Title: CHITOSAN-COATED LIPOSOME DRUG DELIVERY OF ANTIOXIDANT OR ANTI-INFLAMMATORY COMPOUNDS

Abstract: This invention comprises pharmaceutical compositions for administering a nutraceutical, particularly a polycyclic, aromatic, antioxidant or anti-inflammatory compound to an animal. Particularly provided are proliposomal compositions that are advantageously used to deliver nutraceuticals, including polycyclic, aromatic, antioxidant or anti-inflammatory compounds to the gastrointestinal tract after oral administration.
CHITOSAN-COATED LIPOSOME DRUG DELIVERY OF ANTIOXIDANT OR ANTI-INFLAMMATORY COMPOUNDS

BACKGROUND OF THE INVENTION

1. **Field of the Invention**

   This invention relates to delivery of nutraceuticals, particularly antioxidant or anti-inflammatory drugs, nutrients and other compounds to a biological organism. In particular, the invention relates to liposomes and formulations of nutraceuticals, particularly antioxidant or anti-inflammatory drugs, nutrients and other compounds, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds into liposomes to improve or effect delivery of such beneficial compounds to cells and tissues in an organism. Specifically, the invention provides such liposome compositions of antioxidant or anti-inflammatory drugs, nutrients and other compounds in formulations advantageously administered orally to an animal, wherein the formulation comprises a chitosan coating.

2. **Background of the Related Art**

   A nutraceutical is a compound that is a food or a part of a foodstuff that provides medical or health benefits, including the prevention or treatment of disease. DeFelice, 1995, *Trends in Food Science & Technol.* 6: 59-61. Nutraceuticals are also known as functional foods, and include dietary supplements, health foods, phytochemicals and botanical products. They are becoming increasingly more important components of health and nutrition, as the U.S. population ages, diseases have emerged that are chronic and life-style related, and as conventional faith in traditional medicine decreases. In the U.S., nutraceuticals are part of an $18 billion

Diseases and conditions for which nutraceuticals have been or are being developed include numerous types of cancer, coronary heart disease, diabetes, high blood pressure, AIDS, cystic fibrosis, osteoporosis, chronic stress, inflammation, microbial, viral and parasitic infections, psychotic diseases, spasmotic conditions, arthritis, and ulcers, among others. See, for example, Block & Evans, 2000, J. Amer. Nutraceut. Assn 3: 6-16.

Cardiovascular disease (including atherosclerosis, myocardial infarction, ischemia, stroke, pulmonary embolism and other thrombotic diseases) and cancer are major causes of mortality in the U.S., being responsible for up to two-thirds of all deaths per annum. Despite advances in preventative medicine, diet, exercise, diagnostics and therapeutic approaches that have increased the average lifespan of U.S. citizens and reduced the number of premature deaths, these diseases ultimately are no less responsible for mortality than they were a generation ago.

Certain nutraceutical, plant-derived compounds have been recognized as being beneficial both as anticancer and antcardiovascular disease agents. This class of compounds is generally recognized to include polycyclic, aromatic compounds having antioxidant or anti-inflammatory activity, and include the flavonoids (such as flavone, quercetin and chrysin) and derivatives of stilbenes, such as resveratrol. The effects of these compounds as anticancer and antcardiovascular disease agents are both anecdotal and have been subjected to modern scientific scrutiny. One example of anecdotal evidence for the beneficial effects of these compounds is with regard to
what has been termed “French paradox.” The paradox is that, although the French diet is high in nutrients (such as cholesterol and saturated fats) that have been associated with a risk of arteriosclerosis and cardiovascular disease, French citizens have less cardiovascular disease than their counterparts in other Western countries.

The purported reason for this result is that French citizens also consume more red wine that other Western country citizens, and red wine is high (5-50 parts per million) in one particular polycyclic, aromatic antioxidant compound, resveratrol (Siemann & Creasy, 1992, *Am. J. Enol. Vitic.* 43: 49-52; Kopp et al., 1998, *Eur. J. Endocrinol.* 138: 619-620). Resveratrol is also a component of a traditional oriental medicine, Ko-jo-kon, used to treat ailments of the heart, blood vessels and liver. Soleas et al., 1997, *Clin. Biochem.* 30: 91-113. Resveratrol is found in red wine due to its presence in grape skin, and is thought to be a phytoalexin that provides protection against fungi. Celotti et al., 1996, *J. Chromatog.* 730: 47-52. It is also found (at much lower concentrations) in eucalyptus, spruce, lily, mulberry and peanuts.

Resveratrol has also been the subject of several scientific studies. The Copenhagen Heart Study (1995) showed that the relative risk of mortality from coronary artery disease was reduced by 20% when red wine was consumed once a month, by 30% when red wine was consumed once or twice weekly, and by 40% when red wine was consumed 3-5 times per week. Other researchers investigated the physiologic basis for these results. Pendarithi et al. (1999, *Arteriosclerosis Thrombosis and Vascular Biol.* 19: 419-426) showed that resveratrol, dose-dependently, inhibited transcription and expression of tissue factor in endothelial cells. Resveratrol has been shown to have antioxidant (Chanvitayapongs et al.,

As a consequence, the World Health Organization estimates that coronary artery disease could be reduced by up to 40% if resveratrol or resveratrol-containing foods were consumed in proper amounts.

Quercetin (3,3', 4', 5,7-pentahydroxyflavone) is a natural substance found in apples, onions, tea and red wine (like resveratrol, it is derived from grape skins). Quercetin has been shown to be an antioxidant. Cai et al., 1999, *Free Radical Biol. Med.* **27**: 822-829. Quercetin has been shown to be an efficacious agent for preventing and treating prostate cancer by workers at the Mayo Clinic. Xing et al., American Association for Cancer Research, 26th Annual Meeting, March 26, 2001. Prostate cancer is the second leading cause of death in men, with 31,500 fatalities and 200,000 diagnoses per year. Quercetin is currently in therapeutic use for treating inflammatory diseases and disorders.

A major goal in the pharmacological arts has been the development of reagents and methods that reduce the necessity of administering therapeutic compounds, drugs and other agents invasively (i.e., such as by injection). Most preferably, it has been a consistent goal in the art to develop therapeutic compounds, drugs and agents and formulations thereof that permit oral administration (see, for example U.S. Patent No. 4,963,526 to Ecanow issued October 16, 1990), although other reduced-invasiveness formulations such as suppositories have also been developed. Among the various routes of drug administration, the oral intake of drugs is undoubtedly preferred because of its versatility, safety and patient comfort.

In addition, it has been a goal in the nutritional arts to develop preparations that increase transit of certain nutrients through the gastrointestinal tract to increase uptake and delivery of such nutrients into the bloodstream. In particular, such preparations have been developed to permit chemically-labile nutrients (such as vitamins and other sensitive compounds) to pass through the chemically-hostile environment of the stomach for absorption in the intestines (see, for example, U.S.
Patent No. 5,958,450 to Tashiro issued September 28, 1999). Preparations having enhanced intestinal uptake have also been deemed desirable.

One approach known in the prior art for improving efficiency of delivery of therapeutic compounds, drugs and other agents has been to envelop such compounds in a specialized lipid structure termed a liposome (see, for example, U.S. Patent No. 4,744,989 to Payne et al. issued May 17, 1988). Liposomes generically comprise an enclosed lipid droplet having a core, typically an aqueous core, containing the compound. In certain embodiments, the compound is chemically conjugated to a lipid component of the liposome. In other embodiments, the compound is simply contained within the aqueous compartment inside the liposome.

Certain liposome formulations are known in the art.


U.S. Patent No. 5,466,468 to Schneider et al. issued November 14, 1995 discloses parenterally administrable liposome formulations comprising synthetic lipids.

U.S. Patent No. 5,484,809, issued January 16, 1996 to Hostetler et al. discloses taxol and taxol derivatives conjugated to phospholipids.

U.S. Patent No. 5,580,571, issued December 3, 1996 to Hostetler et al. discloses nucleoside analogues conjugated to phospholipids.

U.S. Patent No. 5,626,869 to Nyqvist et al. issued May 6, 1997 discloses pharmaceutical compositions wherein the pharmaceutically active compound is heparin or a fragment thereof contained in a defined lipid system comprising at least
one amphipathic and polar lipid component and at least one nonpolar lipid component.


U.S. Patent No. 5,843,509 to Calvo Salve et al. issued December 1, 1998 discloses stabilization of colloidal systems through the formation of lipid-polysaccharide complexes comprising a water soluble and positively charged polysaccharide and a negatively charged phospholipid.


European Patent Application Publication Number 0350287A2 to Vical discloses conjugates between antiviral nucleoside analogues and polar lipids.


Rahman et al., 1982, Life Sci. 31: 2061-71 found that liposomes which contained galactolipid as part of the lipid appeared to have a higher affinity for parenchymal cells than liposomes which lacked galactolipid.


Although liposomes have conventionally been administered parenterally (see, for example, U.S. Patent No. 5,466,468), reports of oral administration of liposome-related formulations have appeared in the art.

U.S. Patent No. 4,921,757 to Wheatley *et al.* issued May 1, 1990 discloses controlled release of biologically active substances, such as drugs and hormones entrapped in liposomes that are protected from the biological environment by encapsulation within semi-permeable microcapsules or a permeable polymeric matrix.


U.S. Patent No. 5,955,451 to Lichtenberger *et al.* issued September 21, 1999
discloses compositions comprising non-steroid anti-inflammatory drugs (NSAID's) complexed with either zwitterionic or neutral phospholipids, or both, having reduced gastrointestinal irritating effects and enhanced antipyretic, analgesic, and anti-inflammatory activity.

Proliposomes are an alternative to conventional liposomal formulations. Proliposomes are dry, free-flowing granular products, which, on addition of water, disperse to form a multi-lamellar liposomal suspension. The stability problems associated with conventional liposomes such as aggregation, susceptibility to hydrolysis and/or oxidation are avoided by using proliposomes.

U.S. Pat. No. 5,635,206 to Ganter et al. discloses a process for preparing liposomes or proliposomes.

U.S. Pat. No. 5,595,756 to Bally et al. discloses that the bioactive agent concentration in plasma increases when a synergistic effect is induced by lowering the pH (to approximately 2-3) of the solution in which a bioactive agent is entrapped within a liposome and including in the liposomal membrane an amine-bearing lipid.

U.S. Pat. No. 6,093,406 to Alving et al. teaches liposomal derived vaccines that use a liposome and a compound that contains a net negative charge, a net positive charge (via stearylamine) or is neutral in conjunction with liposomes adsorbed to aluminum hydroxide.

Proliposomes of indomethacin were prepared using effervescent granules, which upon hydration yielded liposomes of high encapsulation efficiency and increased anti-inflammatory activity with decreased ulcerogenic index (see, for example, Katare et al., 1991, J. Microencapsulation 81: 1-7).

The proliposomal concept has been extended to administer drugs through
various routes and also to the food industry wherein enzyme immobilization is essential for various food processing regimes. A typical example is the immobilization of the enzyme, chymotrypsin, in liposomes obtained from proliposomes.

There remains a need in the art for a general, inexpensive and effective means for delivering compounds such as nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory or anti-inflammatory compounds to an animal by oral administration. Advantageous embodiments of such delivery means are formulated to efficiently deliver such compounds to the appropriate portion of the gastrointestinal tract for efficient absorption.

SUMMARY OF THE INVENTION

The present invention is directed to an improved method for delivering nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds to an animal by oral administration. This delivery system achieves specific delivery of nutraceuticals such as polycyclic, aromatic, antioxidant or anti-inflammatory compounds through associating the compounds with liposomes and proliposome components.

In preferred embodiments, a nutraceutical is formulated as a proliposomal composition that can be reconstituted in vivo to provide a liposomal preparation. Preferably, the nutraceutical is a polycyclic, aromatic, antioxidant or anti-inflammatory compound. The invention thus also provides pharmaceutical compositions comprising a nutraceutical, particularly the polycyclic, aromatic, antioxidant or anti-inflammatory compound and a lipid formulated as a
proliposomal preparation. In more preferred embodiments, the pharmaceutical compositions of the invention are formulated for oral administration.

The nutraceutical formulations and pharmaceutical compositions of the invention are formulated for oral administration to preferably comprise an enteric coating sufficient to prevent dissolution of the composition in the stomach of an animal. In alternative embodiments, the pharmaceutical compositions also comprise a protective coating between the enteric coating and the core of the composition comprising the proliposomal components thereof. Additional advantageous components of said orally-administrable pharmaceutical compositions further comprise the pharmaceutical compositions as will be understood by those with skill in the art, including *inter alia* excipients, adjuvants, buffers, lubricants, and plasticizers.

In preferred embodiments, the pharmaceutical compositions of the invention further comprise a protective coating that is most preferably a chitosan layer, in certain embodiments wherein the chitosan layer is in between the proliposomes and the enteric coating.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A through 1C depict thermograms produced by differential scanning calorimetry as set forth in Example 1.

Figures 2 and 3 depict transfer rates of glyburide through a Caco-2 cellular
monolayer using the liposomal compositions of the invention, as set forth in Example 2.

Figures 4 and 5 depict total accumulation of glyburide in the receiving chamber of a transwell comprising a Caco-2 cellular monolayer using the liposomal compositions of the invention, as set forth in Example 2.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The present invention provides compositions of matter and methods for facilitating the delivery of nutraceuticals, particularly polycyclic aromatic antioxidant or anti-inflammatory compounds, to the tissues of an animal after oral administration.

As used herein, the terms "nutraceuticals" and "functional foods" include but are not limited to carotenoids, fish oils and omega-fatty acids, green tea, beta-carotene, carnitine, glucosamine, chondroitin sulfate, ginseng, ginko biloba, genistein, saw palmetto, curcumin, lycopene, Echinacea, lipoic acid, calcitonin, hyperforin, flavinoids, and Vitamins A, C and E, and in particular polycyclic, polyaromatic compounds, especially said compounds derived from plant sources such as resveratrol, quercetin and chrysin.

For the purposes of this invention, the term "polycyclic aromatic antioxidant or anti-inflammatory compound" is intended to encompass naturally-occurring, most preferably plant-derived, or synthetic compounds having antioxidant or anti-inflammatory properties and comprising cis-stilbene; trans-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4', 5)-dihydroxy cis-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4', 5)-dihydroxy trans-stilbene; carboxylated derivatives of cis-stilbene or trans-
stilbene; halogenated derivatives of cis-stilbene or trans-stilbene; and resveratrol (trans-3,5,4'-trihydroxystilbene); or flavonoids, most preferably chrysin (5,7-dihydroxyflavone) or quercetin (3,3',4',5,7-pentahydroxyflavone), or biologically-active derivatives thereof.

Formulations, compositions and pharmaceutical compositions comprising nutraceuticals, particularly polycyclic aromatic antioxidant or anti-inflammatory compounds, are preferably provided as proliposomal compositions that can be reconstituted, most preferably in vivo, to produce liposomal compositions of nutraceuticals, especially polycyclic, aromatic, antioxidant or anti-inflammatory compounds. As provided herein, said formulations, compositions and pharmaceutical compositions comprise one or a plurality of nutraceuticalal compounds.

As used herein, the term “proliposome” and “proliposomal” are intended to encompass calcium-free, dry, free-flowing granular products, which, on addition of water, disperse to form multi-lamellar liposomal suspensions comprising one or a plurality of nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds of the invention. The liposomes and preliposomes of the instant invention are not adsorbed to aluminum hydroxide. Advantageously, the stability problems associated with the conventional liposomes (such as aggregation, susceptibility to hydrolysis and oxidation) are avoided by using proliposomes.

The proliposomal compositions provided by the invention are reconstituted, particularly in vivo, to provide liposomal compositions wherein one or a plurality of nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds of the invention are encapsulated in said liposomes. Even more
preferably, the proliposomal composition is reconstituted in the intestines of the animal. When the proliposomes of the instant invention form liposomes in the intestines, the liposomes will be in an environment with a pH of approximately 6.4 (in humans.) This precludes the synergistic effect between the stearylamine and the biologically active component in a low pH (approximately 2-3) that is disclosed and claimed in U.S. Patent No. 5,595,756.

In preparing the proliposomal compositions of the invention, lipid components including neutral lipids, positively-charged lipids or species, negatively-charged lipids or species, amphoteric lipids such as phospholipids, and cholesterol are advantageously used. As defined herein, the “lipid component” of the proliposomal compositions of the invention are intended to encompass a single species of lipid (such as a particular phospholipid) or combinations of such lipids, either of one type such as combinations of phospholipids (for example, a phosphatidylycholine plus a phosphatidylethanolamine) or of different types (such as a phospholipid plus a charged lipid, charged species, a neutral lipid or neutral species). Combinations comprising a multiplicity of different lipid types are also advantageously encompassed by the proliposomal compositions of the invention (see, Lehninger, 1975, *Biochemistry*, 2d ed., Chapters 11 & 24, Worth Publishers: New York; and Small, 1986, “From alkanes to phospholipids,” *Handbook of Lipid Research: Physical Chemistry of Lipids*, Volume 4, Chapters 4 and 12, Plenum Press: New York).

More preferably, the “lipid component” of the proliposomal compositions encompasses at least one lipid, and a positively charged species, that is not calcium. A preferred positively charged species is a primary aliphatic amine, such as
stearylamine. More preferably, the lipid component also contains cholesterol. Most preferably, the lipid component consists essentially of one, two, or three independently selected lipids, a positively charged species and cholesterol. The lipids are independently a phosphatidylcholine, a phosphatidylethanolamine, sphingosine, or ceramide. More preferably, the phosphatidylcholine is distearylphosphatidylcholine, dimyristylphosphatidylcholine or a mixture thereof. Even more preferably, the phosphatidylcholine is distearylphosphatidylcholine or dimyristylphosphatidylcholine. It should also be noted that negatively charged species do not work; they decrease the biological availability of drugs in the present invention. See Figures 2 and 4.

Nutraceuticals such as polycyclic, aromatic, antioxidant or anti-inflammatory compounds that are unstable in the stomach, or that show reduced absorption incident to transit through the stomach or other portions of the gastrointestinal tract, or nutraceuticals such as polycyclic, aromatic, antioxidant or anti-inflammatory compounds that irritate the stomach, and those nutraceuticals including polycyclic, aromatic, antioxidant or anti-inflammatory compounds that are preferentially absorbed in the small intestine are preferred compounds useful with the liposomal formulations of the invention. In preferred embodiments, said compounds include but are not limited to cis-stilbene; trans-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy cis-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy trans-stilbene; carboxylated derivatives of cis-stilbene or trans-stilbene; halogenated derivatives of cis-stilbene or trans-stilbene; and resveratroil (trans-3,5,4'-trihydroxystilbene); or flavonoids, most preferably chrysin (5,7-dihydroxyflavone) or quercetin (3,3',4',5,7-pentahydroxyflavone), or biologically-active derivatives
thereof. Also preferred are carotenoids, fish oils and omega-fatty acids, green tea, beta-carotene, carnitine, glucosamine, chondroitin sulfate, ginseng, ginko biloba, genistein, saw palmetto, curcumin, lycopene, Echinacea, lipoic acid, calcitonin, hyperforin, flavinoids, and Vitamins A, C and E.

The proliposomal preparations of the invention comprising one or a plurality of nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds, are preferably provided in a form that can be orally administered, including but not limited to syrups, elixirs, capsules, tablets, and emulsions. Preferred forms are tablets or capsules, most preferably comprising an enteric coating to prevent premature dissolution under the chemically harsh environment of the stomach. Enteric coatings are prepared as will be understood by one having skill in the art, and preferably include coatings including but not limited to eudragit and cellulose acetate phthalate.

In a preferred embodiment, the tablets or capsules of the invention comprise a protective coating between the enteric coating and the core of the capsule or tablet comprising the proliposomal preparations of the invention. In such embodiments, the protective coating is prepared as will be understood by one having skill in the art, and preferably include coatings including but not limited to hydroxypropyl methylcellulose, polyethylene glycol and ethylcellulose. In additional embodiments, the protective coating further comprises a plasticizing agent, including but not limited to triethylcitrate and polyvinyl pyrrolidone.

The tablets, capsules and other like embodiments of the proliposomal preparations and pharmaceutical compositions of the invention further advantageously comprise particle lubricants that minimize the tendency of the
granular proliposomal compositions to agglomerate. By "particle lubricant" as used herein is meant the class of materials used in the manufacturing of pharmaceutical tablets as lubricants to improve the flowability and prevent agglomeration of an active agent during the tableting process. Examples of particle lubricants include talc, lactose, corn starch, ethyl cellulose, fatty acid salts such as magnesium stearate, agar pectin, fatty acids such as stearic acid, gelatin and acacia.

The formulations, compositions and pharmaceutical compositions of the invention in preferred embodiments comprise a chitosan layer between the proliposomes and the enteric coating. As used herein, the term “chitosan” is used to specifically refer to poly(1,4-β-D-glycopyrano-amine), and to substituted derivatives and analogues thereof having substantially the same properties as the base polymer. Preferably, the chitosan of the invention is hydrolyzed, deacetylated chitin obtained from, inter alia, shrimp, crab or other crustacean shells. As used herein, the chitosan layer can be produced on the proliposome formulations of the invention by phase separation of chitosan from an aqueous solution by a counterion (termed simple coacervation; see Chandy and Sharma, 1992, Biomaterials 13: 949-952). Alternatively, the chitosan layer can be produced on the proliposome formulations of the invention by phase separation of chitosan from an aqueous solution by an oppositely-charged macromolecule (termed complex coacervation; see Polk et al., 1994, Aquacultural Engineer. 13: 311-323). In another embodiment, the chitosan layer can be produced on the proliposome formulations of the invention by emulsification of the chitosan and proliposomes in a non-solvent (see Akbuga and Durmaz, 1994, J. Intl. Pharmaceutics 111: 217-222; Jameela and Jayakrisnan, 1995, Biomaterials 16: 769-775). Chitosan preparations having varying degrees of
deacetylation or average molecular weight are useful in the practice of the invention (see, Chiuo et al., 2001, J. Microencapsul. 18: 613-625).

The invention specifically provides methods for preparing and administering the proliposomal compositions of the invention as disclosed in the Examples below, and pharmaceutical compositions comprising the proliposomal preparations of one or a plurality of nutraceuticals, such as polycyclic, aromatic, antioxidant or anti-inflammatory compounds.

Acceptable formulations preferably are nontoxic to recipients at the dosages and concentrations employed. Effective, non-toxic dosage ranges for nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds, are known in the art. For example, resveratrol is typically administered at dosages of up to 3g/kg/day in rats (Crowell et al., 2004, ToxSci Advance Access), and 4mg/kg/day in rabbits (Zou et al., 2000, Life Sci. 68: 153-163). The administered dose will be affected by the efficiency with which the formulations, compositions and pharmaceutical compositions of the invention deliver one or a plurality of nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds to inter alia the intestine for absorption. Determining a correct concentration of the one or plurality of particular nutraceuticals in the formulation is within the skill of one of skill in the art.

The pharmaceutical composition may contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine,
arginine or lysine); ascorbic acid, sodium sulfite or sodium hydrogen-sulfite; buffers
(such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids);
bulking agents (such as mannitol or glycine); chelating agents (such as
ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine,
polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin);
fillers; monosaccharides, disaccharides, and other carbohydrates (such as glucose,
mannose or dextrins); proteins (such as serum albumin, gelatin or
immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents;
hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight
polypeptides; salt-forming counterions (such as sodium); preservatives (such as
benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol,
methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide);
solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols
(such as mannitol or sorbitol); suspending agents; surfactants or wetting agents
(such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20 and
polysorbate 80, Triton, trimethamine, lecithin, cholesterol, or tyloxapol); stability
enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as
alkali metal halides, preferably sodium or potassium chloride, mannitol, or sorbitol);
delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. See, for
example, REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, (A.R. Gennaro,

Optimal pharmaceutical compositions can be determined by one skilled in
the art depending upon, for example, the desired dosage. See, for example,
REMINGTON'S PHARMACEUTICAL SCIENCES, Id. Such compositions may influence
the physical state, stability, rate of \textit{in vivo} release and rate of \textit{in vivo} clearance of the antibodies of the invention.

Pharmaceutical compositions can comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor. Pharmaceutical compositions of the invention may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (\textit{REMINGTON'S PHARMACEUTICAL SCIENCES, 16th}) in the form of a lyophilized cake or an aqueous solution.

Formulation components are present in concentrations that are acceptable for oral administration. Buffers are advantageously used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

The pharmaceutical compositions of the invention are preferably delivered through the digestive tract. The preparation of such pharmaceutically acceptable compositions is within the skill of the art. One or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds, are administered in this fashion may be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. A capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the one or plurality of nutraceuticals. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.
A pharmaceutical composition comprising one or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds, may involve an effective quantity thereof as disclosed herein or a compound identified in a screening method of the invention in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions may be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions are evident to those skilled in the art, including formulations involving one or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds. For example, sustained-release preparations may include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules, polyesters, hydrogels, polylactides (U.S. 3,773,919 and EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., 1983, Biopolymers 22: 547-556), poly (2-hydroxyethyl-methacrylate) (Langer et al., 1981, J. Biomed. Mater. Res. 15: 167-277) and Langer, 1982, Chem. Tech. 12: 98-105), ethylene vinyl acetate (Langer et al., id.) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained release compositions may also include liposomes, which can be prepared by any of several methods known in the art. See e.g., Eppstein et al., 1985, Proc. Natl. Acad. Sci. USA 82: 3688-3692; EP 036,676; EP 088,046 and EP 143,949.

The pharmaceutical composition to be used for in vivo administration
typically is sterile. In certain embodiments, this may be accomplished by filtration through sterile filtration membranes. In certain embodiments, where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. In certain embodiments, the composition for parenteral administration may be stored in lyophilized form or in a solution. In certain embodiments, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once pharmaceutical compositions of the invention has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations may be stored either in a ready-to-use form or in a form (e.g., lyophilized) that is reconstituted prior to administration.

The effective amount of a pharmaceutical composition of the invention to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments, will thus vary depending, in part, upon the molecule delivered, the indication for which the pharmaceutical composition is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. A clinician may titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. Typical dosages range from about 0.1 μg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In certain embodiments, the dosage may range from 0.1 μg/kg up to about 100 mg/kg.
or 1 µg/kg up to about 100 mg/kg; or 5 µg/kg up to about 100 mg/kg.

The dosing frequency will depend upon the pharmacokinetic parameters of a FoxM1B inhibitor disclosed herein or compound identified in a screening method of the invention in the formulation. For example, a clinician administers the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

Acceptable formulations preferably are nontoxic to recipients at the dosages and concentrations employed. Effective, non-toxic dosage ranges for nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds, are known in the art. For example, resveratrol is typically administered at dosages of up to 3g/kg/day in rats (Crowell et al., 2004, ToxSci Advance Access), and 4mg/kg/day in rabbits (Zou et al., 2000, Life Sci. 68: 153-163). The administered dose will be affected by the efficiency with which the formulations, compositions and pharmaceutical compositions of the invention deliver one or a plurality of nutraceuticals, particularly polycyclic, aromatic, antioxidant or anti-inflammatory compounds to *inter alia* the intestine for absorption. Determining a correct concentration of the one or plurality of particular nutraceuticals in the formulation is within the skill of one of skill in the art.

The pharmaceutical composition may contain formulation materials for
modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); ascorbic acid, sodium sulfite or sodium hydrogen-sulfite; buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose or dextrins); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20 and polysorbate 80, Triton, trimethamine, lecithin, cholesterol, or tyloxapol); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride, mannitol, or sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. See, for example, REMINGTON’S PHARMACEUTICAL SCIENCES, 18th Edition, (A.R. Gennaro,

Optimal pharmaceutical compositions can be determined by one skilled in the art depending upon, for example, the desired dosage. See, for example, REMINGTON'S PHARMACEUTICAL SCIENCES, Id. Such compositions may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the antibodies of the invention.

Pharmaceutical compositions can comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor. Pharmaceutical compositions of the invention may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (REMINGTON'S PHARMACEUTICAL SCIENCES, Id.) in the form of a lyophilized cake or an aqueous solution.

Formulation components are present in concentrations that are acceptable for oral administration. Buffers are advantageously used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

The pharmaceutical compositions of the invention are preferably delivered through the digestive tract. The preparation of such pharmaceutically acceptable compositions is within the skill of the art. One or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds, are administered in this fashion may be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. A capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-
systemic degradation is minimized. Additional agents can be included to facilitate absorption of the one or plurality of nutraceuticals. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

A pharmaceutical composition comprising one or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds, may involve an effective quantity thereof as disclosed herein or a compound identified in a screening method of the invention in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions may be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions are evident to those skilled in the art, including formulations involving one or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds. For example, sustained-release preparations may include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules, polyesters, hydrogels, polylactides (U.S. 3,773,919 and EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., 1983, Biopolymers 22: 547-556), poly (2-hydroxyethyl-methacrylate) (Langer et al., 1981, J. Biomed. Mater. Res. 15: 167-277) and Langer, 1982, Chem. Tech. 12: 98-105), ethylene vinyl acetate (Langer et al., id.) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained release
compositions most preferably comprise liposomes, which can be prepared by any of
several methods known in the art. See e.g., Eppstein et al., 1985, Proc. Natl. Acad.

The pharmaceutical composition to be used for in vivo administration
typically is sterile. In certain embodiments, this may be accomplished by filtration
through sterile filtration membranes. In certain embodiments, where the
composition is lyophilized, sterilization using this method may be conducted either
prior to or following lyophilization and reconstitution. In certain embodiments, the
composition for parenteral administration may be stored in lyophilized form or in a
solution. In certain embodiments, parenteral compositions generally are placed into
a container having a sterile access port, for example, an intravenous solution bag or
vial having a stopper pierceable by a hypodermic injection needle.

Once pharmaceutical compositions of the invention has been formulated, it
may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a
dehydrated or lyophilized powder. Such formulations may be stored either in a
ready-to-use form or in a form (e.g., lyophilized) that is reconstituted prior to
administration.

The effective amount of a pharmaceutical composition of the invention to be
employed therapeutically will depend, for example, upon the therapeutic context and
objectives. One skilled in the art will appreciate that the appropriate dosage levels
for treatment, according to certain embodiments, will thus vary depending, in part,
upon the molecule delivered, the indication for which the pharmaceutical
composition is being used, the route of administration, and the size (body weight,
body surface or organ size) and/or condition (the age and general health) of the
patient. A clinician may titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. Typical dosages range from about 0.1 μg/kg to up to about 100 mg/kg or more, depending on the factors mentioned above. In certain embodiments, the dosage may range from 0.1 μg/kg up to about 100 mg/kg; or 1 μg/kg up to about 100 mg/kg; or 5 μg/kg up to about 100 mg/kg.

The dosing frequency will depend upon the pharmacokinetic parameters of one or a plurality of nutraceuticals, particularly polycyclic, aromatic antioxidants or anti-inflammatory compounds according to the invention, or compound identified in a screening method of the invention in the formulation. For example, a clinician administers the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

Pharmaceutical compositions of the invention can be administered alone or in combination with other therapeutic agents.

Animals to be treated with the proliposomal preparations and pharmaceutical compositions of the invention are intended to include all vertebrate animals, preferably domesticated animals, such as cattle, horses, goats, sheep, fowl, fish, household pets, and others, as well as wild animals, and most preferably humans.

One advantage of orally-administered liposomal formulations over
parenterally-administered formulations is that oral administration reduces uptake of liposomes by the liver, thus reducing liver toxicity (which is a particular liability of parenterally-administered liposomal formulations). Oral formulations are targeted to deliver nutraceuticals, including polycyclic, aromatic, antioxidant or anti-inflammatory compounds, to the intestine, which is a large surface for absorption and results in slow release of the administered compound. Finally, oral administration avoids transport-mediated saturation of nutraceutical absorption, particularly with regard to polycyclic, aromatic, antioxidant or anti-inflammatory compounds of the invention.

The following Examples illustrate certain aspects of the above-described method and advantageous results. The following examples are shown by way of illustration and not by way of limitation.

**EXAMPLE 1**

Proliposomal formulations useful for oral administration were developed using an *in vitro* model system. Human Caco-2 cells (colon adenocarcinoma cells), grown on semipermeable filters, provide a simple and reliable *in vitro* model for studying drug transport across the intestinal mucosa. Caco-2 cells are recognized in the art for yielding useful predictions on oral absorption of new drug formulations.

1. **Preparation of proliposomal formulations**

   In order to assay the proliposomal tablets of the invention, glyburide (glybenclamide), an oral blood-glucose-lowering drug of the sulfonylurea class, was used as model drug, because uptake in the CaCo-2 system can be monitored by
measuring transport across monolayers formed by this cell line.

Proliposomal tablets were prepared as follows. The identities and amounts of each of the reagents used to prepare the tablets of the invention are shown in Table I. Phospholipids DMPC and DSPC were obtained from Avanti Polar Lipids (Alabaster, AL); glyburide, cholesterol, stearamine, dicetylphosphate and all tissue culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO); purified talc and anhydrous lactose were obtained from J.T. Baker (Phillipsburg, NJ) and Quest, Int’l. (Hoffman Estates, IL); chloroform, methanol and ethanol were obtained from Fisher Scientific (Fairlawn, N.J.); Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA; Accession No. HTB 37); and transwell culture chambers were obtained from Costar (Cambridge, MA).

Glyburide, lipid and cholesterol were dissolved at room temperature in 10mL chloroform. Lactose (25mg/tablet) was suspended in the organic mixture and the suspension evaporated to dryness at 60EC in a conventional coating pan (pan drying method). The solid residue was collected and sifted through a #60 mesh screen. The sifted residue was then mixed with Explotab® (3mg/tablet), lactose (50mg/tablet) and talc (2mg/tablet) and compressed into tablets using a Manesty B3B 16 station press. The tablets were then coated with a solution of hydroxypropyl methylcellulose in ethyl alcohol (3% w/v) containing triethyl citrate (15% of polymer weight) as a plasticizer. Eudragit L30 D-55 (7% w/w) was then applied on the coated tablets.

Table I provides a formulary for preparing proliposomal tablets according to the invention.

In alternative methods, proliposomal formulations can be prepared by
lyophilization. In these embodiments, mixtures of lipids and drug are prepared in aqueous solution and then sonicated, causing small unilamellar liposomes to form and resulting in an optically-clear solution. Such a solution is then freeze-dried and mixed with the other components of the tablets as described above. This method has the advantages that it can be performed in five steps, and avoids the use of organic solvents, which can be toxic, in preparing the formulation.

**TABLE I**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Quantity of each ingredient used (mg/tablet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyburide</td>
</tr>
<tr>
<td>DSPC/Neu</td>
<td>5.0</td>
</tr>
<tr>
<td>DSPC.Neu.Cho</td>
<td>5.0</td>
</tr>
<tr>
<td>DSPC/Pos</td>
<td>5.0</td>
</tr>
<tr>
<td>DSPC/Pos/Cho</td>
<td>5.0</td>
</tr>
<tr>
<td>DSPC/Neg</td>
<td>5.0</td>
</tr>
<tr>
<td>DSPC/Neg/Cho</td>
<td>5.0</td>
</tr>
<tr>
<td>DMPC/Neu</td>
<td>5.0</td>
</tr>
<tr>
<td>DMPC/Neu/Cho</td>
<td>5.0</td>
</tr>
<tr>
<td>DMPC/Pos/Cho</td>
<td>5.0</td>
</tr>
</tbody>
</table>

DSPC = distearylphosphatidylcholine  
DMPC = dimyristylphosphatidylcholine
STA = stearylamine (Pos: positively charged species)
CHO = cholesterol (Neu: neutral lipid)
DCP = dicetylphosphate (Neg: negatively charged species)

In other alternative methods, proliposomal formulations can be prepared by spray-drying. In these embodiments, mixtures of lipids and drug are prepared in aqueous solution. To such a mixture is added a surfactant such as Tween 80®, and then dried using a spray dryer. The resulting dried proliposomal preparation is mixed with the other components of the tablets as described above. This method has the advantages that it can be performed in five steps, is suitable for use with temperature-sensitive materials, and avoids the use of organic solvents, which can be toxic, in preparing the formulation.

In another embodiment of this alternative method, a mechanical mixer is used instead of using a surfactant. The mechanical mixer produces a proliposomal composition in the absence of a surfactant that can be spray-dried as described above. This embodiment is particularly advantageous because it avoids the use of both surfactants and organic solvents in preparing proliposomal formulations according to the invention.

2. **Chemical assays of reagents and proliposomal formulations**

The purity of the reagents used to make the proliposome tablets of the invention described herein was tested using differential scanning calorimetry. Samples were prepared by dissolving lipid with glyburide and cholesterol separately at a ratio of 1:1 (w/w) in an excess of chloroform. The organic layer was removed and thermograms obtained using a differential scanning calorimeter (TA Instruments, New Castle, DE, Model 2910). Each component was scanned both
individually and using a mixture comprising glyburide, lipid and cholesterol at a ratio of 1:1:1 (w:w:w). 2-5mg of sample was scanned at a rate of 20EC per minute over a suitable temperature range (25-225EC) in a hermetically-sealed aluminum pan. The peak transition temperatures of the dispersion were compared with the pure compounds. The results of these experiments are shown in Figures 1A through 1C.

Figure 1A shows a thermogram of DMPC alone compared with mixtures of DMPC and cholesterol (DMPC/CHOL), DMPC and glyburide (DMPC/GLYB) and DMPC, cholesterol and glyburide (DMPC/CHOL/GLYB). Peak transition temperatures are shown in the Figure. In contrast to the simple and easily-recognizable peak transition temperature obtained for DMPC, the mixtures are heterogeneous, having more than one localized peak region where a thermal transition occurs.

Figure 1B shows a thermogram of DSPC alone compared with mixtures of DSPC and cholesterol (DSPC/CHOL), DSPC and glyburide (DSPC/GLYB) and DSPC, cholesterol and glyburide (DSPC/CHOL/GLYB). Peak transition temperatures are shown in the Figure. A similar pattern is observed herein, where there is a simple and easily-recognizable peak transition temperature obtained for DSPC, but the mixtures are heterogeneous, having more than one localized peak region where a thermal transition occurs.

Thermograms were also obtained individually and in mixtures for glyburide and cholesterol, and these results are shown in Figure 1C. From these thermograms, it is evident that the presence of cholesterol acts as an “impurity” in the drug, lowering its melting point. The same effect is observed in mixtures of the drug and
lipid. In the presence of both cholesterol and lipid, the melting point of glyburide is further decreased, demonstrating a synergistic effect. These results also indicate that the amount of heat required to melt the drug in a pure state is far higher than the amount needed when the drug is combined with cholesterol or lipid. This explains the increased solubility of the drug when prepared in a solid dispersion of lipid and/or cholesterol.

Liposomes were reconstituted from proliposomal tablets by adding one tablet to 1mL phosphate buffered saline in a sterile glass vial. The tablet was allowed to stand at 37EC for 1 hour with shaking, which was sufficient to dissolve the tablet and reconstitute the liposomal preparation.

Reconstituted liposomes were characterized for size distribution by large-angle dynamic light scattering using a particle size analyzer (Brookhaven Instruments, Model BI-90). Each preparation was diluted with filtered saline to an appropriate concentration to achieve a medium viscosity of 0.089 centipoise and a medium relative refractive index of 1.332 at room temperature. Measurements obtained under these condition are shown in Table II. These results indicated that the particle size of the resulting liposomes varied both with the presence or absence of cholesterol and with the identity of the phospholipid component. The mean diameter of the liposomes was greater in neutral liposome embodiments than in charged liposome embodiments, and can be explained by the greater propensity of neutral liposomes to aggregate or fuse with one another.

Encapsulation efficiency, defined as the percentage of the glyburide encapsulated in liposomes, was determined using the protamine-induced aggregation method as described in Kulkarni et al. (1995, Pharm. Sci. 1: 359-362). Briefly, each
tablet was disintegrated in 1 mL of phosphate-buffered saline (PBS, pH 7.4) to give a concentration of 10 mg/mL of lipid. To 100:1L of the preparation, equal

**TABLE II**

<table>
<thead>
<tr>
<th>Formulation/Charge</th>
<th>Lipid Type</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSPC</td>
<td>DMPC</td>
</tr>
<tr>
<td>Neutral</td>
<td>1413</td>
<td>1825</td>
</tr>
<tr>
<td>Neutral/Cholesterol</td>
<td>1035</td>
<td>748</td>
</tr>
<tr>
<td>Positive</td>
<td>1059</td>
<td>N.D.</td>
</tr>
<tr>
<td>Positive/Cholesterol</td>
<td>867</td>
<td>629</td>
</tr>
<tr>
<td>Negative</td>
<td>3633</td>
<td>N.D.</td>
</tr>
<tr>
<td>Negative/Cholesterol</td>
<td>800</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D.: not determined

quantities of a protamine solution (50 mg/mL) in PBS was added and vortexed for about 1 min. The mixture was then incubated for about 12 hours at room temperature. After incubation, the mixture was centrifuged at about 16,000 x g for about 5 minutes. 100:1L of the supernatant was removed and the pellet was dissolved in about 1 mL of reagent-grade alcohol (95% ethanol) and sonicated for 5 minutes.

The quantity of glyburide in the pellet and the supernatant was determined by HPLC analysis using the Star® 9010 solvent system and Star 9095® variable-wavelength ultraviolet/visible spectrum spectrophotometric detector (Varian Associates, Walnut Creek, CA) and the data analyzed by a Dynamax®
MacIntegrator (Rainin Instrument Co., Woburn, MA). HPLC analysis was performed using a C18 column (Phenominex®) packed with 5μm particles and having dimensions of 250mm in length and an internal diameter of 4.6mm. The mobile phase was a solution of methanol in 0.1M phosphate buffer, pH 3.5 at a ratio of 75:25 by volume. Column flow rate was 1.0 mL/min and the output was scanned at a wavelength of 225nm.

The results of these characterization experiments are shown in Table III.

These results demonstrated that a slightly higher percentage of the drug was encapsulated in DMPC. These results are consistent with a slightly higher amount of the drug being encapsulated in "fluid" liposomes (i.e., those comprising DMPC) than liposomes in a gel state (i.e., those comprising DSPC) at 37EC.

**TABLE III**

**Drug Encapsulation Efficiency (%) ± s.d.**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lipid Type</th>
<th>DSPC</th>
<th>DMPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>81.6 ± 0.4</td>
<td>86.7 ± 2.7</td>
</tr>
<tr>
<td>Neutral</td>
<td></td>
<td>80.4 ± 0.6</td>
<td>88.8 ± 1.2</td>
</tr>
<tr>
<td>Neutral/Cholesterol</td>
<td></td>
<td>78.4 ± 0.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>81.0 ± 1.2</td>
<td>87.6 ± 0.6</td>
</tr>
<tr>
<td>Positive/Cholesterol</td>
<td></td>
<td>81.2 ± 0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>80.4 ± 0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Negative/Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D.: Not determined
EXAMPLE 2

Caco-2 cell cultures were prepared as monolayers on polycarbonate transwells having a membrane pore size of 4nm. Caco-2 cells were first grown in T-150 flasks (Falcon, Lincoln Park, NJ) at 37EC under an atmosphere of 5% CO2 and 95% air in Dulbecco’s modified Eagle’s medium (pH 7.2, Sigma Chemical Co., St. Louis, MO), with conventional supplements. The medium was changed every other day until the monolayers reached about 90% confluency. Media was removed and the cells were washed with Hank’s balanced salt solution (HBSS, Sigma). The cells were trypsinized by adding 0.5mL of a 0.25% trypsin solution containing 1mM EDTA to each flask and incubating the monolayers for 10 min at 37EC. The separated cells were removed from the flasks and collected into centrifuge tubes, centrifuged at 200 x g for 10 min, the supernatant removed and the pellet resuspended in a sufficient amount of Dulbecco’s modified Eagle medium to yield a suspension that would produce about 60,000 cells/cm² on plating. The Caco-2 cells were then seeded into Transwell semipermeable membrane inserts having 4:m pore size. In the transwells, media was changed every other day until the cells were used for the transport studies described below.

Caco-2 cell cultures on transwell membranes prepared as described above were used for transport studies about 17 days after plating. Proliposome tablets were dissolved as described above by incubation for 1h with shaking at 37EC in 2mL HBSS. As a control, pure glyburide treated with chloroform was compressed into tablet form with lactose and Explotab®; all controls were treated exactly as experimental.
The medium from the transwell plates was gently removed using a micropipette. 0.5 mL of the reconstituted liposomal suspension was gently added to the donor compartment of the transwell and 1.5 mL of HBSS was added to the receiver compartment. 100:1 of FITC-Dextran was then added to the donor compartment to a final concentration of 10:g/mL of FITC-Dextran in the donor side. FITC-Dextran was used as a marker to test for the presence of leaks, if any, on the monolayers covering the semipermeable transwell membranes. Samples (300:L) were carefully withdrawn from the receiver side at 50, 120, 180, 240, 300 minutes after addition, and the receiver side was replenished with 300:L of fresh HBSS each time the sample was taken. Cells were incubated at 37EC in a 5% CO2/95% air atmosphere at all times during these assays. Sampling was done under aseptic conditions in a laminar air-flow hood.

The amount of glyburide transported during each sampling interval was determined by injecting 90:L of the sample onto the HPLC system described above in Example 1 and peak areas were recorded. These experiments were performed in triplicate and the average of the results was reported. The results of the experiments are shown in Figures 2 through 5.

Figure 2 shows the results of glyburide transit across Caco-2 cell monolayers in formulations containing distearilphosphatidylcholine (DSPC). Control experiments performed in the absence of DSPC had a flow rate of almost 1:g/hr X cm². Formulations of glyburide with DSPC (a “neutral” lipid at physiological pH) showed a similar level of flux across the monolayer, although the addition of cholesterol to these formulations increased the flux about two-fold. Formulations of
glyburide with negatively-charged lipid, on the other hand, in either the presence or absence of cholesterol were transported across the monolayer at a lower rate. In contrast, formulations of glyburide with positively-charged lipid were transported across the membranes at a rate about fourfold higher than control, and the addition of cholesterol increased this to a rate of about fivefold higher than control.

Figure 3 shows the results of parallel experiments using dimyristolphosphatidylcholine (DMPC) as the lipid component. A similar pattern of glyburide flux was seen in these experiments; however, the degree of enhancement of transit across the Caco-2 cell monolayer was much higher for formulations containing DMPC. For example, glyburide formulations containing DMPC and positively-charged lipid had a transit rate almost thirty-fold higher than control. Formulations of neutral lipid were elevated to a lesser degree; in the presence of cholesterol such formulations had a transit rate about eightfold higher than control, and in the absence of cholesterol this rate was about fivefold higher than control.

Figures 4 and 5 show the cumulative amount of transported glyburide using DSPC- and DMPC-containing formulations over a five hour period. Figure 4 shows DSPC-containing formulations, wherein the highest accumulation levels were achieve with glyburide formulations containing DSPC and positively-charged lipid (about 27:g). Similar formulations additionally containing cholesterol had lower total amounts (about 13:g). DSPC formulations containing neutral lipid and cholesterol showed slower kinetics but achieved essentially the same total accumulation as DSPC/positive lipid/cholesterol formulations. Formulations containing DSPC and neutral lipids in the absence of cholesterol showed the same total accumulation as control (about 2.5:g), while DSPC formulations with
negatively-charged lipid (in the presence or absence of cholesterol) showed lower total accumulation amounts.

Figure 5 shows the results of similar experiments performed with DMPC formulations. Total accumulation levels were noticeably higher than control only for formulations containing DMPC, positively-charged lipid and cholesterol (about 34:9), while DMPC formulations with neutral lipid (in the presence or absence of cholesterol) resulted in total accumulation at levels equivalent to control (about 2-5:9).

These results demonstrated that liposomes can be successfully prepared for oral administration in the form of enteric-coated proliposome tablets. The presence of cholesterol reduces the particle size of the formulation. Proliposomes provide a stable system of production of liposomes for oral administration. Degradation of proliposome contents of the tablet in the stomach can be effectively avoided by administering the proliposomes as enteric-coated tablets. Enhanced transport of glyburide across Caco-2 cells was observed with such liposomal formulations.

Although the transport of glyburide with DMPC formulations is higher than transport in the DSPC formulation in vitro, DSPC formulations are better suited for in vivo conditions because of the rigidity and increased stability of the membrane against the attack of bile salts and enzymes of the intestine. Since in vitro transport across Caco-2 cells is an indication of bioavailability, an increased transport with the liposome formulation suggests an increased bioavailability of compounds that are poorly absorbed otherwise. For example, using a suitable polymer coating for the proliposomal tablets of the invention, colonic delivery of drugs, especially peptides may be possible.
Proliposomes are ideally suited for lipophilic compounds, since the majority of such a polycyclic, aromatic, antioxidant or anti-inflammatory compound will partition into the lipid phase. These results also have implications for developing formulations that stabilize the encapsulated drug.

EXAMPLE 3

Producing a Chitosan Layer

A 3-5% chitosan layer is produced on the proliposomal formulations of the invention as follows. Chitin is prepared according to conventional methods, for example, as disclosed in Chen et al. (1994, Carbohydrate Polym. 24: 41-46), and chitosan is prepared from chitin, for example, as disclosed in Yamamoto and Amaiike (1997, Macromolecules 30: 3936-3937). Proliposomes are prepared as described in Example 1 above. A 3% or 5% chitosan solution is prepared in 1% acetic acid solution, and about 2mg of the proliposomes of the invention are poured into the chitosan solution and stirred until completely mixed. Spherical gels are produced from this mixture by adding a solution of 1N sodium hydroxide/26% ethyl alcohol using a small-bore (about 27Ga) syringe and incubated for about 30 min. The chitosan-encased proliposomes are removed and rinsed in distilled water under neutral conditions and then dried for at least 24 h at room temperature.

EXAMPLE 4

Using the methods set forth herein, the following formulations are prepared for oral administration:

• Calcitonin or resveratrol + excipients + lipids + 3% Ethyl Cellulose coating
+ 3% Chitosan coating + 3% enteric coating

- 3% Calcitonin or 3% resveratrol + excipients + lipids + 3% Ethyl Cellulose coating + enteric coating + Chitosan coating

5

- Calcitonin or resveratrol + excipients + lipids + 5% Ethyl Cellulose coating + 5% enteric coating

10

- Calcitonin or resveratrol + excipients + lipids + 5% Ethyl Cellulose coating + 5% Chitosan coating

- Calcitonin or resveratrol + excipients + 3% Ethyl Cellulose coating + 3% Chitosan coating + 3% enteric coating

- Calcitonin or resveratrol + excipients + 3% Ethyl Cellulose + 3% enteric coating + 3% Chitosan coating

15

- Calcitonin or resveratrol + excipients + 5% Ethyl Cellulose coating + 5% enteric coating

- Calcitonin or resveratrol + excipients + 5% Ethyl Cellulose coating + 5% Chitosan coating

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.
What is claimed is:

1. A pharmaceutical composition comprising a proliposomal preparation of a nutraceutical in a capsule or tablet comprising an enteric coating, and a protective coating, wherein the proliposomal preparation consisting essentially of one, two or three lipids, a primary aliphatic amine and cholesterol, and wherein the protective coating comprises chitosan.

2. A pharmaceutical composition according to claim 1 wherein the nutraceutical is an antioxidant or anti-inflammatory compound,

3. A pharmaceutical composition of claim 2 wherein the antioxidant or anti-inflammatory compound is a polycyclic aromatic antioxidant or anti-inflammatory compound.

4. A pharmaceutical composition according to claims 3 wherein the polycyclic aromatic antioxidant or anti-inflammatory compound is cis-stilbene; trans-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy cis-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy trans-stilbene; carboxylated derivatives of cis-stilbene or trans-stilbene; halogenated derivatives of cis-stilbene or trans-stilbene; resveratrol (trans-3,5,4'-trihydroxystilbene); chrysin (5,7-dihydroxyflavone) or quercetin (3,3',4',5,7-pentahydroxyflavone), or biologically-active derivatives thereof.

5. A pharmaceutical composition according to claims 1, 2, 3 or 4 wherein the enteric coating is cellulose acetate phthalate or a poly(acrylate, methacrylate) copolymer.

6. A pharmaceutical composition according to claims 1, 2, 3, 4 or 5 further comprising a sustained-release coating between the chitosan layer and the proliposomes, wherein the sustained-release coating is hydroxypropyl methylcellulose, polyethylene glycol or ethylcellulose.

7. A pharmaceutical composition according to claims 1, 2, 3, 4, 5 or 6 wherein the primary aliphatic amine is stearylamine.

8. A pharmaceutical composition according to claims 1, 2, 3, 4, 5, 6, or
7 wherein the lipid is a phospholipid.

9. A pharmaceutical composition according to claim 6 wherein the sustained-release coating further comprises a plasticizer.

10. A pharmaceutical composition according to claim 9 wherein the plasticizer is triethylcitrate or polyvinyl pyrrolidone.

11. A pharmaceutical composition according to claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 further comprising a particle lubricant that is talc, lactose, corn starch, ethyl cellulose, fatty acids or salts thereof, agar, pectin, gelatin or acacia.

12. A pharmaceutical composition according to claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 wherein the nutraceutical is resveratrol, quercetin, chrysin or calcitonin.

13. A pharmaceutical composition according to claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 wherein the lipid is a phosphatidylcholine, a phosphatidylethanolamine, sphingosine, or ceramide.

14. A pharmaceutical composition according to claim 13 wherein the phosphatidylcholine is distearlyphosphatidylcholine, dimyristylphosphatidylcholine or a mixture thereof.

15. A method for increasing the bioavailability of a nutraceutical, said method comprising orally administering to an animal in need thereof a proliposomal preparation of the nutraceutical in a capsule or tablet comprising an enteric coating, and a protective coating in between the proliposomal preparation and the enteric coating, wherein the proliposomal preparation consisting essentially of a lipid, a primary aliphatic amine and cholesterol, and wherein the protective coating comprises chitosan.

16. A method according to claim 1 wherein the nutraceutical is an antioxidant or anti-inflammatory compound.

17. A method according to according to claim 16 wherein the antioxidant or anti-inflammatory compound is a polycyclic aromatic antioxidant or anti-inflammatory compound.
18. A pharmacological composition according to claims 17 wherein the polycyclic aromatic antioxidant or anti-inflammatory compound is cis-stilbene; trans-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy cis-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy trans-stilbene; carboxylated derivatives of cis-stilbene or trans-stilbene; halogenated derivatives of cis-stilbene or trans-stilbene; resveratrol (trans-3,5,4'-trihydroxystilbene); chrysin (5,7-dihydroxyflavone) or quercetin (3,3',4',5,7-pentahydroxyflavone), or biologically-active derivatives thereof.

19. A method according to claims 15, 16, 17 or 18 wherein the animal is a human.

20. A method according to claims 15 wherein the nutraceutical is resveratrol, quercetin, chrysin or calcitonin.

21. A method according to claims 15, 16, 17, 18, 19 or 20 wherein the enteric coating is cellulose acetate phthalate or a poly(acrylate, methacrylate) copolymer.

22. A method according to claims 15, 16, 17, 18, 19, 20 or 21 wherein the proliposomal preparation further comprises a sustained-release coating between the chitosan layer and the proliposomes, wherein the sustained-release coating is hydroxypropyl methylcellulose, polyethylene glycol or ethylcellulose.

23. A method according to claims 15, 16, 17, 18, 19, 20, 21 or 22 wherein the primary aliphatic amine is stearylamine.

24. A method according to claims 15, 16, 17, 18, 19, 20, 21, 22 or 23 wherein the lipid is a phospholipid.

25. A method according to claims 22 wherein the sustained-release coating further comprises a plasticizer.

26. A method according to claim 25 wherein the plasticizer is triethylenecitrate or polyvinyl pyrrolidine.

27. A method according to claims 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26, wherein the proliposomal preparation further comprises a particle lubricant.
that is talc, lactose, corn starch, ethyl cellulose, fatty acids or salts thereof, agar, pectin, gelatin or acacia.

28. A method according to claims 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or 27 wherein the lipid is a phosphatidylcholine, a phosphatidylethanolamine, sphingosine, or ceramide.

29. A method according to claims 28 wherein the phosphatidylcholine is distearylphosphatidylcholine, dimyristylphosphatidylcholine or a mixture thereof.

30. A method of preventing coronary heart disease, myocardial infarction, ischemia, stroke, thrombosis, pulmonary embolism, or cancer, said method comprising administering a pharmaceutical composition of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 to human in need thereof.

31. A method for delivering a nutraceutical to the intestine or colon, said method comprising orally administering to an animal in need thereof a proliposomal preparation of a nutraceutical in a tablet comprising an enteric coating, and a protective coating in between the proliposomal preparation and the enteric coating, wherein the proliposomal preparation consisting essentially of a lipid, a primary aliphatic amine and cholesterol, and wherein the protective coating comprises chitosan.

32. A method according to claim 31, wherein nutraceutical is an antioxidant or anti-inflammatory compound.

33. A method according to claim 32, wherein the antioxidant or anti-inflammatory compound is a polycyclic aromatic antioxidant or anti-inflammatory compound.

34. A method according to claim 33 wherein the polycyclic aromatic antioxidant or anti-inflammatory compound is cis-stilbene; trans-stilbene; 3, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy cis-stilbene; 3-, 4-, or 4'-hydroxy, or (3,4')- or (4',5)-dihydroxy trans-stilbene; carboxylated derivatives of cis-stilbene or trans-stilbene; halogenated derivatives of cis-stilbene or trans-stilbene; resveratrol (trans-3,5,4'-trihydroxy stilbene); chrysins (5,7-dihydroxyflavone) or quercetin (3,3',4',5,7-pentahydroxyflavone), or biologically-active derivatives thereof.
36. A method according to claims 31, 32, 33 or 34 wherein the animal is a human.

37. A method according to claim 31 wherein the nutraceutical is resveratrol, quercetin, chrysin or calcitonin.

38. A method according to claims 31, 32, 33, 34, 35, 36 or 37 wherein the enteric coating is cellulose acetate phthalate or a poly(acrylate, methacrylate) copolymer.

39. A method according to claims 31, 32, 33, 34, 35, 36, 37, or 38, wherein the proliposomal preparation further comprises a sustained-release coating between the chitosan layer and the proliposomes, wherein the sustained-release coating is hydroxypropyl methylcellulose, polyethylene glycol or ethylcellulose.

40. A method according to claims 31, 32, 33, 34, 35, 36, 37, 38, or 39, wherein the primary aliphatic amine is stearylamine.

41. A method according to claims 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 wherein the lipid is a phospholipid.

42. A method according to claim 39 wherein the sustained-release coating further comprises a plasticizer.

43. A method according to claim 42 wherein the plasticizer is triethylcitrate or polyvinyl pyrrolidine.

44. A method according to claims 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, or 43 wherein the proliposomal preparation further comprises a particle lubricant that is talc, lactose, corn starch, ethyl cellulose, fatty acids or salts thereof, agar, pectin, gelatin or acacia.

45. A method according to claim 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44 wherein the lipid is a phosphatidylcholine, a phosphatidylethanolamine, sphingosine, or ceramide.

46. A method according to claim 45, wherein the phosphatidylcholine is distearylphosphatidylcholine, dimyristylphosphatidylcholine or a mixture thereof.

47. A pharmaceutical composition according to claims 1, 2, 3, 4, 5, 6, 7, - 47 -
8, 9, 10, 11, 12, 13 or 14, wherein the protective coating is in between the proliposomal preparation and the enteric coating.
NOT TO BE TAKEN INTO CONSIDERATION FOR THE PURPOSES OF INTERNATIONAL PROCESSING
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**INTERNATIONAL SEARCH REPORT**

**PCT/US2004/00781**

**A. CLASSIFICATION OF SUBJECT MATTER**


According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>X</td>
<td>WO 03/009261 A (ORADEL MEDICAL LTD; YATVIN, MILTON, B) 4 December 2003 (2003-12-04) page 7, line 12 - page 8, line 14 page 14 - page 27; examples 1-3</td>
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<td>WO 02/13782 A (HYUNDAI PHARMACEUTICAL IND. CO., LTD; KIM, HACK-JOO; AN, HEUNG-MAN; CH) 21 February 2002 (2002-02-21) abstract page 11; example 1 page 14 - page 16; example 5 claims 1-5</td>
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<td>WO 03/059288 A (ENZREL, INC; YATVIN, MILTON, B) 24 July 2003 (2003-07-24) the whole document</td>
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

**A** document defining the general state of the art which is not considered to be of particular relevance

**E** earlier document but published on or after the International filing date

**L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

**O** document referring to an oral disclosure, use, exhibition or other means

**P** document published prior to the international filing date but later than the priority date claimed

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other documents or the invention is obvious from such document

* member of the same patent family

**Data of the actual completion of the international search**

4 August 2005

**Date of mailing of the international search report**

19/08/2005

**Name and mailing address of the ISA**

European Patent Office, P.B. 5818 Patentisset 2 NL – 2280 HV Rijswijk
Tel. (431-70) 540-0040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016

**Authorized officer**

Muller, S
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<td>US 6 761 901 B1 (BETAGERI GURU V ET AL) 13 July 2004 (2004-07-13) column 6 - column 9; example 1 claims 1-29</td>
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# INTERNATIONAL SEARCH REPORT

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<td>1.</td>
<td><strong>X</strong> Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:</td>
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<td>Although claims 15-46 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.</td>
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<td>3.</td>
<td><strong>☐</strong> Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
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<td>2.</td>
<td><strong>☐</strong> As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.</td>
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<td>3.</td>
<td><strong>☐</strong> As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:</td>
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<td>4.</td>
<td><strong>☐</strong> No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:</td>
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| Remark on Protest | □ The additional search fees were accompanied by the applicant's protest. |
|                  | □ No protest accompanied the payment of additional search fees. |

Form PCT/SA/210 (continuation of first sheet (2)) (January 2004)
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