17α–DIHYDROEQUILENIN FOR USE AS A MEDICAL ANTIOXIDANT

BACKGROUND OF THE INVENTION

Biologically generated free radicals have been implicated in a large number of disease states. The survival of aerobic organisms in an oxygen environment involves a complicated interplay between the biological generation of these very reactive chemical species and the ability of the organism to control them (Del Maestro RF, Acta Phy Scan Suppl. 492:153-68 (1980)). This interplay between the host organism and biologically generated free radicals results in profound biochemical alterations which culminate in cellular injury and death of the organism. The accumulated products of free radical reactions result in some of the large number of disease conditions which have been suggested to result, in part, from cellular injury induced by an increased flux of intracellular free radicals. These include, but are not limited to cancers, cardiovascular disease, central nervous system disorders, bone disease, aging, Alzheimer's dementia, inflammatory disorders, rheumatoid arthritis, autoimmune diseases, respiratory distress and emphysema.

The association of free radical damage with many disease states is well documented and many cellular constituents, including enzymes, ion channels, structural proteins and membrane lipids are potential targets for reactive free radical species (Rice-Evans C, Mol Aspects of Med 13(1):1-111 (1992)). The antioxidant status at the appropriate site will limit the damage. Free radical reaction with these potential targets may compromise a range of cellular functions leading to pathological change and ultimately cell death. The antioxidant status at the potential reaction site will limit damage. Antioxidants play an important role in protecting DNA, proteins (including lipoproteins) and membrane lipids against oxidative damage.

There is strong evidence that free radical damage contributes to the etiology of many chronic health problems. For most human diseases, oxidant formation from endogenous sources is secondary to the initial disease process, but oxidative damage exacerbates the primary lesion. For example, reperfusion injury can be defined as the damage that occurs to an organ during the resumption of blood flow following an episode of ischaemia. Oxygen restoration, although necessary, causes increased oxidant formation in the damaged tissue and temporarily worsens the injury (Uraizee A,

Circulation 75(6):1237-1248 (1987)). The decline in the antioxidant defenses in the hypoxic myocardium followed by an increase in lipid peroxidation upon reoxygenation was documented by Guanieri (Biochim-Biophys-ACTA 718(2):157-164 (1982)). In reperfusion injury, the inflammatory response at the site of injury on the endothelium after the ischemic insult generates superoxide from adhesion and activation of neutrophils. In a number of different clinical conditions, the production of oxygen free radicals in the liver is also increased. In viral hepatitis and in chronic active hepatitis, a high number of stimulated macrophages accumulate in the liver, and they produce free radicals. A large number of toxic chemicals cause toxic liver injury, due to increased free radical generation in the liver, frequently mediated by the cytochrome P-450. It can be concluded that hydroxyl radical formation catalyzed by iron released from ferritin is a mechanism incidental to many liver diseases (Lee WM, N Eng J of Med ; Review P.1118 (1995)).

Oxidation and the use of antioxidants is also important for the treatment of numerous inflammatory disease states. Rheumatoid arthritis (RA) is the most common chronic inflammatory disease. Epidemiological studies reveal a prevalence rate of classical and definite RA between 0.3 and 1.5 percent. Joint disease with chronic persistent inflammation is accompanied by the formation of H_2O_2 in the inflamed rheumatoid joint. During inflammation, oxygen free radicals are also produced, especially by polymorphonuclear leukocytes (PMN) and macrophages. In any chronic or acute inflammatory disease, PMN and macrophages will produce both O_2^- and H_2O_2. Tuberculosis, psoriasis, systemic lupus erythematosus, other autoimmune diseases, and adult respiratory distress syndrome can also be mentioned as inflammatory diseases with oxidation as a contributor, and many others can be added to this list.

The generation of oxygen radicals and the process of lipid peroxidation have also become a focus of attention for investigators in the fields of central nervous system (CNS) trauma and stroke (e.g., ischemia). Numerous studies have provided considerable support for the occurrence of free radical and lipid peroxidation reactions in the injured or ischemic CNS (Hall ED, J-Neurotrauma 9(Suppl. 1):S165-S172 (1992)).

Antioxidants have been suggested to be protective against breast cancer and other cancers including those of the brain and liver, as well as to protect against

cardiovascular disease and osteoporosis (Wiseman H, Free Radical Res 21(3):187-94 (1994)). They have been demonstrated to protect model and cellular membranes including the nuclear membrane against potentially carcinogenic free radical intermediates and the products of lipid peroxidation. Severe complications associated with atherosclerosis and its common incidence have focused attention on prevention and therapy of this vascular disease state, possibly through their ability to protect low density lipoproteins (LDL) against oxidative damage (Steinberg D, N Engl J of Med 14:915-924 (1989)).

DESCRIPTION OF THE INVENTION

In accordance with this invention, there is provided a method of treating or inhibiting free radical induced disease states by administering an antioxidant amount of 17α-dihydroequilenin or its pharmaceutically acceptable salt of the 3-sulfate ester, to a mammal in need thereof. As a corollary of that process, this invention provides a process for treating free radical reactions with enzymes, ion channels, structural proteins and membrane lipids in a mammal, which comprises administering 17α-dihydroequilenin or a pharmaceutically acceptable sulfate ester salt thereof, as a sacrificial substrate, in an amount sufficient to selectively react with and inhibit free radical reaction with the patients enzymes, ion channels, structural proteins or membrane lipids. Specific situations in which antioxidant therapy is indicated to be warranted are with cancers, central nervous system disorders, bone disease, aging, inflammatory disorders, peripheral vascular disease, rheumatoid arthritis, autoimmune diseases, respiratory distress, emphysema, prevention of reperfusion injury, viral hepatitis, chronic active hepatitis, tuberculosis, psoriasis, systemic lupus erythematosus, adult respiratory distress syndrome, central nervous system trauma and stroke.

As used in accordance with this invention, treating covers treatment of an existing condition, ameliorating the condition, or providing palliation of the condition and inhibiting includes inhibiting or preventing the progress or development of the condition.

Pharmaceutically acceptable salts of 17α-dihydroequilenin 3-sulfate ester include, but are not limited to, the alkali metal salts, alkaline earth metal salts, ammonium salts, alkylamine salts containing 1-6 carbon atoms or dialkylamine salts containing 1-6 carbon atoms in each alkyl group.

The antioxidant properties of 17α-dihydroequilenin were established in a standard pharmacological test procedure that measured the ability to inhibit the formation of oxidatively modified low density lipoprotein (LDL) induced by exposure to either Cu++ ions or cultured endothelial cells (Parthasarathy S, Proc Natl Acad Sci USA 86:1046-1050 (1989)) by the TBARS (thiobarbituric acid reactive substances) method for analysis of free aldehydes (Yagi K., Biochem Med 15:212-216 (1976)).

The results obtained in this standard pharmacological test procedure demonstrate that 17α-dihydroequilenin is a potent inhibitor of LDL oxidation, inhibiting the process by up to 100%. IC_{50}s of 0.17 μM and 0.065 μM were obtained in the Cu++ mediated and the porcine aortic endothelial cell mediated oxidations, respectively. By comparison, an IC_{50} of 0.56 μM was obtained for estrone in the porcine aortic endothelial cell mediated oxidation test procedure. It was also demonstrated in this test procedure that 17α-dihydroequilenin is a potent inhibitor of HDL and plasma oxidation. IC_{50}s of 0.065 μM and 0.07 μM were obtained, respectively.

Antioxidant properties of 17α-dihydroequilenin was also assesed for its effect on the kinetics involved in the oxidation of LDL, HDL, and plasma that occurs in the presence of Cu++, a standard technique that has been used to induce LDL modification (Esterbauer H, Ann NY Acad Sci 1989;570:254-267, Huber LA, Free Rad Res Comms 1990;8:167-173, Vossen Rcrm, Lipids 1993;8:857-861, Jialal I, J Lipid Res 1992;33:899-906). The formation of conjugated dienes, a major initial lipid peroxidation product, was followed spectrophotometrically. The following parameters were assessed: the lag phase or T_{min}: the time it takes for oxidation to begin; T_{50}: the time it takes for 50% of the conjugated dienes to form; and T_{max}: the time it takes to reach maximum oxidation.

17α-Dihydroequilenin when tested at a concentration of 25nM was able to extend the lag phase in the formation of conjugated dienes in human LDL by 28%. T_{50} and T_{max} were extended by 31% and 30%, respectively. In the same assay, estrone at 25 nM increased all parameters by only 7-10%. 17α-Dihydroequilenin at 25 nM had a marked inhibitory effect on the formation of conjugated dienes in human HDL. It extended the lag phase by 299%. T_{50} and T_{max} were extended by 134% and 118%, respectively. Estrone at the same concentration effected the formation of conjugated dienes in HDL by extending the lag phase by 20% , T_{50} by 38% and T_{max} by 46%.

17α-Dihydroequilenin at 25 nM had no effect on the lag phase in the formation of conjuagted dienes in human plasma. However, it did extend the T_{50} and T_{max} by 19% and 32%, respectively, indicating that although it did not effect the initiation of oxidation it has slowed its rate. Estrone in this system also had no effect on the lag phase, and only a 5% and 10% effect on its T_{50} and T_{max}, repectively.

To further demonstrate that the antioxidant properties of 17α-dihydroequilenin, two additional standard pharmacological test procedures were conducted using cells in culture. In the first test procedure, radiolabeled-LDL (^{125}I-LDL) (McFarlane AS, In: Munro HN, Allison JB, eds. Mammalian Protein metabolism, Vol. 1. New York: Academic Press 297-341 (1964)) was modified by exposure to Cu++ in the presence and absence of 17α-dihydroequilenin. Next, J774 macrophages, which express scavenger lipoprotein receptors which bind oxidatively modified-LDL, were exposed to the treated ^{125}I-LDL. The results of this experiment demonstrate that binding of the Cu++-treated-LDL that was oxidized in the presence of 17α-dihydroequilenin was reduced by 70% and 41% (2.5 and 0.25μM 17 α-dihydroequilenin respectively). By comparison, the same concentrations of estrone reduced the binding of LDL that was oxidized by 39% and 0%, respectively. Since binding and metabolism of oxidized LDL by macrophages is though to contribute strongly to the development of foam cells and therefore, atherosclerotic plaque, this effect of reducing LDL oxidation and subsequent binding to scavenger receptors is thought to be of significant benefit.

In the second test procedure, porcine aortic endothelial cells (PAEC) were exposed to LDL that had been modified as above, by exposure to Cu++ in the presence and absence of 17α-dihydroequilenin. Oxidized LDL has been demonstrated to be cytotoxic to endothelial cells, and this process has also been strongly implicated in the atherogenic process. Subsequent to a 24 hr incubation of the cells with the treated LDL, an MTT assay was performed to assess cytotoxicity (Hansen MB, J Immu Methods 119:203-210 (1989)). This test procedure assesses the percent of cells that are viable (live) in a given assay. In the assay, following exposure to 25 ug/ml LDL oxidized in the absence of compound, only 2% of the cells remained viable. In contrast, the percent live cells following exposure to LDL Cu++ treated in the presence of 17 α-dihydroequilenin (0.25μM) was 45% or greater. Other compounds tested in this same assay had minimal effects on protection of PACE (17β-estradiol = 11% living; Equilin = 4% living; Estrone = 37% living. The results of this test procedure demonstrate that LDL modified in the presence of 17α-dihydroequilenin was not

cytotoxic, and therefore, the data is in agreement with the inhibition of oxidative modification by 17α-dihydroequilenin as demonstrated by the TBARS method above.

Based on the results obtained in these test procedures, 17α-dihydroequilenin and the pharmaceutically acceptable salts of its sulfate ester, such as the alkali metal salts, alkaline earth metal salts, ammonium salts, alkylamine salts containing 1-6 carbon atoms, or dialkylamine salts containing 1-6 carbon atoms in each alkyl group, are therefore useful as antioxidants, and in the treatment or inhibition of free radical induced disease states.

The antioxidants of this invention can be formulated neat or with a pharmaceutical carrier for administration, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmacological practice. The pharmaceutical carrier may be solid or liquid.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium

carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, lethicins, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compounds of this invention can also be administered orally either in liquid or solid composition form.

The antioxidants of this invention may be administered rectally in the form of a conventional suppository. For administration by intranasal or intrabronchial inhalation or insufflation, the antioxidants of this invention may be formulated into an aqueous or partially aqueous solution, which can then be utilized in the form of an aerosol. The compounds of this invention may also be administered transdermally through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semipermiable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

In addition, the antioxidants of this invention may be employed as a solution, cream, or lotion by formulation with pharmaceutically acceptable vehicles containing 0.1 - 5 percent, preferably 2%, of active compound which may be administered to a fungally affected area.

The dosage requirements vary with the particular compositions employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Based on the results obtained in the standard pharmacological test procedures, projected daily dosages of active compound would be 0.02 μg/kg - 500 μg/kg. Treatment will generally be initiated with small dosages less than the optimum dose of the compound. Thereafter the dosage is increased until the optimum effect under the circumstances is reached; precise dosages for oral, parenteral, nasal, or intrabronchial administration will be determined by the administering physician based on experience with the individual subject treated. Preferably, the pharmaceutical composition is in unit dosage form, e.g. as tablets or capsules. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form.

WHAT IS CLAIMED IS:

1. A method of inhibiting or treating free radical induced disease states by administering an antioxidant amount of 17α-dihydroequilenin or a pharmaceutically acceptable salt of 17α-dihydroequilenin-3-sulfate ester, to a mammal in need thereof.

2. The method of claim 1 wherein the pharmaceutically acceptable salt of the 3-sulfate ester is an alkali metal salt, alkaline earth metal salt, ammonium salt, alkylamine saltcontaining 1-6 carbon atoms, or dialkylamine salt containing 1-6 carbon atoms in each alkyl group.

3. A method of inhibiting free radical reactions with enzymes, ion channels, structural proteins and membrane lipids in a mammal in need of such treatment, which comprises administering 17α-dihydroequilenin or a pharmaceutically acceptable salt of 17α-dihydroequilenin-3-sulfate ester, as a sacrificial substrate, in an amount sufficient to selectively react with and inhibit free radical reaction said mammals enzymes, ion channels, structural proteins or membrane lipids.

4. A method of inhibiting endogenous free radical involvement in the development of cancers, central nervous system disorders, Alzheimer's disease, bone disease, aging, inflammatory disorders, peripheral vascular disease, rheumatoid arthritis, autoimmune diseases, respiratory distress, emphysema, prevention of reperfusion injury, viral hepatitis, chronic active hepatitis, tuberculosis, psoriasis, systemic lupus erythematosus, adult respiratory distress syndrome, central nervous system trauma and stroke, or injury during reperfusion procedures which comprises administering 17 α-dihydroequilenin or a pharmaceutically acceptable salt of 17α-dihydroequilenin-3-sulfate ester.

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