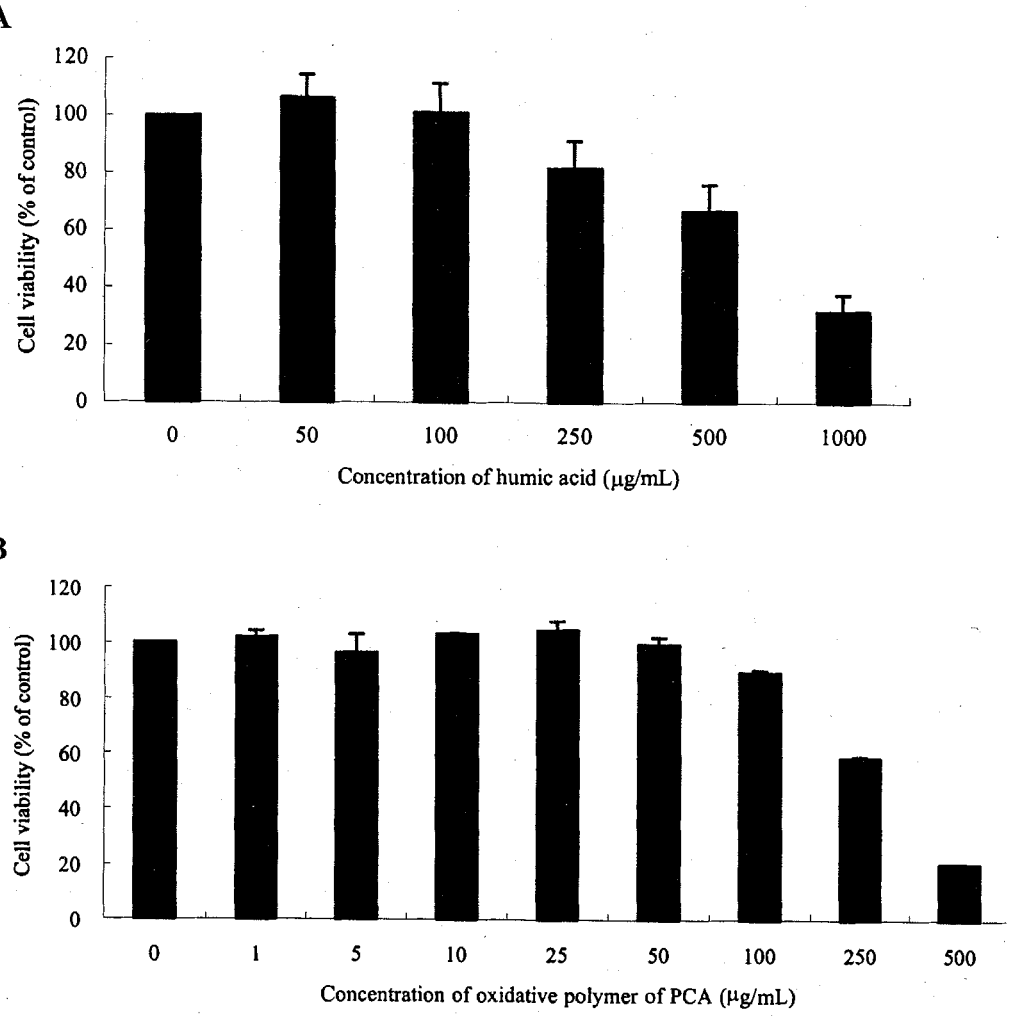


Fig. 2. The effects of tested compounds on cell viability. **A** The effect of humic acid on the viability of MDCK cells. **B** The effect of oxidative product of protocatechuic acid (PCA) on the viability of MDCK cells. Each experiment involves triplicate wells per concentration

1–100 μg/ml did not show significant reduction of cell viability base on the tetrazolium-based colorimetric-MTT assay (Fig. 2A). No visible changes in cell morphology or cell density was observed at these concentrations. The estimated dose that reduced cell viability by 50%, i.e., 50% cytotoxic concentration (CC_{50}) was 737.8 ± 12.6 μg/ml. The oxidative polymer of protocatechuic acid (OP-PCA) at concentrations of 1–50 μg/ml did not show obvious inhibition of cell viability. The CC_{50} for OP-PCA was 320 ± 7.5 μg/ml.

Inhibition of plaque formation

We investigated the effectiveness of test compounds on the plaque formation of infected-MDCK cells. Humic acid inhibits over 90% plaque formation at the

concentration of 100 μg/ml no matter this compound was added to cells either at the same time as viruses added or after viruses adsorption (Fig. 3A). The estimated dose that inhibits plaque formation by 50% (IC_{50}) was 48.61 ± 7.32 μg/ml and 55.27 ± 5.46 μg/ml respectively when the compound was added at the stage of viral adsorption or post adsorption. Virus adsorption was performed at 4 °C for 1 h. The oxidative polymer of protocatechuic acid (OP-PCA) inhibits 60% plaque formation at the concentration of 10 μg/ml when the compound was added at virus adsorption stage. When the concentration of oxidative polymer of protocatechuic acid increases to 100 μg/ml, plaque formation is inhibited to proximately 100% no matter this compound was added to cells either at the same time as viruses added or after viruses adsorption (Fig. 3B). The IC_{50} for OP-PCA were 6.59 ± 1.02 μg/ml and 55.27±8.12 μg/ml respectively when the compound was added at the stage of viral adsorption or post-adsorption. The monomer of protocatechuic acid shows no inhibition of plaque formation (Fig. 3C). The tested compounds were also evaluated the anti-viral activity of a clinical influenza isolate A/Taiwan/174/2000 (H3N2). Similar results were obtained as in the laboratory strain, A/WSN/33 (H1N1). For humic acid, the IC_{50} were 42.81±6.42 μg/ml and 53.37±6.86 μg/ml respectively when the compound was added at the stage of viral adsorption or post adsorption. For OP-PCA, the IC_{50} were 5.59±1.82 μg/ml and 59.08±8.32 μg/ml respectively when the compound was added at the stage of viral adsorption or post-adsorption. The monomer of protocatechuic acid shows no inhibition of plaque formation.

Inhibition of viral protein synthesis

We further measured the effects of tested compounds on the viral protein synthesis. The humic acid, when added at a concentration of 100 μg/ml at the stage of viral adsorption, is found inhibiting the synthesis of ^{35}S-methionine labeled virus protein. The OP-PCA, on the other hand, demonstrates the inhibition at a concentration as low as of 10 μg/ml. The PCA, however, shows no inhibition at all (Fig. 4A). When the compounds were added after viral adsorption, both humic acid and oxidative PCA are found to inhibit the viral protein synthesis at concentration of 100 μg/ml, whereas the PCA shows no inhibition (Fig. 4B). These results are consistent with that in plaque inhibition assay.

Inhibition of endonuclease activity of RNA polymerase complex

To further understand the mechanism of inhibition of influenza virus replication by humate-like compounds tested in his report, we evaluated the effects of these compounds on the endonuclease activity of RNA polymerase complex that plays an important role on transcription of viral RNA. As shown in Fig. 5, the humic acid at a concentration of 100 μg/ml strongly inhibits the cleavage activity of the RNA polymerase complex purified from viral cores (lane 4); whereas oxidative polymer of protocatechuic acid and its monomer, protocatechuic acid, show no effect (lanes 2 and 3).

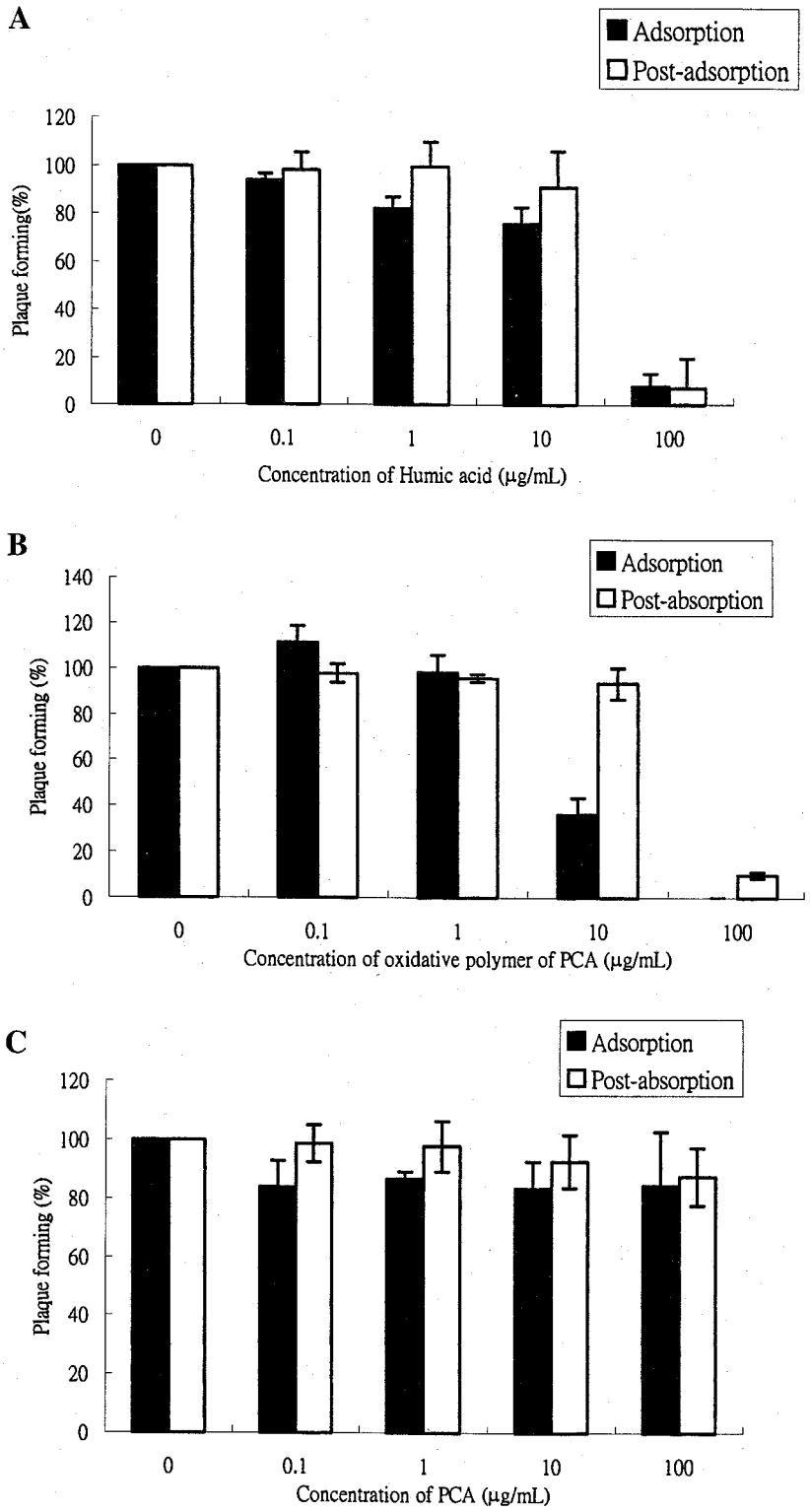


Fig. 3. Inhibition of influenza virus replication in vitro. **A** The effect of humic acid on the plaque forming of influenza virus. **B** Plaque reduction by oxidative protocatechuic acid. **C** The effect of protocatechuic acid on the plaque forming. Each experiment involves triplicate wells per concentration. Viral adsorption was performed at 4 °C for 1 h

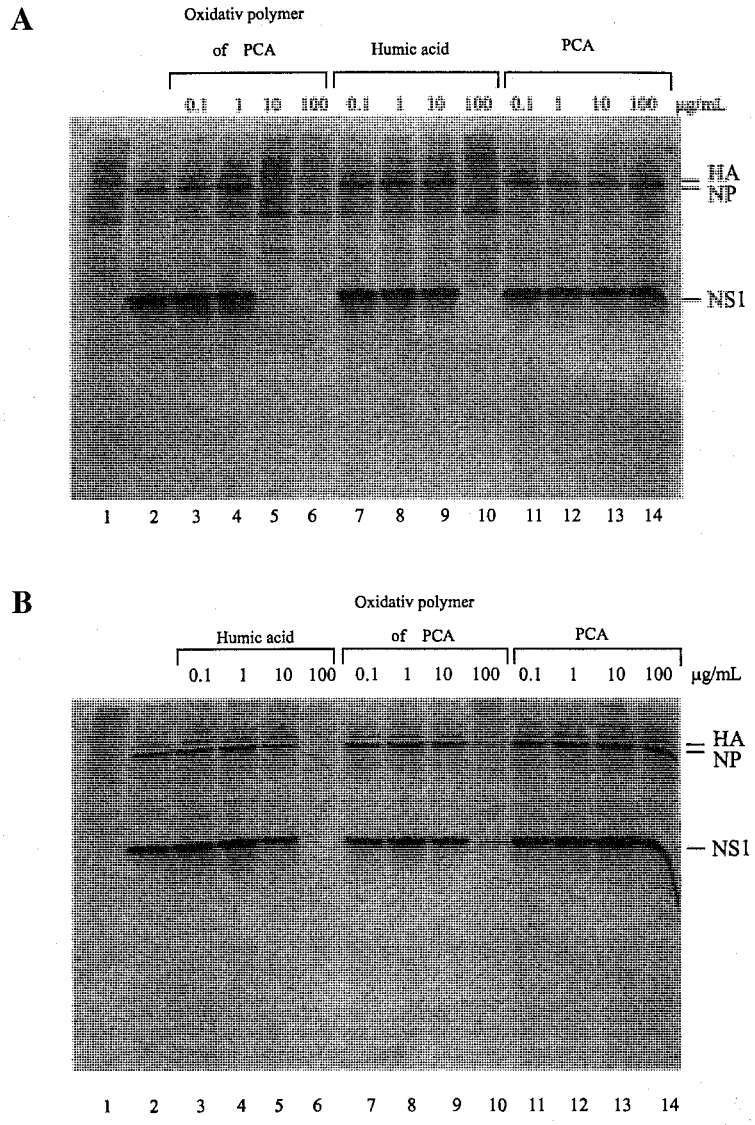
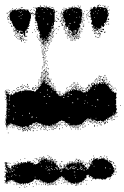


Fig. 4. Inhibition of viral protein synthesis. **A** Compounds were added at the stage of viral adsorption. **B** Compounds were added after viral adsorption. *1* was the protein extract from non-infected cells. *2* was the protein extract from flu-infected cells without adding any compound

Inhibition of influenza hemagglutinin

The envelope of influenza virion possesses the glycoproteins (HA) that are responsible for hemagglutination, that is, agglutination of erythrocytes. The activity of hemagglutinin reflects the adsorption ability of influenza virus. In plaque inhibition assay, the OP-PCA shows higher inhibitory effect of viral plaque formation when it was added at the stage of viral adsorption in comparison to being added after adsorption. This implies that the OP-PCA interferes with viral



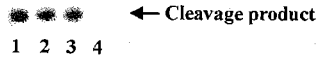


Fig. 5. Inhibition of endonuclease activity. Virus core was purified and added to ^{32}P-cap-labeled AIMV RNA4 (*1*). 100 μg/ml of the oxidative polymer of protocatechuic acid (*2*), protocatechuic acid (*3*) and humic acid (*4*) were individually added to the same reaction as *1*. The cleavage product is indicated by arrow

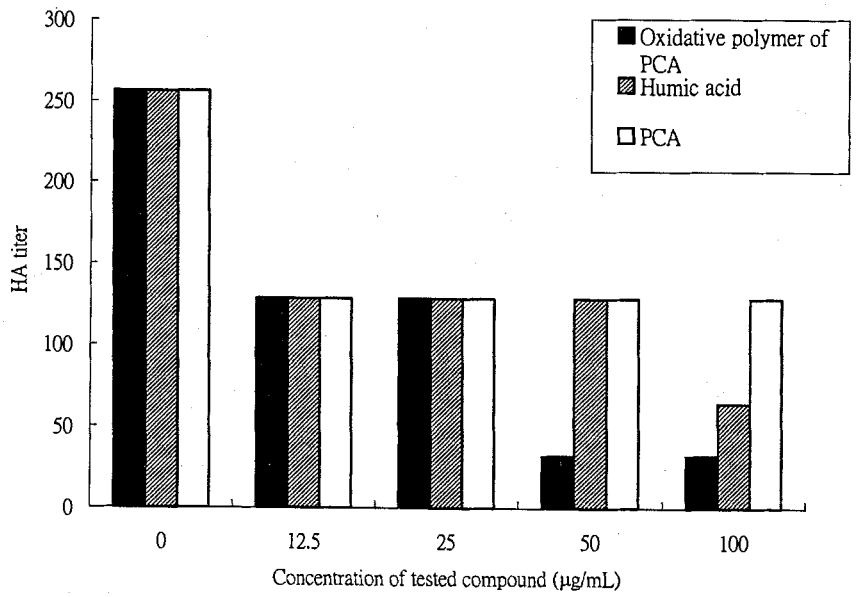


Fig. 6. HA titers of influenza A virus treated with various compounds. Results represent means of two separate experiments

adsorption. In Fig. 6, we found that the OP-PCA exhibits distinguished inhibition of hemagglutination at concentrations of 50–100 μg/ml. Humic acid inhibits hemagglutination at a concentration of 100 μg/ml, whereas the monomer of PCA only shows slight change.

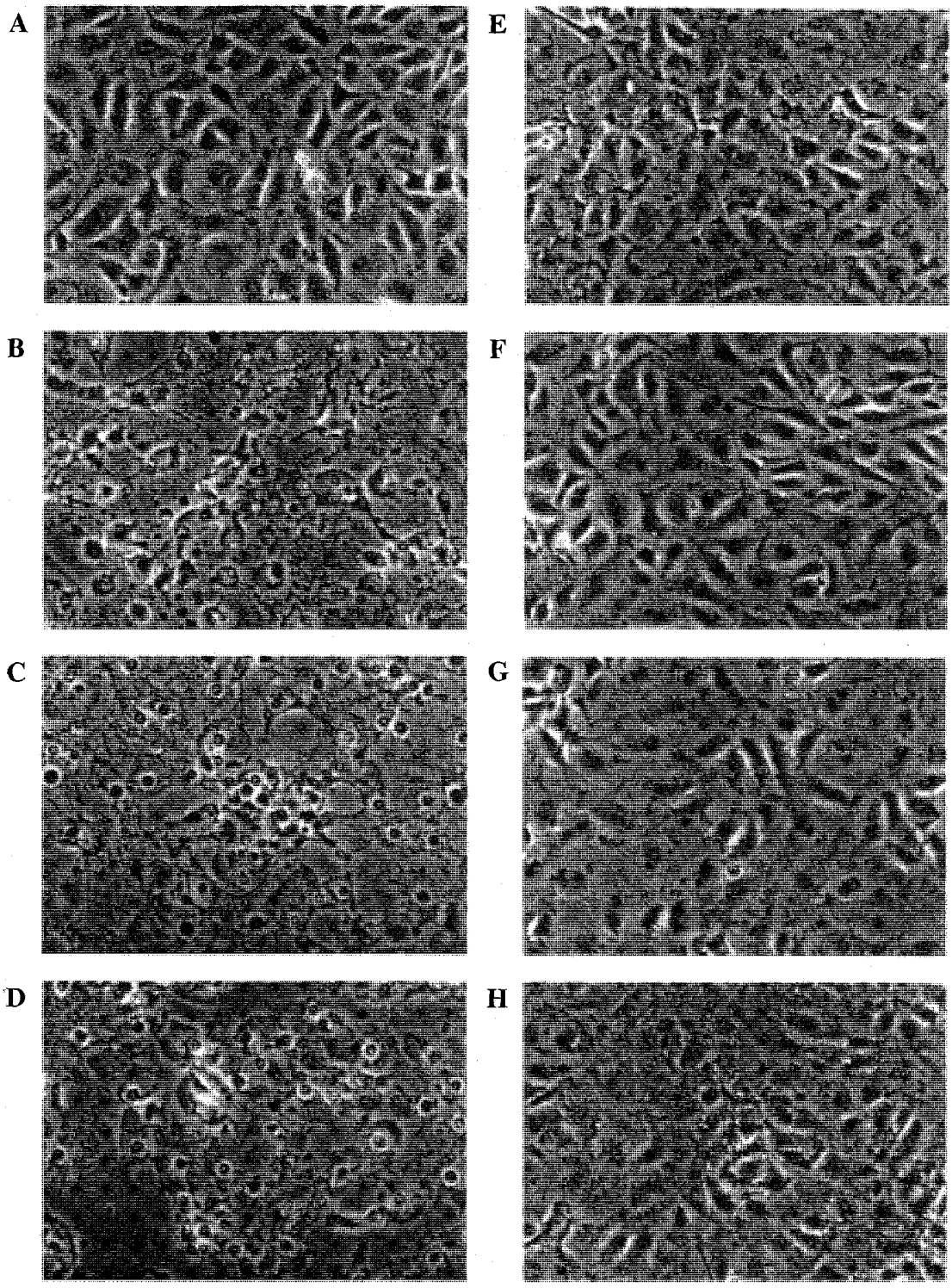


Fig. 7 (*continued*)

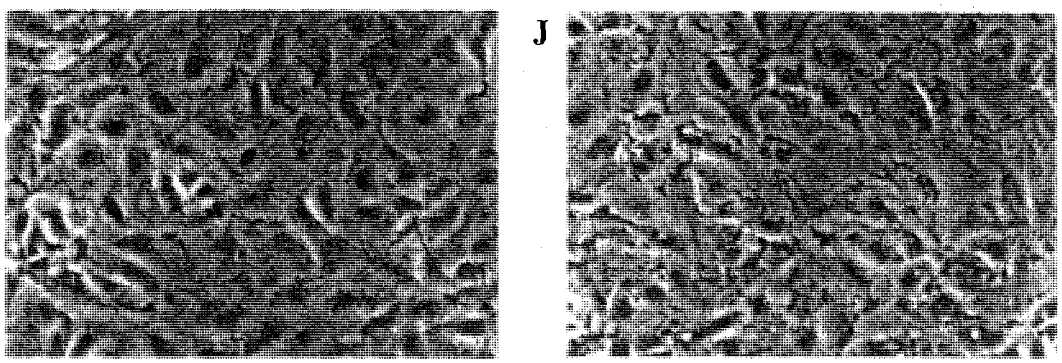


Fig. 7. Inhibition of low-pH-induced cell–cell fusion of infected CV-1 cells. **A** uninfected, pH 5; **B** infected, pH 5; **C** infected plus humic acid (100 μg/ml), pH 5; **D** infected plus PCA (100 μg/ml), pH 5; **E** infected plus the oxidative polymer of PCA (100 μg/ml), pH 5; **F** uninfected, pH 7; **G** infected, pH 7; **H** infected plus humic acid (100 μg/ml), pH 7; **I** infected plus PCA (100 μg/ml), pH 7; **J** infected plus the oxidative polymer of PCA (100 μg/ml), pH 7

Inhibition of cell–cell fusion

Like many other enveloped viruses, the influenza virus uses membrane fusion to inject its genome into host cells. The low-pH environment in the endosome triggers a sequence of changes in the conformation of the influenza virus hemagglutinin, which leads to fusion. Here we evaluate the effects of test compounds on the fusion mediated by low pH (pH 5). The oxidative polymer of PCA, at a concentration of 100 μg/ml inhibits completely the cell–cell fusion (Fig. 7E), whereas the fusions still can clearly be observed when humic acid or PCA are added at the same concentration (Fig. 7C and D).

Discussion

We have found that the oxidative polymer of PCA (OP-PCA), in contrast to its monomer, inhibits influenza plaque formation at a concentration that shows no cytotoxicity. The inhibitory effect is higher when the OP-PCA was added at the stage of viral adsorption in comparison with the post-adsorption, which implies that the major anti-viral mechanism of this compound occurs in the stage of viral adsorption. This assumption is confirmed by the fact that the oxidative polymer of PCA inhibits hemagglutination and low pH-induced cell–cell fusion. In contrast, the humic acid shows lower inhibition of hemagglutination and no inhibition on acid-induced cell–cell fusion, whereas humic acid exhibits distinguished inhibitory effect on the endonuclease activity of viral RNA polymerase. Influenza viral RNA polymerase plays an important role on viral RNA synthesis that occurs after viral entering the cells. This reflects the similar inhibitory effect on plaque reduction assay no matter humic acid was added at the stage of adsorption or after virus entering the host cells.

It is known that the influenza viral RNA transcriptase complex is composed of three viral RNA polymerases, PA, PB1 and PB2 proteins. These three polymerases

form complex in the first step. The complex then binds to viral RNA and acquires endonuclease that cleaves capped host cellular mRNA as a primer for synthesis viral mRNA. In this report, we found that humic acid inhibits the endonuclease activity of this viral RNA polymerase complex. It has been demonstrated that metal ion catalyzes RNA cleavage by the influenza virus endonuclease [17]. It has also been studied that the ion chelating agents can inhibit influenza viral RNA polymerase [18]. In gel-mobility-shift assay, we found that humic acid interferes viral polymerase complex binding to viral RNA (data not shown). Here we speculate that the humic acid may play a role as a metal chelating agent and then inhibits viral RNA polymerase by binding to ions. Cleavage of RNA by viral polymerase complex is occurred in host cell nucleus. We recently have proven that humic acid can enter nucleus (data not shown).

There are several replicative targets that can be considered for chemotherapeutic intervention. Transcription of influenza virus (the "cap-snatching" model), however, represents a unique antiviral target [16]. PA, PB1 and PB2 proteins are highly conserved among influenza viruses, and this complex activity has no known cellular counterpart. The propitious nature of this viral RNA polymerasse complex renders it a good target for developing anti-flu agents. A substituted 2,6-diketopiperazine compound that was originally identified in the extract of *Delitschia confertaspora* (a fungus) has been shown to inhibit influenza virus transcriptase [19]. However, this compound is pretty difficult to have a large scale of preparation (personal communication with Dr. Krug). Here we found that humic acid can inhibit the endonuclease activity of viral RNA polymerase. It may be a potential lead compound for development of anti-flu agent.

As previously described, several structurally resembling phenolic complexes possess anti-flu activity [11–13]. In this report, we found that two novel humic-like substances, the oxidative polymer of PCA and humic acid possess the in vitro anti-flu activity. Although these two compounds show structural resemblance, they exhibit different actions of anti-flu. It is interesting to know the detail mechanism. Chemical modification of these two compounds may give a precious information for drug discovery of anti-flu in the future.

Acknowledgements

The investigation was supported by a grant from National Health Research Institute (NHRI-CN-BP8901S). The authors thank Dr. Guang-Wu Chen for reviewing this manuscript.

References

1. Blick TJ, Sahasrabudhe A, McDonald M, Owens IJ, Morley PJ, Fenton RJ, McKimm-Breschkin JL (1998) The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. Virology 246: 95–103
2. Sidwell RW, Huffman JH, Barnard DL, Bailey KW, Wong MH, Morrison A, Syndergaard T, Kim CU (1998) Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor. Antiviral Res 37: 107–120

3. Li Y, Yamakita Y, Krug RM (1998) Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein. Proc Natl Acad Sci USA 95: 4864–4869
4. Mendel DB, Tai CY, Escarpe PA, Li W, Sidwell RW, Huffman JH, Sweet C, Jakeman KJ, Merson J, Lacy SA, Lew W, Williams MA, Zhang L, Chen MS (1998) Bischofberger N. Kim CU. Oral administration of a prodrug of the influenza virus neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. Antimicrob Agents Chemother 42: 640–646
5. Waghorn SL, Goa KL (1998) Zanamivir. Drugs 55: 721–727
6. Dunn CJ, Goa KL (1999) Zanamivir: a review of its use in influenza. Drugs 58: 761–784
7. Bardsley-Elliot A, Noble S (1999) Oseltamivir. Drugs 58: 851–862
8. Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, Singh S, Kinnersley N, Ward P, Mills RG (2000) Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. J Am Med Assoc 283: 1016–1024
9. Tanaka T, Kojima T, Kawamori T, Mori H (1995) Chemoprevention of digestive organs carcinogenesis by natural product protocatechuic acid. Cancer 75: 1433–1439
10. Mori H, Sugie S, Rahman W, Suzui N (1999) Chemoprevention of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine-induced mammary carcinogenesis in rats. Cancer Lett 143: 195–198
11. Serkedjieva J (1995) Inhibition of influenza virus protein synthesis by a plant preparation from Geranium sanguineum L. Acta Virol 39: 5–10
12. Sidwell RW, Huffman JH, Moscon BJ, Warren RP (1994) Influenza virus-inhibitory effects of intraperitoneally and aerosol-administered SP-303, a plant flavonoid. Chemotherapy 40: 42–50
13. Nakayama M, Suzuki K, Toda M, Okubo S, Hara Y, Shimamura T (1993) Inhibition of the infectivity of influenza virus by tea polyphenols. Antiviral Res 21: 289–299
14. Liang HJ, Tsai CL, Chen PQ, Lu FJ (1999) Oxidative injury induced by synthetic humic acid polymer and monomer in cultured rabbit articular chondrocytes. Life Sci 65: 1163–1173
15. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J, De Clercq E (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods 20: 309–321
16. Shih SR, Krug RM (1996) Surprising function of the three influenza viral polymerase proteins: selective protection of viral mRNAs against the cap-snatching reaction catalyzed by the same polymerase proteins. Virology 226: 430–435
17. Doan L, Handa B, Roberts NA, Klumpp K (1999) Metal ion catalysis of RNA cleavage by the influenza virus endonuclease. Biochemistry 38: 5612–5619
18. Oxford JS, Perrin DD (1974) Inhibition of the particle-associated RNA-dependent RNA polymerase activity of influenza viruses by chelating agents. J Gen Virol 23: 59–71
19. Tomassini JE, Davies ME, Hastings JC, Lingham R, Mojena M, Raghoobar SL, Singh SB, Tkacz JS, Goetz MA (1996) A novel antiviral agent which inhibits the endonuclease of influenza viruses. Antimicrob Agents Chemother 40: 1189–1193

Author's address: Dr. S.-R. Shih, School of Medical Technology, Chang Gung University, 259 Wen-Hua 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan; e-mail: srshih@mail.cgu.edu.tw

Received April 24, 2001

Archivum Immunologiae et Therapiae Experimentalis, 1993, **41**, 95–97
PL ISSN 0004-069X

A Randomised, Double-Blind Study on the Efficacy of Tołpa* Torf Preparation (TTP*) in the Treatment of Recurrent Respiratory Tract Infections

ADAM JANKOWSKI, BARBARA NIENARTOWICZ, BOŻENA POLAŃSKA and ALEKSANDRA LEWANDOWICZ-USZYŃSKA

Department of Pediatric Propedeutics, University Medical School, Kasprowicza 64/66, 51-146 Wrocław, Poland

Abstract. TTP* is a new immunomodulating drug of natural origin, registered in Poland for human use. In the randomised, double blind study to assess the therapeutic efficacy of the Tołpa* Torf Preparation (TTP*) in the recurrent respiratory tract infectious participated 39 young patients (age 16–22). TTP* was administered orally, 5 mg daily for three weeks. During 3 months follow-up period favourable results of treatment were obtained in 14 of 20 TTP* treated patients and in 8 of 19 of the placebo patients. The therapeutic effects were seen even after the 6 months follow-up period. The phagocytic activity of granulocytes was significantly stimulated in the TTP* – treated patients but not in the placebo-treated patients. The results suggest that TTP* is effective drug in the treatment of recurrent respiratory tract infections with undefined infectious etiology. In the period of the treatment as well as during 6 months observation no side effects were noticed.

Key words: Tołpa* Torf Preparation; recurrent infections; respiratory tract infections; immunomodulation biotherapy in humans.

Introduction

Recurrent infections of the respiratory tract are connected, especially in children and young people, with disturbances of the immune system. It is well established that this syndrome is associated with an increase of T-suppressor lymphocyte number, decreased ratio of CD4 to CD8 and a deficiency of immunoglobulin synthesis[4,6,7,8]. Among the factors contributing to the development of recurrent respiratory infections are pollution of environement and the excessive use of antibiotics.

The aim of this paper is to evaluate the efficacy of the Tołpa* Torf Preparation (TTP*)[9], new immunomodulating drug of natural origin in the treatment of recurrent respiratory tract infections in young people.

Materials and Methods

The trial was performed on 39 patients (22 males and 17 females) 16–22 years old. The diagnosis of recurrent respiratory infections was based on the following clinical criteria. At least once during 9 months of observation of every patient a bout of respiratory disease occurred. The major symptoms

Tołpa* Torf Preparation (TTP*) is the trade mark of Torf Corporation.

Preliminary results of this paper were presented in the monograph A. DANYSZ (ed.): "Clinical investigations on Tołpa* Torf Preparation", Torf Corporation, Wrocław 1992, pp. 1–88.

were: purulent pharyngitis, bronchitis or pneumonia with the frequency not less than once monthly. All these patients had been previously treated with several different antibiotics eg. aminoglycosides (gentamycin), erythromycin, cephalosporins, ceporan with only transient improvement.

The study was double blind. The patients were randomly divided into two groups. Group 1 with 20 patients received the preparation decoded after completion of the trial as TTP*, group 2 with 19 patients received another preparation decoded finally as placebo.

The preparations were administered orally once daily in the dose of 5 mg on empty stomach, during 3 weeks. All patients made notes every day about their general feeling, a frequency of bouts of cough, common cold, elevated temperature etc. They registed also the adverse reactions.

The clinical results were based on the analysis of frequency of illnesses made 3 months and then 6 months after the end of the treatment. The following criteria were used: very good result – during the period of observation not a single episode of respiratory tract infection occurred. Good result – during the time of observation only one episode of respiratory tract infection occurred. No effect – during of observation two or more episodes of respiratory tract infection were motified. Worsening – the frequency of respiratory tract infections increased or more severe course of the episodes were observed.

In the analysis of drug efficacy the subjective evaluation of the patient, especially the wellbeing and appetite were also taken into consideration. In accordence with the good clinical practice (GCP) recommendations before starting of the treatment all patients were informed, that they would be treated with Tołpa* Torf Preparation. They also were informed about the results of preclinical investigations. They signed an agreement for this treatment. In a few cases the agreement was signed by their legal curators.

Phagocytosis test with the peripheral blood leukocytes of the patients and *Staphylococcus aureus* 209 P Oxford, and *Escherichia coli* strains was performed as described[4].

Clinical data were analyzed with chi-square and results of phagocytosis with Student's *t*-test. The criterion for significance was a P value less than 0.05.

Results and Discussion

In the group of patients receiving TTP* and observed for 3 months very good clinical results were obtained in 14 of 20 patients whereas in the placebo group in 8 of 19 patients there was the improvement. In not a single patient and adverse reaction was observed (Table 1). In two patients receiving TTP* and in one patient from the placebo group increased appetite was registered. During the 6 months follow-up there was still a significant difference between the TTP* and placebo group. Patients were submitted to detailed clinical and laboratory investigation in the period of remission. No clinical syndromes were found. In all patients following laboratory tests were performed: RBC sedimentation rate, blood morphology, general urine test, ALAT activity and serum proteins electrophoresis. In the acute phase of disease following effects of infection were observed: an enhancement of leucocytosis and appearance of lypochromic anemia. After the end of the infection only anemia with a tendency to decline was observed.

Table 1. Clinical results TTP* administration in patients with recurrent respiratory tract infection

Follow up period	TTP*, series 10490				Placebo			
	very good	good	no effect	worse-ning	very good	good	no effect	worse-ning
3 months	14	4	2	0	8	6	5	0
6 months	9	7	4	0	4	5	10	0

5 mg TTP* was administered daily for 3 weeks. The frequency of bouts of infection observed during the 3 and 6 months follow-up periods is shown. There were significant differences between the study groups with the very good results ($P < 0.005$).

These studies were performed twice: at the beginning and after the treatment. In these studies only symptoms of acute inflammation were found. No side effects were noticed.

In the case of purulent pharyngitis, *Staphylococcus aureus* and *Streptococcus viridans* were isolated. Additionally, in two cases the presence of *Hemophilus influenzae* was established. No other bacteriological and virological studies were performed in the cases of pneumomia and bronchitis.

No one of the patients studied required hospitalization during the period of the treatment.

In the patients receiving TTP* significantly increased phagocytic activity of their peripheral blood granulocytes. Such effect was not observed in patients from the placebo group (Table 2).

It should be emphasized, that patients included into the trial had been reviously treated, with various antibiotics, with a transient improvement only. We have shown previously, that the possible cause of the recurrent respiratory tract infections in children is

Table 2. Phagocytic activity of granulocytes of patients with recurrent respiratory tract infections treated with TTP* or placebo

Phagocytosis of:	PI of granulocytes from patients: TTP* treated I	TTP* treated II	placebo I	placebo II
S. aureus	10.500±890	13.500±950	10.810±680	12.100±790
S. coli	10.150±880	12.690±910	12.150±810	12.180±790

The phagocytosis indices (PI, ±SD) were calculated shortly before the beginning of the study (I) and after the termination of the 3-week treatment (II). The significant differences ($P < 0.05$) between the I and II values of the phagocytic indices were found in the TTP* group but not in the placebo.

impairment of T-cell and B-cell functions as well as reduced number of cells in T-cell subpopulations. It was demonstrated in the pre-clinical investigations, that Tołpa* Torf Preparation has immunomodulatory activity[9].

Our observations appear to confirm the results obtained by others. In patients receiving TTP* the number of very good results was significantly greater then after placebo administration.

The beneficial effect of TTP* on the phagocytic activity of granulocytes deserves special attention because the administration of antibiotics may inhibit phagocytosis[5]. Low toxicity of TTP* was remarkable. The TTP* treated patients had improved well being, and increased appetite.

The results obtained after the prophylactic administration of such immunomodulating drugs as TFX, Broncho-Vaxom isoprinosine and lavamisole suggest that immunostimulation is beneficial for fast recovery[1-4,6-9].

Taking into account the good clinical results and the lack of adverse reactions we conclude that TTP* can be recommended and used for treatment of recurrent respiratory tract infections in young people.

References

1. Drachman R., Schlesinger M. and Alon U. (1988): Immunomoregulation with levamisole in children with frequently relapsing steroid responsive neuphrotic syndrome. Acta Paedioatr. Scand., **77**, 721–726.
2. Giełdanowski J. (1981): Immunoregulators – thymus factor (TFX) and levamisole in immune reactions and inflammatory processes. Arch. Immunol. Ther. Exp., **29**, 121–132.
3. Gohla S. and Neth R. (1988): Gezietle Immunostimulation durch eine Polysaccharidfraction aus Thuja occidentalis L. Tpk Therapeutikon, **10**, 717–725.
4. Jankowski A., Drabik E., Szyszko Z., Stasiewin W. and Brzosko W. (1986): Die Behandlung rezidivierender Atemwesinfektionen bei Kindern durch Aktivierung des Immunosystems. Therapiewoche Schweiz, **1**, 25–32.
5. Meleżyńska-Matej M., Grzybek-Hryncewicz K., Kotlarek-Haus S. and Kaiser A. (1981): Studies on phagocytosis in leukaemia. III. Phagocytosis inhibiting factor in sera of leukaemic patients. Folia Haematol., **108**, 559–562.
6. Prusek W., Jankowski A., Radomska G., Wieczorek E. and Podwysocka M. (1987): Immunostimulation in recurrent respiratory tract infections therapy in children. Arch. Immunol. Ther. Exp., **35**, 289–302.
7. Puigollers J., Rodes S. and Hernandes R. (1980): Immunoglobulin production in man stimulated by an orally administered bacterial lysate (Broncho-Vaxom). Respiration, **40**, 142–149.
8. Shinichi K. and Kitamura K. (1983): Suppresor cell in levamisole – treated mice: a possible role of T-cell-mediated feedback suppression in the drug-induced suppression of the humoral immune response. Immunopharmacology, **10**, 279–287.
9. Skopińska-Różewska E., Brajczewska-Fiszer W., Żukowska M., Małkowska-Zwierz W., Polakowski I., Pazdur J., Radomska D., Mościcka M., Sokolnicka I., Kolewska D., Marczak M., Morawska I., Nowakowska E., Rządkowska-Bodalska H. and Olechnowicz-Stępień W. (1991): Immunomodulujące działanie Preparatu Torfowego Tołpy (PTT). Reumatologia, **29**, 162–171.
10. Tołpa S., Kukla S., Rządkowska-Bodalska H., Olechowicz-Stępień W., Czyżewski W., Adamek W., Dec J., Dudek Z. and Wróbel-Pieciul H. (1983): Antineoplastic preparation from the acidified alkaline hydrolizate from peat. Polish Patent, No. P-184428.

Received in June 1992
Accepted in November 1992

VIROLOGY 218, 389–395 (1996)
ARTICLE NO. 0208

SHORT COMMUNICATION

Inhibition of HIV-1 in Cell Culture by Synthetic Humate Analogues Derived from Hydroquinone: Mechanism of Inhibition

JOSEF SCHNEIDER,*[,1] ROLAND WEIS,* CHRISTINE MÄNNER,* BEATE KARY,* ALBRECHT WERNER,† BERNHARD J. SEUBERT,‡ and URS N. RIEDE§

*Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene der Universität, Hermann-Herder-Str. 11, D-79104 Freiburg, Germany; †Paul-Ehrlich-Institut, Paul-Ehrlich-Str. 51-59, D-63225 Langen, Germany; ‡Weyl GmbH, Sandhofer Str. 96, D-68305 Mannheim, Germany; and §Pathologisches Institut der Universität, Albertstr. 19, D-79104 Freiburg, Germany

Received September 5, 1995; accepted February 13, 1996

Humic acids are natural constituents of soil and ground water and mainly consist of mixtures of polycyclic phenolic compounds. A similar complex of compounds with a mean size of about 1000 Da, designated HS-1500, was synthesized by oxidation of hydroquinone. HS-1500 inhibited HIV-1 infection of MT-2 cells with an IC_{50} of 50–300 ng/ml and showed a mean cell toxicity of about 600 μg/ml. Inhibition of HIV-induced syncytium formation was observed at 10–50 μg/ml. Treatment of free and cell-attached HIV with HS-1500 irreversibly reduced its infectivity, whereas the susceptibility of target cells for the virus was not impaired by treatment prior to infection. The HIV envelope protein gp120SU bound to sepharose-coupled HS-1500 and could be eluted by high salt and detergent. HS-1500 interfered with the CD4-induced proteolytic cleavage of the V3 loop of virion gp120SU. Furthermore, binding of V3 loop-specific antibodies was irreversibly inhibited, whereas binding of soluble CD4 to gp120SU on virus and infected cells was not affected. In conclusion, our data suggest, that the synthetic humic acid analogue inhibits the infectivity of HIV particles by interference with a V3 loop-mediated step of virus entry.

The progression of the HIV infection toward AIDS appears to be correlated to the virus burden which is sustained by continuously high virus replication and reinfection of target cells (*1, 2*). Thus, virus-receptor binding and internalization represent promising targets for antiviral therapy. Antibodies to the viral envelope proteins, gp120SU and gp41TM (*3, 4*), to the receptor CD4 as well as soluble forms of the receptor itself (*5*) inhibit primary steps of infection *in vitro*. Yet the type-specificity of V3 loop-reactive neutralizing antibodies (*6, 7*) and the low efficiency of soluble CD4 against primary virus isolates (*8*) limit their therapeutic applicability. In contrast, polyanionic compounds with various chemical structures inhibit a broader spectrum of HIV subtypes (*9*). Here we report on the inhibition of HIV-1 by HS-1500, a stable complex that was synthesized by oxidation of hydroquinone at high pH as described in the European patent No. 0 537 430 B1. HS-1500 consists of aliphatically linked phenolic rings with carboxylic functional groups. The pattern of bands obtained by isoelectric focussing of HS-1500 was remarkably similar to that of natural humic acid complexes (*10*). The lyophilized product has a virtually unlimited shelf life. Neutral aqueous solutions retain their virus inactivating capacity at 4° for at least three months. The compound is related to the previously described polyhydroxy-carboxylates (PHCs) by its complexity, basic chemical structure, and functional groups (*11*). However, HS-1500 is distinct from the group of PHCs by its lower mean molecular mass, a significantly higher efficiency against HIV-1, and an inhibition mechanism that mainly affects virus–cell fusion.

We have first observed the inhibitory potential of HS-1500 in a syncytium assay with MT-2 cells (*12*). The potential mode of inhibition was elucidated by inhibition of virus antigen production and the demonstration of direct influence of HS-1500 on the fusion of infected with uninfected MT-2 cells. The inhibitory concentrations in these assays, which were derived from repeated dose response experiments (not shown) are summarized in Table 1. Upon pretreatment of virus and continuous presence of HS-1500 an inhibitory concentration 50 (IC_{50}) of 0.3 μg/ml was observed in the syncytium assay. The complete inhibition at 10 μg/ml appeared to be irreversible, since syncytium formation was not resumed, when cells of the completely protected microcultures were further subcultured in the absence of HS-1500 for up to 50 days. In the virus antigen assay, an IC_{50} of 0.05 μg/ml was observed, indicating inhibition of virus production. Fusion of infected with uninfected MT-2 cells was sup-

[1] To whom correspondence and reprint requests should be addressed. Fax: 0761-203-6639. E-mail: schf@sun1.ukl.uni-freiburg.de.

0042-6822/96 $18.00

TABLE 1

Efficient Inhibition of HIV-1 in Cell Culture by HS-1500

Assay	Syncytia[a]	Antigen[b]	Cell fusion[c]
IC_{50} [μg/ml]	0.3	0.05	10–30
IC_{90} [μg/ml]	2–5	0.3	50–100

[a] 1.25×10^4 MT-2 cells (*33*) were incubated in triplicate for 5 days with 150 syncytium-inducing units of HIV-1_{IIIB} from H9 cells (*34*) and appropriate dilutions of HS-1500 in the wells of microtiter plates. Dose response curves were obtained by counting of syncytia with an inverted microscope 5 days later.

[b] Inhibition of virus replication was measured in the cell-free supernatant by the p24 core protein ELISA (DuPont).

[c] To determine syncytium formation by fusion of infected with uninfected lymphocytes, 10% of HIV-1_{IIIB}-infected and 90% of uninfected MT-2 cells were cocultivated for 2 days with varying concentrations of HS-1500 before syncytia counting.

pressed with an IC_{50} of 10–30 μg/ml and completely inhibited at 300 μg/ml. Thus, for direct interference with HIV-induced cell fusion about 30- to 100-fold more HS-1500 was needed than for inhibition of fusion as a consequence of infection by free virus. The differences in the two syncytium assays suggest that incubation of HIV with HS-1500 may affect an early step of the virus replication cycle, which is more sensitive to the synthetic humate, than the virus-induced syncytium formation itself. Similar differences have been observed with other inhibitors (*13, 14*). HIV-induced cell fusion may be less sensitive to inhibition by HS-1500 because of the higher multiplicity of g120-CD4 interactions and additional cellular proteins such as LFA-1 that are exclusively involved in cell to cell fusion (*15*). The 50% anticellular toxicity (TC_{50}) of HS-1500 was found to be about 600 μg/ml in MT-2 cells by the MTT assay (*16*). Comparison of this result with the IC_{50}-values (Table 1) yields a selectivity index (SI; ratio of IC_{50} to TC_{50}) of 2,000 (syncytium assay) to 12,000 (antigen assay).

In quantitative terms HS-1500 resembles the most efficient polyanionic polysaccharides such as dextran sulfate (*13*) and curdlan sulfate (*17*). In particular, HS-1500 inhibits HIV at least 10 times more efficiently and selectively than the PHCs (*11*), although the compounds are structurally related. Due to the chemical complexity of these two groups of inhibitors, no correlations between their chemical structure and efficacy can be drawn at present.

To study the influence of the synthetic humate on the infectivity of HIV particles, three dilutions of the virus stock in cell culture medium were treated with HS-1500 and then separated from the inhibitor by sedimentation. Separate sedimentation of either untreated virus or of HS-1500 only served as controls. Reduction of the viral infectivity was visualized by the syncytium assay. As shown in Fig. 1A, pretreatment of the virus for 30 min with 0.4 μg/ml of HS-1500 reduced its syncytium inducing activity by more than 50% as compared to the virus stock that was sedimented without prior treatment. HS-1500 could not be concentrated by sedimentation. Because almost the same IC_{50} was observed, when cells and virus were treated simultaneously for 5 days (Table 1), we are tempted to conclude that HS-1500 rapidly inactivated the HIV particle in an irreversible mode. Thus, the virion appeared as the primary and most sensitive target. Inhibition of syncytium formation by continuous presence of HS-1500 in the cell culture can be explained by inactivation of the virus, because it follows the same dose response as the inactivation of cell-free virus. Inactivation of the input virus would also suffice to explain suppression of virus antigen production.

Pretreatment of MT-2 cells with HS-1500 concentrations of 1.5 to 44 μg/ml and washing prior to infection resulted in a 20–30% increase of syncytium formation (data not shown). Therefore, CD4 and other cell surface structures relevant for virus attachment and entry are not significantly involved in the inhibition by HS-1500.

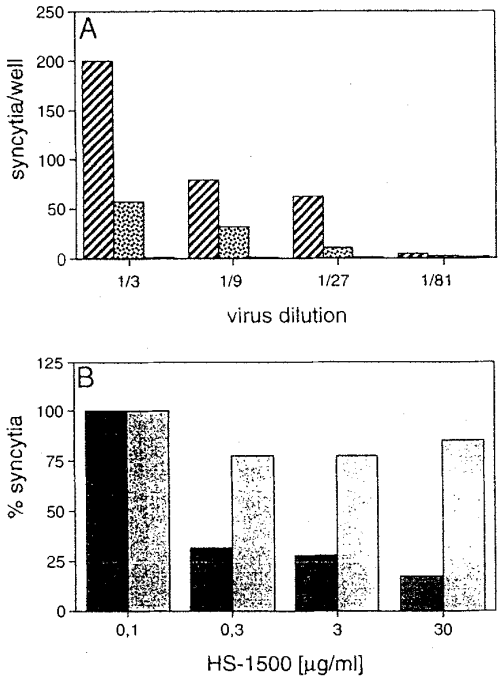


FIG. 1. Inactivation of HIV-1 virions before and after binding to target cells. (A) Pretreatment of the virus. The indicated dilutions of the HIV-1_{IIIB} virus stock were incubated with two concentrations of HS-1500 for 15 min at room temperature (▨, mock-treated; □, 0.4 μg/ml; and ■, 40 μg/ml HS-1500). Virus was washed twice before determining the infectious units in aliquots of the resuspended virus pellet by the syncytium assay. (B) Postbinding inhibition of HIV by HS-1500. MT-2 cells were incubated with 200-fold dilutions of virus stock at 0° (▨ or 37° (□). Unbound virus was removed by washing of the cells. These were then incubated with the indicated concentrations of HS-1500 for 12 hr and washed again. 1.25×10^4 cells were plated in triplicates into wells of microtiter plates and incubated without HS-1500 for 6 days followed by counting of syncytia.

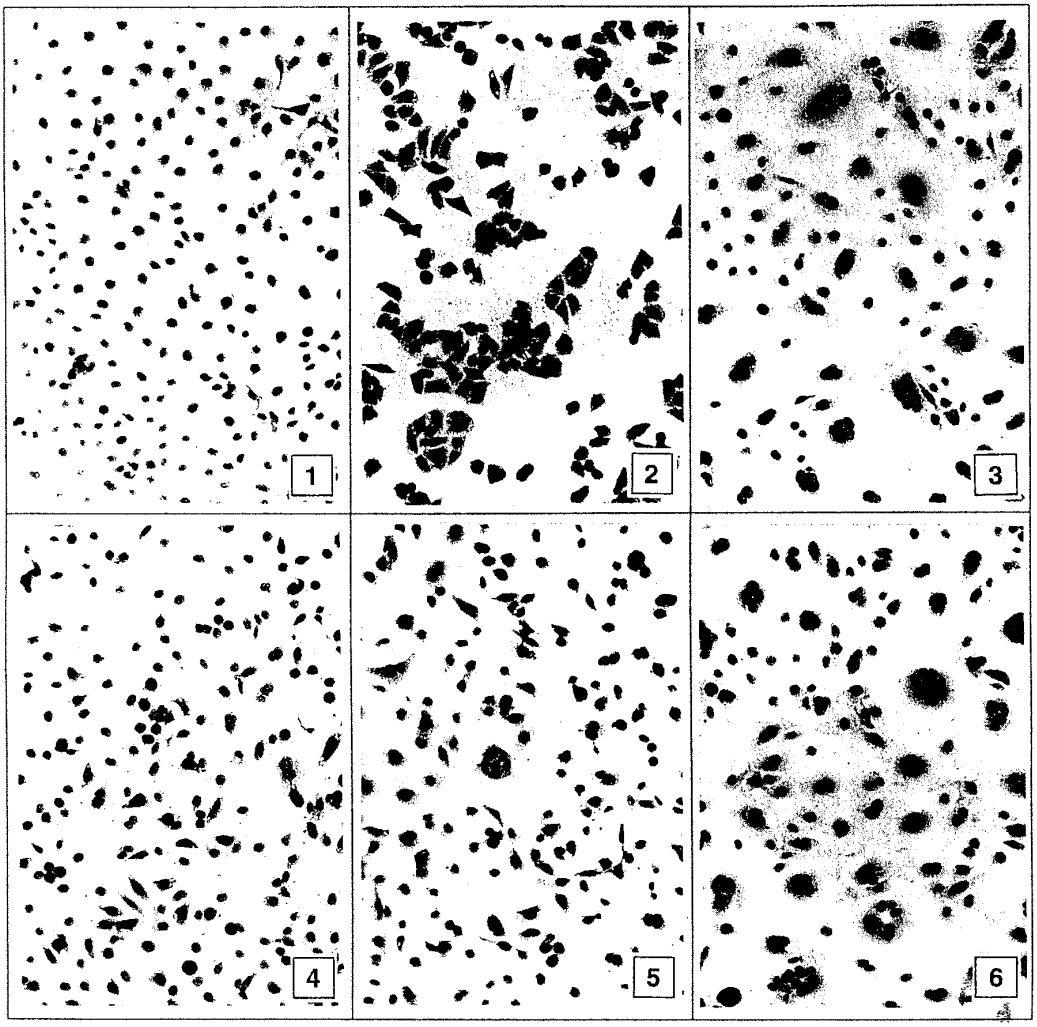


FIG. 2. Inhibition of HIV-envelope-induced syncytium formation. Gp120SU expressing CL4-cells (section 1) and HeLa-CD4 cells (section 2) were incubated alone or mixed at a 2/1 ratio (section 3) and cultured for 20 hr without HS-1500 (sections 1–3). In the other mixed cultures (sections 4–6) HS-1500 was present at 25, 12.5, or 6.25 μg/ml from the start of the coculture. Staining with hemocolor (Merck, Darmstadt) facilitated the detection and counting of multinucleated giant cells.

To discriminate between interference at pre- and post-binding events, virus was incubated with MT-2 cells for 1 hr at 0°, thus, allowing binding without entry (*18*). After removal of unbound virus, cells were treated with HS-1500. The cells were further cultured at 37° for 12 hr in the presence and then 6 days in the absence of inhibitor. Incubation of cells and virus in the cold vs 37° reduced the mean number of syncytia/well from 140 to 45 in the inhibitor-free control cultures (not shown). As illustrated in Fig. 1B, syncytium formation was suppressed to about 25% of the inhibitor-free cultures, when HS-1500 was applied after attachment of virus to the cells in the cold. In contrast, incubation of the cells with virus for 1 hr at 37° prior to treatment with HS-1500 only weakly affected syncytium formation. This temperature shift experiment showed that a step of the infection process occurring within the first hour after virus–cell attachment is inhibitor sensitive. The presence of HS-1500 for 12 hr was sufficient for inhibition, indicating, that the majority of cell-attached virions were irreversibly inactivated. With regard to IC_{50}, HS-1500 is equally effective irrespective of its addition before or after binding of the virus to the target cell (see Table 1). However, cell-attached virus is incompletely inhibited by 30 μg/ml of HS-1500, a concentration sufficient to render cell-free virus preparations noninfectious. Because in contrast to membrane fusion, the CD4-induced conformational change of gp120SU is not influenced by low temperature (*19*), HS-1500 most probably affects a later step of virus internalization.

To further examine a potential direct effect of the inhibi-

'or on the HIV-induced cell to cell fusion, interference was studied in a virus-free model system (*20*). CD4-expressing HeLa cells (*35*) fuse with the HIV envelope protein expressing CL4 cells to form multinucleated syncytia within 20 hr (Fig. 2, sections 1–3). Formation of syncytia was completely inhibited at 25 μg/ml of HS-1500 (section 4). Sporadic syncytia were observed at 12.5 μg/ml (section 5), and the inhibitory effect was diluted out at 6.25 μg/ml (section 6). Counting syncytia/well yielded an IC_{50} of about 10 μg/ml. This value is in the range of the IC_{50} (10–30 μg/ml) estimated for fusion of infected with uninfected cells (Table 1). Thus, fusion-inhibition is independent of virus replication and cell type. Because in infection experiments treatment of the CD4-bearing target cells failed to inhibit syncytium formation, this experiment suggested an interaction of HS-1500 with the viral envelope protein complex.

We therefore studied the binding of gp120SU to an affinity matrix consisting of HS-1500 covalently coupled to sepharose. Culture supernatants of radiolabeled H9/HIV-1_{IIIB} cells served as source of gp120SU. HIV-specific polypeptides were immunoprecipitated by an HIV-specific serum (*36*) from the respective culture supernatants before and after adsorption to HS-1500-sepharose. The autoradiograph in Fig. 3A shows that serum from an AIDS-patient immunoprecipitated polypeptides of about 20, 24, and 18 kDa from the unabsorbed supernatant (lane 5), which represent the respective viral polypeptides gp120SU, p24CA, and p18MA of HIV. Absorption of the culture fluid with HS-1500-bound sepharose preferentially removed gp120SU (lane 1). The bands of the other *bona fide* virion proteins of 18, 24, and 65 kDa are also decreased, suggesting a certain degree of unspecific binding. Absorption with humate-free sepharose had no effect (lane 2). Normal control serum did not recognize any polypeptide in the absorbed or unabsorbed culture supernatants (lanes 3 and 4). Thus exhausting absorption demonstrated that gp120SU selectively bound to HS-1500, whereas other immunoreactive HIV-polypeptides mainly remained in the supernatant. The excess of the serum proteins from cell culture medium present during binding supports the notion of selective binding of gp120SU to HS-1500. To exclude a potential proteolysis or unspecific loss of gp120SU by incubation with HS-1500-sepharose, gp120SU was eluted from the affinity matrix and subsequently immunoprecipitated. Gp120SU could be eluted from HS-1500-sepharose by high salt washing buffer (Fig. 3B, lane 2), by its constituents NP-40 and desoxycholate (lane 4), and to a lesser extent by 0.5 *M* sodium chloride (lane 5), but not by 30% sucrose (lane 3). Altogether these experiments demonstrate, that soluble gp120SU binds reversibly to humate without impairment of major antigenic determinants. The fact that binding was reverted by detergents and high salt suggests a hydrophobic and/or ionic nature of interaction.

The inactivation of the virion was neither caused by interference with gp120-CD4 binding, nor by forced shedding of gp120SU, since pretreatment of virus particles with HS-1500 did not impair attachment of a recombinant form of soluble CD4 (*5*) to virus or detection of gp120SU in virus pellets (data not shown). However, two indepen-

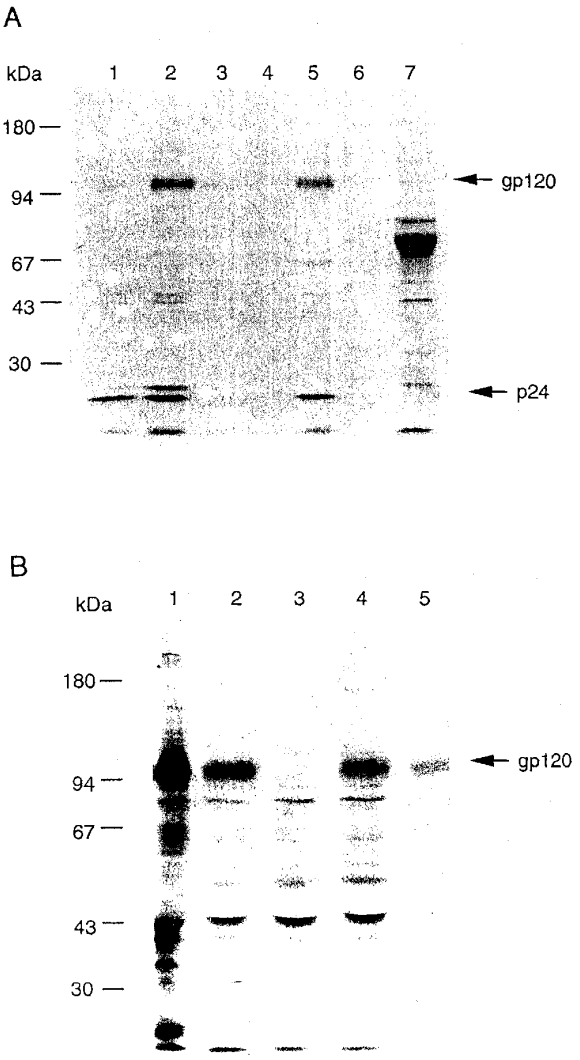


FIG. 3. Specific adsorption of gp120SU to HS-1500 sepharose. HS-1500 was covalently coupled to epoxy sepharose (Pharmacia, Freiburg, Germany) as described by the manufacturer. Culture supernatant of ^{35}S-cysteine-labeled HIV-1_{IIIB}-infected H9 cells was preabsorbed with HS-1500-sepharose (A, lanes 1 and 3) or uncoupled sepharose (A, lanes 2 and 4). Unabsorbed HIV-polypeptides were immunoprecipitated with an AIDS-patient serum (A, lanes 1 and 2) or a negative control serum (A, lanes 3 and 4). For control, supernatant was immunoprecipitated with the same sera prior to absorption (A, lanes 5, 6; B, lane 1). HIV-polypeptides were eluted from HS-1500-sepharose by high salt washing buffer (B, lane 2), 30% sucrose (B, lane 3), 0.5% desoxycholate, and 0.5% NP-40 (B, lane 4), and 0.5 *M* NaCl (B, lane 5). Immunoprecipitates and a sample of the culture supernatant (A, lane 7) were analyzed by SDS–PAGE and autoradiography.

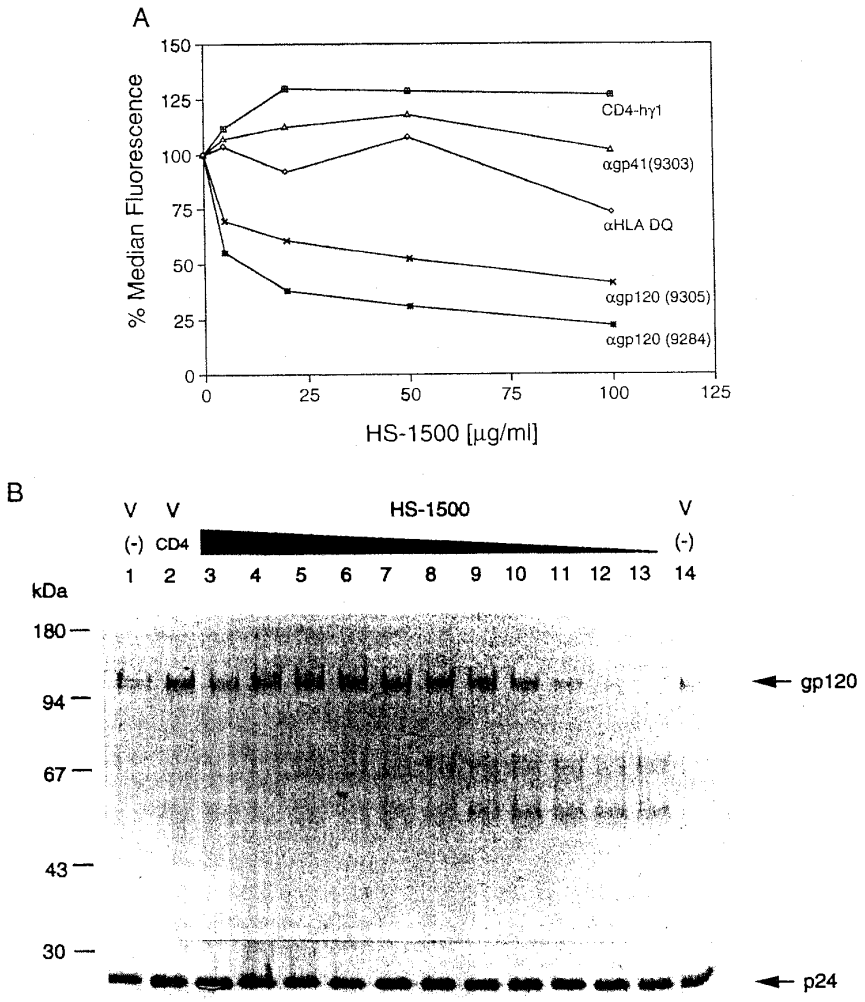


FIG. 4. Interaction of HS-1500 with the V3 loop of gp120SU. (A) Permanent and dose-dependent inhibition of V3 loop-specific antibody binding. HIV-1_{IIIB}-infected H9 cells were treated with the indicated serial dilutions of humate, washed, and incubated individually with mouse monoclonal antibodies specific for either the N-terminal part (NEA9284) or the β-turn of the V3 loop (NEA9305), for gp41 (NEA9303) or HLA-DQ (0416, Dianova Hamburg); (NEA antibodies: DuPont, Bad Homburg). After staining with a FITC-labeled goat-anti-mouse antibody respective FITC-labeled donkey-anti-human antibody for CD4-hγ 1 (*5*), cells were characterized by FACS analysis. Medians of respective histograms are provided as a function of HS-1500 concentrations. (B) Inhibition of the proteolytic cleavage of the V3 loop by HS-1500. HIV-1_{SF2} was incubated with rsCD4 and HS-1500, followed by immunoblot analysis of the proteins gp120SU and p24CA as described previously. Lane 1, untreated virus; lane 2, virus with 20 ng of rsCD4; lane 3 virus with 20 ng of rsCD4 and 20 μg/ml HS-1500; lanes 1–3 no incubation; lanes 4–13, virus with 20 ng of rsCD4 and 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 μg/ml HS-1500, or without inhibitor; lane 14, virus incubated without rsCD4 and HS-1500.

dent observations support the notion that HS-1500 inactivated HIV mainly by binding to the V3 loop of gp120SU: HS-1500 specifically prevented (i) the binding of antibodies to the V3 loop and (ii) the proteolytic cleavage of the V3 loop. HS-1500 significantly inhibited the binding of the V3 loop-specific antibodies 9284 (*21*) and 9305 (*22*) to HIV-infected H9 cells. In this assay, binding of recombinant soluble CD4 was slightly enhanced, the binding of the gp41TM-specific antibody 9303 (*23*) was not affected, and the reaction of the HLA-specific antibody was only weakly impaired at 50 μg/ml of HS-1500 (Fig. 4A). These data illustrate that HS-1500 specifically suppressed the recognition of V3-specific epitopes on infected cells in a dose-dependent manner. Since the gp41TM-specific and the V3 loop-specific antibodies are of the same murine IgG subclass, a direct impairment of the reactivity of antibodies by HS-1500 appears most unlikely. Because the inhibitor-treated cells had been washed prior to incubation with the antibodies, HS-1500 seems to bind permanently to the native form of gp120SU in the multimeric

envelope complex at the cell surface. The results of this and the temperature shift experiment (Fig. 1B) suggest that HS-1500 by binding to the V3 loop interferes with the same function of gp120SU as V3 loop-specific antibodies (*24*).

Incubation of highly purified virions with soluble CD4 resulted in cleavage of the V3 loop by a protease inherent to these virus preparations. The cleavage could be blocked by monoclonal antibodies to the crest of the V3 loop (*25*). Incubation of virus with soluble CD4 and HS-1500 and subsequent analysis of viral proteins by immunoblotting as described (*25*) demonstrated that the presence of HS-1500 interfered with the cleavage of gp120SU into fragments of approximately 70 and 50 kDa (Fig. 4B). Spontaneous cleavage was observed in the purified particles (Fig. 4B, lane 1) prior to incubation, and cleavage was enhanced by incubation at 37° even without CD4 (lane 14). Due to this background, the efficacy of the CD4-induced cleavage can be evaluated more precisely from the decrease of the gp120SU band rather than from the appearance of the cleavage products. Incubation of the virus preparation with 20 ng of recombinant soluble CD4 (rsCD4) yielded complete cleavage (lane 13). Addition of HS-1500 in twofold serial dilutions inhibited the cleavage in a dose-dependent manner with an IC_{50} between 0.3 and 0.075 μg/ml. Thus, inhibition of the cleavage and inhibition of HIV infectivity are observed at about the same concentration of HS-1500, which further supports the hypothesis that interaction of HS-1500 with the V3 loop represents the predominant inhibition mechanism of the HS-1500 complex.

In further support of a direct interaction of HS-1500 with the V3 loop, we observed a positive correlation between the surplus of basic vs acidic amino acids in the V3 loop and the sensitivity of individual virus isolates to inhibition. Along this line, HS-1500 inactivated HIV-1_{IIIB} and HIV-1_{NDK} with similar efficiency. The surplus of 9 basic amino acids is conserved in their V3 loops, while in the V3 loop amino acid sequences only 17 of 38 positions are identical (*26, 27*). In agreement, HIV-1_{BaL}, characterized by a surplus of only 3 basic amino acids in the V3 loop, is inhibited at a 30–100 times higher IC_{50} although the V3 loop sequences of HIV-1_{BaL} and HIV-1_{IIIB} are more closely related than those of HIV-1_{NDK} and HIV-1_{IIIB}. These data also suggest that HS-1500 inhibits a broader range of HIV-isolates than the mainly type-specific V3 loop-reactive antibodies (*28*). Interestingly, the high cytopathogenicity and efficient replication in lymphocytes of HIV-1 isolates appears to be correlated to a certain excess of basic vs acidic amino acids in the V3 loop (*29, 30*). Therefore, development of resistance to HS-1500 may select for HIV variants with low cytopathogenicity.

For other polyanionic HIV-inhibitors interaction with the V3 loop is suggested as one of several potential mechanisms of HIV-inhibition. However, all polyanionic polysaccharides tested so far fail to inactivate free virus (*17, 31, 32*). Even the PHCs, which resemble HS-1500 structurally, inhibit HIV by a different mechanism. Most PHC preparations efficiently interfere with binding of gp120SU to CD4 and block the binding of virus to cells completely (*11*), whereas HS-1500 slightly increases the binding of recombinant soluble CD4 to HIV-infected cells and mainly inactivates the virus particle.

In conclusion, we describe the inhibition of HIV-1 by a synthetic complex of compounds related to natural humic acids. Isolation of the active component(s) of the mixture by affinity binding to V3 loop oligopeptides could yield a valuable tool to study the functions of the V3 loop within the process of virus–cell fusion. Therapeutic approaches involving a mixture of closely related compounds rather than a singular compound of defined chemical structure may represent a suitable strategy against a variable target such as HIV. The low toxicity of HS-1500 in various mammalian species (A. Haase, personal communication) and lack of mutagenicity (U. N. Riede, unpublished) will facilitate its evaluation in animal models for human AIDS.

ACKNOWLEDGMENTS

We thank Monika Konanz for excellent technical assistance. We are indebted to R. C. Gallo, M. Popovic, V. Bosch, K. Karjalainen, A. Traunecker, and H.-J. Schlaeger for providing cell lines and to O. Haller and O. Herchenröder for critical reading of the manuscript. This work was supported by the Forschungsschwerpunkt Baden-Württemberg (AZ II-7532).

REFERENCES

1. Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M., *Nature* 373, 123–126 (1995).
2. Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S., and Shaw, G. M., *Nature* 373, 117–122 (1995).
3. Dalgleish, A. G., Beverley, P. C., Clapham, P. R., Crawford, D. H., Greaves, M. F., and Weiss, R. A., *Nature* 312, 763–767 (1984).
4. Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J. C., and Montagnier, L., *Nature* 312, 767–768 (1984).
5. Traunecker, A., Schneider, J., Kiefer, H., and Karjalainen, K., *Nature* 339, 68–70 (1989).
6. McKeating, J. A., Gow, J., Goudsmit, J., Pearl, L. H., Mulder, C., and Weiss, R. A., *AIDS* 3, 777–784 (1989).
7. Kliks, S. C., Shioda, T., Haigwood, N. L., and Levy, J. A., *Proc. Natl. Acad. Sci. USA* 90, 11518–11522 (1993).
8. Daar, E. S., Li, X. L., Moudgil, T., and Ho, D. D., *Proc. Natl. Acad. Sci. USA* 87, 6574–6578 (1990).
9. De Clercq, E., *Adv. Virus Res.* 42, 1–55 (1993).
10. Kutsch, H., and Schumacher, B., *Biol. Fertil. Soils* 18, 163–167 (1994).
11. Schols, D., Wutzler, P., Klöcking, R., Helbig, B., and De Clercq, E., *J. AIDS* 4, 677–685 (1991).
12. Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., De Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F., and Tersmette, M., *J. Virol.* 66, 1354–1360 (1992).
13. Baba, M., Schols, D., Pauwels, R., Nakashima, H., and De Clercq, E., *J. AIDS* 3, 493–499 (1990).
14. Pleskoff, O., Seman, M., and Alizon, M., *J. Virol.* 69, 570–574 (1995).

15. Pantaleo, G., Butini, L., Graziosi, C., Poli, G., Schnittman, S. M., Greenhouse, J. J., Gallin, J. I., and Fauci, A. S., *J. Exp. Med.* **173**, 511–514 (1991).
16. Mosmann, T., *J. Immunol. Methods* **65**, 55–63 (1983).
17. Jagodzinski, P. P., Wiaderkiewicz, R., Kurzawski, G., Kloczewiak, M., Nakashima, H., Hyjek, E., Yamamoto, N., Uryu, T., Kaneko, Y., Posner, M. R., and Kozbor, D., *Virology* **202**, 735–745 (1994).
18. Grewe, C., Beck, A., and Gelderblom, H. R., *J. AIDS* **3**, 965–974 (1990).
19. Sattentau, Q. J., and Moore, J. P., *J. Exp. Med.* **174**, 407–415 (1991).
20. Kräusslich, H. G., Ochsenbauer, C., Traenckner, A. M., Mergener, K., Fäcke, M. G., and Bosch, V., *Virology* **192**, 605–617 (1993).
21. Skinner, M. A., Ting, R., Langlois, A. J., Weinhold, K. J., Lyerly, H. K., Javaherian, K., and Matthews, T. J., *AIDS Res. Hum. Retroviruses* **4**, 187–197 (1988).
22. Matsushita, S., Robert-Guroff, M., Rusche, J., Koito, A., Hattori, T., Hoshino, H., Javaherian, K., Takatsuki, K., and Putney, S., *J. Virol.* **62**, 2107–2114 (1988).
23. Jabbar, M. A., and Nayak, D. P., *J. Virol.* **64**, 6297–6304 (1990).
24. Skinner, M. A., Langlois, A. J., McDanal, C. B., McDougal, J. S., Bolognesi, D. P., and Matthews, T. J., *J. Virol.* **62**, 4195–4200 (1988).
25. Werner, A., and Levy, J. A., *J. Virol.* **67**, 2566–2574 (1993).
26. Ratner, L., Fisher, A., Jagodzinski, L. L., Mitsuya, H., Liou, R. S., Gallo, R. C., and Wong-Staal, F., *AIDS Res. Hum. Retroviruses* **3**, 57–69 (1987).
27. Spire, B., Sire, J., Zachar, V., Rey, F., Barre-Sinoussi, F., Galibert, F. H., and Chermann, J. C., *Gene* **81**, 275–284 (1989).
28. Profy, A. T., Salinas, P. A., Eckler, L. I., Dunlop, N. M., Nara, P. L., and Putney, S. D., *J. Immunol.* **144**, 4641–4647 (1990).
29. De Jong, J. J., De Ronde, A., Keulen, W., Tersmette, M., and Goudsmit, J., *J. Virol.* **66**, 6777–6780 (1992).
30. Fouchier, R. A., Groenink, M., Kootstra, N. A., Tersmette, M., Huisman, H. G., Miedema, F., and Schuitemaker, H., *J. Virol.* **66**, 3183–3187 (1992).
31. McClure, M. O., Moore, J. P., Blanc, D. F., Scotting, P., Cook, G. M., Keynes, R. J., Weber, J. N., Davies, D., and Weiss, R. A., *AIDS Res. Hum. Retroviruses* **8**, 19–26 (1992).
32. Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., and De Clercq, E., *Proc. Natl. Acad. Sci. USA* **85**, 6132–6136 (1988).
33. Miyoshi, I., Kubonishi, I., Yoshimoto, S., and Shiraishi, Y., *Gann* 72, 978–981 (1981).
34. Popovic, M., Read Connole, E., and Gallo, R. C., *Lancet* **2**, 1472–1473 (1984).
35. Maddon, P. J., McDougal, J. S., Clapham, P. R., Dalgleish, A. G., Jamal, S., and Weiss, R. A. A., *Cell* **54**, 865–874 (1988).
36. Schneider, J., Kaaden, O., Copeland, T. D., Oroszlan, S., and Hunsmann, G., *J. Gen. Virol.* **67**, 2533–2538 (1986).

Investigation of the Immunostimulatory Properties of Oxihumate

Gisela Käthe Jooné[a], Johan Dekker[b], and
Constance Elizabeth Jansen van Rensburg[a,*]

[a] Department of Pharmacology, Faculty of Medical Sciences, University of Pretoria, PO Box 2034, Pretoria, 0001, South Africa. Fax: +27-12-3192411.
E-mail: cmedlen@postillion.up.ac.za
[b] Enerkom (Pty) Ltd, Pretoria, South Africa

* Author for correspondence and reprint requests

Z. Naturforsch. **58 c**, 263–267 (2003); received September 10/October 22, 2002

A unique process has been developed to convert bituminous coal by controlled wet oxidation followed by base treatment to a water-soluble humate called oxihumate. The effects of oxihumate on the proliferative response of lymphocytes has been studied *in vitro* and *ex vivo*. Oxihumate increased the proliferative response of phytohaemagglutinin-stimulated human lymphocytes, from a concentration of 20 μg/ml and upwards. This response was even more striking in the case of lymphocytes from HIV-infected patients and was not limited to the *in vitro* setting since similar effects were observed *ex vivo* following administration of a non-toxic dosage of 4 g oxihumate per day to HIV-positive individuals for two weeks. Mechanistic studies revealed that stimulation of the proliferative response of lymphocytes by oxihumate is associated with an increased production of IL-2, as well as expression of the IL-2 receptor in the setting of decreased production of IL-10. Oxihumate therefore holds promise for the treatment of immunocompromized patients.

Key words: Oxihumate, Immunostimulation, IL-2

Introduction

Humic substances are widely spread in nature. They occur mainly in heavily degraded peat but also in all natural environments in which organic materials and microorganisms are, or have been present (Visser, 1973; Hartenstein, 1981). Peat extracts have been used from ancient times in therapeutic baths for the treatment of various conditions for many years (Brandt, 1964; Eichelsdörfer, 1976; Klöcking, 1994). The antiseptic properties of peat were first recognized during World War I when it was applied directly on to wounds to prevent infection (Haanel, 1924).

More recently, humate has been used in the treatment of Von Willebrand disease (Lopez-Fernandez *et al.*, 1992). Patients were treated with an infusion of 35 mg/kg body weight after which normal factor VIII levels were achieved.

A unique process has been developed to convert bituminous coal by controlled wet oxidation, followed by base treatment to water-soluble humates, called oxihumate (the potassium salt of oxihumic acid) (Bergh *et al.*, 1997). The possible application of coal-derived humic and fulvic acid as antimicrobials, has been described by Cloete *et al.* (1990) and Van Rensburg *et al.* (2000) whereas the anti-inflammatory properties of coal-derived fulvic acid has been reported recently by Van Rensburg *et al.* (2001) and Snyman *et al.* (in press).

Antiviral properties, at a concentration of 100 μg/ml of ammonium humate (the ammonium salt of humic acid) *in vitro* has been described by Thiel *et al.* (1981) resulting in the successful use of this agent as a topical treatment for herpes virus-induced skin diseases (Klöcking *et al.*, 1983). Schneider *et al.* (1996) reported on the anti-HIV activity of synthetic humate analogues derived from hydroquinone. These compounds inhibited HIV-1 infection of MT-2 cells with an impressively low IC_{50} of 50–300 ng/ml. The infectivity of HIV particles was inhibited by interference of a V3 loop-mediated step of virus entry. Similar results were found with oxihumate (Van Rensburg *et al.*, 2002). In this study we investigated the effects of oxihumate on human lymphocyte functions.

Abbreviations: PHA, Phytohaemagglutinin A; MNL, mononuclear leukocytes.

Materials and Methods

Oxihumate

Oxihumate was provided as a 1% solution in water by Enerkom (Pty) Ltd, Pretoria, South Africa. Average values for the elemental composition of oxihumate are 40%, 2.5% and 1% for carbon, hydrogen and nitrogen respectively whereas the approximate molecular weight of humic acid obtained from oxidized coal is between 57 and 70 kD (Piccolo *et al.*, 2000).

Mononuclear leukocytes (MNL)

These were prepared as described previously (Anderson *et al.*, 1993) by density centrifugation on Histopaque-1077 (Sigma Diagnostics, St Louis, MO, USA) of blood taken either from healthy adult human volunteers or HIV-infected individuals (with a CD4 count between 209 and 504 X 10^6/l) before treatment or after a 2-week treatment of either placebo, 4 g or 6 g oxihumate taken orally per day. These patients participated in a pilot study to evaluate the therapeutic efficacy of oxihumate in HIV-infected individuals. This trial was carried out in accordance with the World Medical Association Declaration of Helsinki. All ethical and legal standards were followed as determined by the University of Pretoria, as well as the Medicine Control Council of South Africa.

The cells were then either resuspended to 2 × 10^6/ml in complete RPMI 1640 medium (supplemented with 1% glutamine, penicillin and streptomycin at 100 µg/ml and 10% heat inactivated fetal calf serum obtained from Bio Whittaker, Walkersville, Maryland) or incubated first in complete medium in 5 ml tissue culture flasks for 30 min to remove adherent cells from the suspension before re-suspending to 2 × 10^6/ml.

Mitogen-activated MNL proliferation

Fifty microliters of MNL suspension (1 × 10^5 cells/well), were added to 110 µl of complete RPMI 1640 medium in the wells of microtiter culture plates (96 wells) followed by 20 µl of various oxihumate concentrations (5–100 µg/ml) and 20 µl of the mitogen phytohaemagglutinin (PHA, 5 µg/ml final concentration). Control systems without oxihumate and/or mitogen were included and the final volumes of all the wells were adjusted to 200 µl. After 72 hours incubation (37 °C in ar atmosphere of 5% CO_2) the extent of lymphocyte proliferation was assayed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reactivity which detects only viable cells (Mosman, 1983). The plates were read on a Ceres UV 900 micro-ELISA reader using a test wavelengtl of 540 nm and a reference wavelength of 620 nm.

Measurement of cytokines

These experiments were set up as describec above. MNL suspensions (1 ml) obtained from healthy donors were treated in the presence o PHA (5 µg/ml) with 60, 80 and 100 µg/ml oxihumate for 72 h in 5 ml plastic tubes and the supernatants collected and stored at – 70 °C for subsequent IL-2 and IL-10 determination using the relevant Biotrak TM human ELISA systems from Amersham TM (Amersham International Plc Buckinghamshire, England).

Expression of CD25

MNL suspensions obtained from healthy donor were treated in the presence of PHA (5 µg/ml with or without 100 µg/ml oxihumate for 72 h and CD25 expression measured with an FITC-conjugated monoclonal antibody against CD25. Flow cytometric analysis was performed using a Coulter Epics XL-MLC flow cytometer (Coulter Corp Miami, Florida, USA) equipped with a 488 nm air cooled argon laser.

Results

MNL proliferation

Oxihumate had no effect on resting lymphocyte up to a concentration of 100 µg/ml, but increased the proliferative response of PHA-stimulated monocyte depleted lymphocytes at 20 µg/ml and upwards in a dose-related manner (Fig. 1A). Similar effects were seen when monocyte rich MNL were used (Fig. 1B). This response was even more striking in the case of monocyte rich lymphocyte (MNL) from HIV-infected patients (Fig. 1B).

Significant ($p < 0.05$) increases in PHA-stimulated proliferation of MNL of HIV-infected individuals were also observed *ex vivo* followin

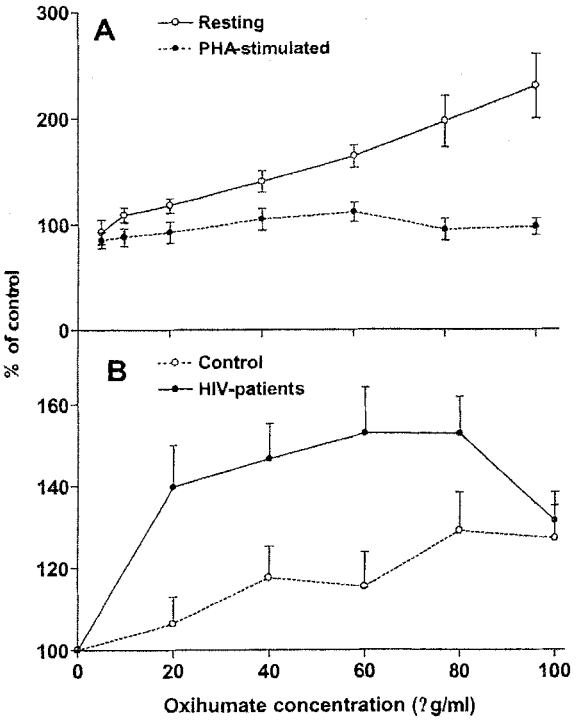


Fig. 1. The effects of a 72 h treatment with various concentrations of oxihumate on **[A]** resting and phytohaemagglutinin (PHA)-stimulated, monocyte-depleted, human lymphocyte proliferation and **[B]** PHA-stimulated mononuclear lymphocyte (MNL) cultures from normal donors and HIV-infected individuals. Results expressed as percentage of control ± SEM of 5–18 different experiments.

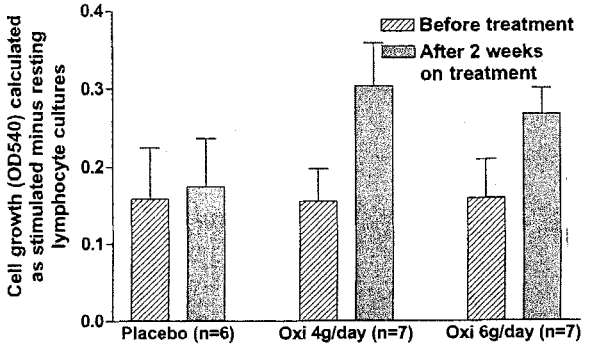


Fig. 2. The effects of a two week treatment of HIV-positive individuals with oxihumate on phytohaemagglutinin-stimulated mononuclear lymphocyte cultures *ex vivo*.

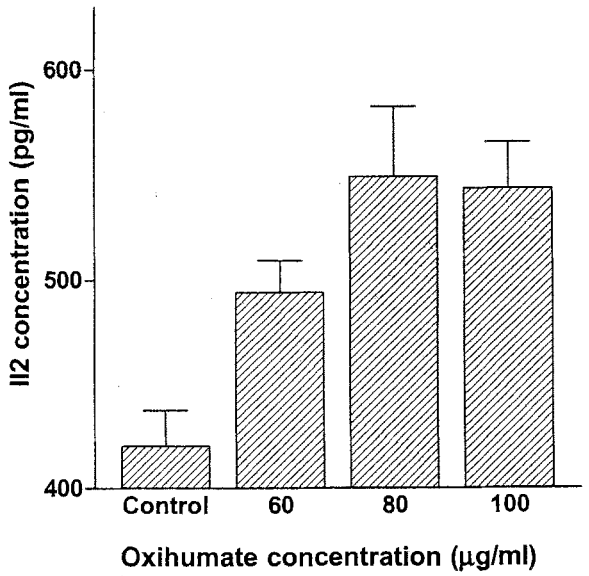


Fig. 3. The effects of a 72 h treatment of various concentrations of oxihumate on IL-2 production by phytohaemagglutinin-stimulated mononuclear lymphocyte cultures. Results expressed as the mean ± SEM of 4 different experiments.

administration of 4 g (but not 6 g) oxihumate per day for 2 weeks, compared to the placebo-treated group (Fig. 2).

IL-2 secretion

Oxihumate increased the secretion of IL-2 by PHA-stimulated MNL significantly ($p < 0.05$) at all three concentrations tested (Fig. 3).

IL-10 secretion

The effects of oxihumate treatment on IL-10 secretion by PHA-stimulated MNL are shown in Fig. 4. Oxihumate decreased the secretion of IL-10 at all three concentrations to the level observed in resting cells ($p < 0.001$).

CD25 expression

Oxihumate (at 100 µg/ml) increased the expression of the IL-2 receptor CD25 by PHA stimulated MNL significantly ($p < 0.05$) but had no effect on the levels of CD25 on resting MNL. Control values (median) for PHA stimulated MNL were 17.3 ± 0.3, whereas the values for MNL treated with 100 µg/ml oxihumate were 27.6 ± 0.2.

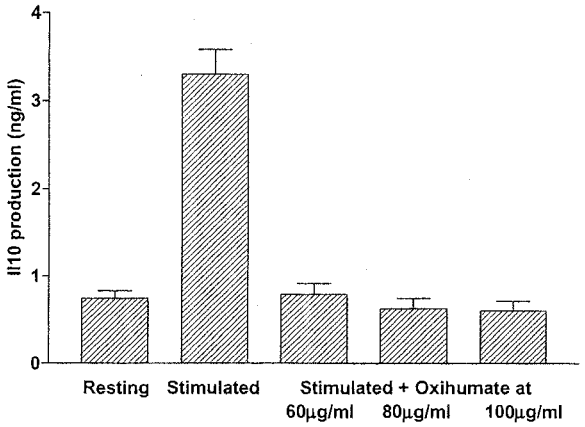


Fig. 4. The effects of a 72 h treatment of various concentrations of oxihumate on IL-10 production by phytohaemagglutinin-stimulated mononuclear lymphocyte cultures. Results expressed as the mean ± SEM of 4 different experiments.

Discussion

Although the HIV disease is associated with an increased rate of T-cell turnover, the loss of $CD4^+$ cell numbers exceeds the capacity to replenish with the resultant loss of cellular immune function (Losso *et al.*, 2000). A function of interleukin-2 (IL-2), a T-cell-derived cytokine, is to promote T-cell growth and maturation. Although IL-2 does not reduce viral replication *in vitro* (Kovacs *et al.*, 1996) it might counteract the virus-induced loss of $CD4^+$ cells in HIV infected individuals by increasing the proliferation of T-cells. IL-2, given in conjunction with a combination of highly active antiretroviral therapy (HAART), causes dramatic increases in mean CD4 counts compared to HAART alone (Davey *et al.*, 2000; Shearer *et al.*, 1998).

Oxihumate, a water-soluble humate derived from coal, increased the proliferative response of PHA-stimulated MNL as well as monocyte d pleted human lymphocytes, at 20 µg/ml and u wards. This response was even more striking in t case of lymphocytes from HIV-infected patier and was therefore not limited to the *in vitro* s ting. Similar effects were observed *ex vi* following administration of 4 g oxihumate per d to HIV positive individuals for two weeks. Tl increase can be attributed to increased producti of IL-2, as well as expression of the IL-2 recept (CD25) on lymphocytes. Oxihumate therefo seems to enhance the activity of T_{H1} cells (IL producing cells) whilst decreasing, at the sar time, IL-10, a T_{H2}-associated cytokine. The abili of some HIV-positive individuals to maintain nc mal T_{H1} type responses has long term protecti effects on the survival of these patients as disea progression is attributed to a T_{H1} to T_{H2} cytoki shift (Shearer *et al.*, 1998; Altfeld *et al.*, 2000).

Oxihumate therefore possesses both immunos mulatory, as well as anti-viral activity (Van Rer burg *et al.*, 2002) and did not produce any meas able toxicity in experimental animals during eith sub-chronic or acute oral or dermal exposu (Progress Report: Biochon (Pty) Ltd, Pretor South Africa, July 1999), nor did it produce a measurable toxicity in HIV-infected individu treated with oral doses of up to 8 g per day f two weeks (Botes *et al.*, 2002). This combinati of properties in one compound appears to unique and merits further evaluation in immur compromized patients such as those infected w HIV.

Acknowledgements

This study was supported by a grant from En kom (Pty) Ltd as well as a grant from the Techn ogy and Human Resources for Industry Progra of the National Research Foundation and the D partment of Trade and Industry.

Altfeld M., Addo M. M., Kreuzer K. A., Rockstroh J. K., Dumoulin F. L., Schliefer K., Leifeld L., Sauerbruch T., and Spengler U. (2000), T(H)1 to T(H)2 shift in peripheral blood of HIV-infected patients is detectable by reverse transcriptase polymerase chain reaction but not by enzyme-linked immunosorbent assay under nonstimulated conditions. J. Acquir. Immune. Defec. Syndr. **23**, 287–294.

Anderson R., Smit M. J., and Van Rensburg C. E. J. (1993), Lysophospholipid-mediated inhibition of Na^+, K^+-adenosine triphosphatase is a possible mechanism of immunosuppressive activity of cyclosporine A. Mol. Pharmacol. **44**, 605–619.

Bergh J. J., Cronje I. J., Dekker J., Dekker T. G., Gerritsma L. M., and Mienie L. J. (1997), Non-catalytic oxidation of water-slurried coal with oxygen: identification of fulvic acids and acute toxicity. Fuel. **76**, 149–154.

Botes M. E., Dekker J., and Van Rensburg C. E. J. (in press), A phase I trial with oral oxihumate in HIV-infected patients. Drug Development Res.

Brandt H. (1964), Die Behandlung degenerativer Erkrankungen der Wirbelsäule und der Gelenke mit salizylierten Huminsäurebädern. Fortschr. Med. **82**, 110.

Cloete T. E., Swart H., Cronje I. J., and Dekker J. (1990), Oxidized coal products as industrial bactericides. Third International Symposium on Gas, Oil, Coal and Environmental Biotechnology. New Orleans, Louisiana, 3–5 December.

Davey R. T., Murphy R. L., Graziano F. M., Boswell S. L., Pavia A. T., Cancio M., Nadler J. P., Chaitt D. G., Dewar R. L. Sahner D. K., Duliege A. M., Capra W. B., Leong W. P., Giedlin M. A., Lane H. C., and Kahn J. O. (2000), Immunologic and virologic effects of subcutaneous interleukin 2 in combination with antiretroviral therapy: A randomized controlled trial. J. Amer. Ass. **284**, 183–189.

Eichelsdörfer D. (1976), Moor in der Heilkunde. In: Moor- und Torfkunde (K. Göttlich, ed.). Schweizerbart, Stuttgart.

Haanel B. F. (1924), Facts about peat. In: Mines Brand Publ no 614, Can. Dept. Mines, Ottawa.

Hartenstein R. (1981), Sludge decomposition and stabilization. Science. **212**, 743–749.

Klöcking R. (1994), Humic substances as potential therapeutics. In: Humic Substances in the Global Environment and Implications on Human Health (Senesi N. and Miano T. M., eds.). Elsevier Science B. V. Amsterdam, The Netherlands.

Klöcking R., Sprössig M., Witzler P., Thiel K. D., and Helbig B. (1983), Antiviral wirksame huminsäureähnliche Polymere. Z. Physiother. **33**, 95–101.

Kovacs J. A., Vogel S., Albert J. M., Falloon J., Davey R. T. Jr., Walker R. E., Polis M. A., Spooner K., Metcalf J. A., Baseler M., Fyfe G., and Lane H. C. (1996), Controlled trial of interleukin-2 infusion in patients infected with the human immunodeficiency virus. N. Engl. J. Med. **335**, 1350–1356.

Lopez-Fernandez M. F., Blanco-Lopes M. J., Castineira M. P., and Batlle J. (1992), Further evidence for recessive inheritance of Von Willebrand disease with abnormal binding of Von Willebrand factor to factor VIII. Am. J. Hematol. **40**, 20–27.

Losso M. H., Belloso W. H., Emery S., Benetucci J. A., Cahn P. E., Lasala M. C., Lopardo G., Salomon H., Saracco M., Nelson E., Law M. G., Davey R. T., Allende M. C., and Lane H. C. (2000), A randomized, controlled, phase II trial comparing escalating doses of subcutaneous interleukin-2 antiretrovirals versus antiretrovirals alone in human immunodeficiency virus-infected patients with CD4+ cell counts ≥ 350/mm^3. J. Infect. Dis. **181**, 1614–1621.

Mosman T. (1983), Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth. **65**, 55–62.

Piccolo A., Conte P., and Cozzolino A. (2000), Differences in high performance size exclusion chromatography between humic substances and macromolecular polymers. In: Humic Sustances Versatile Components of Plants, Soils and Water (Ghabbour E. A. and Davies G., eds.). M. P. G. Books Ltd., Cornwall, UK.

Schneider J., Weis R., Manner C., Kary B., Werner A., Seubert B. J., and Riede U. N. (1996), Inihbition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: mechanism of inhibition. Virology. **218**, 389–395.

Shearer G. M., and Clerici M. (1998), Cytokine profiles in HIV type 1 disease and protection. AIDS Res. Hum. Retroviruses. **14**, 149–152.

Snyman J. R., Dekker J., Malfeld S. C. K., and Van Rensburg C. E. J. (in press), A pilot study to evaluate the safety and therapeutic efficacy of topical oxifulvic acid in atopic volunteers. Drug Development Res.

Thiel K. D., Helbig B., Klöcking R., Wurtzer P., Sprössig M., and Schweizer H. (1981), Comparison of the *in vitro* activities of ammonium humate and of enzymatically oxidized chlorogenic and caffeic acids against type 1 and type 2 human herpes virus. Pharmazie **36**, 50–53.

Van Rensburg C. E.J, Dekker J., Weiss R., Smith T.-L., Janse van Rensburg E., and Schneider J. (2002), Investigation of the anti-HIV properties of oxihumate. Chemotherapy **48**, 138–143.

Van Rensburg C. E. J., Malfeld S. C. K., and Dekker J. (2001), Topical application of oxifulvic acid suppresses the cutaneous immune response in mice. Drug Development Res. **53**, 29–32.

Van Rensburg C. E. J., van Straten A., and Dekker J. (2000), An *in vitro* investigation of the antimicrobial activity of oxifulvic acid. J. Antimicrob. Chemother. **46**, 853–854.

Visser S. A. (1973), Some biological effects of humic acids in the rat. Acta Biol. Med. Germ. Band **31**, 569–581.

eJIAS
eJOURNAL OF THE INTERNATIONAL AIDS SOCIETY

XV INTERNATIONAL AIDS CONFERENCE
BANGKOK, 11-16 JULY 2004

Abstract Number: A10360

New humic acid derivative as potent inhibitors of HIV-1 replication

Author: G Kornilaeva[1], A Bercovich[2], T Pavlova[1], E Karamov[1]
[1]Ivanovsky Institute of virology, RAMS, Moscow, Russian Federation; [2]Ligfarm, Moscow, Russian Federation

Background: The anti-HIV-1 activity of the new humic acid derivative has been determined in vitro, and the serial passages at low concentrations have been performed to investigate the emergence of resistance to this drug candidate.
Methods: The antiviral efficiency of the humic acid derivative was tested against HIV-1 T-tropic laboratory strain and HIV-1 M-tropic AZT-resistant wild-type strain. CEM cells were infected and cultivated in the presence of compound at the concentrations 250-0.0025 µg/ml. The level of virus production in infected cells was detected with p24 HIV-1 antigen ELISA detection system. The serial virus passages were performed at the concentration - 0.0025 µg/ml of compound. The virus generation of each passage was analyzed for HIV-1 p24 antigen and the infectious activity.
Results: The IC50 values of the humic acid derivative against HIV-1 T-tropic laboratory strain and HIV-1 M-tropic AZT-resistant wild-type strain were 0.85 and 3.5 µg/ml respectively. The combination of humic acid derivative and AZT intensifies their anti virus activity in 30-100 times. The determined p24 HIV-1 antigen value of the first passage virus generation at the presence of 0.0025 µg/ml of compound was identical to that of the control while the infectious activity approached to zero. The virus generations of second and third passages possessed the low values of p24 HIV-1 antigen and the undetectable infectious activity.
Conclusions: The new acid humic derivative is effective inhibitor of various HIV-1 variants with high selectivity indexes (5500-3000). According to our date this compound disorder HIV-1 replication and yield the defective unifectious virions.

Med. Gen. Med. 2004 July 11; 6(3), A10360