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Prospects for introducing deferiprone as potent pharmaceutical antioxidant

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1. ABSTRACT

Free radical formation is primarily initiated from metal catalytic centers involving iron and copper. Under certain conditions, free radical reactions can lead to free radical cascades and oxidative stress, which can cause biomolecular, cellular and tissue damage (FRD). The use of natural antioxidants to prevent FRD is in most cases not effective. Many chelators have been shown to inhibit free radical reactions and toxicity in experimental models of both *in vitro* and *in vivo*. Deferiprone (L1) has been shown to be effective and safe in the reversal of accelerating oxidative stress related tissue damage in iron loading and non iron loading conditions such as cardiomyopathy in thalassaemia, acute kidney disease and Friedreich ataxia. The selection of chelating drugs and their combinations could be used as new strategies for antioxidant therapies. *In vitro*, *in vivo* and clinical data suggest that L1 is the most potent drug antioxidant because of its high therapeutic index, ability to reach extracellular and intracellular compartments of many tissues and ability to inhibit both iron and copper catalysed free radical reactions.

2. INTRODUCTION

Free radical formation is a natural process leading to the production of highly reactive free radical species such as the superoxide, nitric oxide and hydroxyl radicals. Under certain conditions free radicals can cause chain reactions and cascades. Many metabolic pathways are utilizing free radicals for the production of essential biomolecules such as prostaglandins and leukotrienes, which are derived from the metabolic pathway of arachidonic acid. These and many other biomolecules are produced through radical reactions, which are involved in essential physiological pathways and biological activities in cells. Free radicals and other oxygen activated products (FRP) such as hydrogen peroxide are also involved in many biological processes such as the oxidation of food products, the metabolism of natural products, drugs and other xenobiotic molecules, the destruction of invading microbes through phagocytosis, the destruction of senescent and other cells and of cellular components, endothelium-derived relaxation (by nitric oxide), etc. Free radicals and other oxygen activated products can also cause

modification or damage to virtually all known organic biomolecules including DNA, sugars, proteins and lipids (1-3).

Under normal conditions the production of free radicals is well regulated and controlled by specific metabolic pathways and antioxidants. However, under certain conditions free radicals can cause diseases such as cancer or acceleration of tissue and organ damage eg in cardiomyopathy and ageing and also total irreversible damage in processes similar to rancidity. The latter resembles the period after death, where the body's antioxidant processes collapse and the cellular oxidation processes are accelerated and cannot be controlled leading to the complete destruction of organic biomolecules.

There are more than a quarter of a million publications in relation to the properties, role and mode of action of antioxidants. Many natural and synthetic molecules have been shown to have antioxidant activity using both in vitro and in vivo models. However, there is not yet almost any specific and effective antioxidant treatment prescribed for any disease or condition associated with damage due to free radicals or related byproduct reactive molecular species. Major drawbacks in the prospects of introducing any form of antioxidant therapy, is the lack of antioxidant specificity, tissue targeting and possible toxicity. Other important considerations for introducing any form of antioxidant therapy is the dose and timing of administration of the antioxidant (s), the assessment and monitoring methods of the antioxidant effects, the possibility of interference with other treatments related to the underlying condition and the duration of administration including the possibilities for prophylactic, long and short term uses.

Classical antioxidants are usually aiming for the neutralization of free radicals and other related FRP, which arise mainly from iron and copper catalytic centers. A more effective way of increasing the antioxidant activity of antioxidants and the prevention of free radicals and free radical cascades is the inhibition of the redox catalytic centers involving iron and copper. Iron and copper chelating drugs can achieve this goal to a great extent by binding these metal ions and rendering them redox inactive. The strategy for the design of improved targeting antioxidant therapies in different clinical conditions is based on the design of specific chelating drugs and other antioxidants with free radical inhibitory activity, which can reach high therapeutic concentrations and have low toxicity in humans (3-5).

3. METABOLIC IMBALANCE OF FREE RADICAL PRODUCTION LEADING TO TOXICITY AND BIOLOGICAL DAMAGE

Under normal physiological conditions the formation of free radicals and other oxygen and nitrogen activated products such as the hydroxyl radical, superoxide, nitrogen oxide, hydrogen peroxide and lipid peroxides is primarily depended on iron and copper catalytic centers involved in redox reactions. Some of these catalytic centers are present within proteins which are involved in the production or modification of free radicals and other oxygen activated species. Examples of such proteins include oxygenases, which are involved in the incorporation of oxygen in organic substrate, hydroxylases (monooxygenases), which catalyse the incorporation of one atom of elemental oxygen in organic substrate and oxidases, which are involved in the oxidation of organic substrate by the reduction of oxygen to peroxides. The latter are either decomposed by catalase or utilized by a peroxidase (1, 2). Both catalase and peroxidases contain iron which is used in their catalytic centers. Iron and copper can also function as redox catalytic centers when bound to side chain ligands of proteins and other high molecular weight biomolecules or when present in the form of low molecular weight complexes with natural ligands or chelators. The latter are mainly formed in the transit metal pools of the metabolic pathways of these metals, during cell damage and also in conditions of copper and iron overload, where the capacity of the metal transporting proteins is saturated (6).

Specific proteins of iron and copper metabolism have been evolved, which can control efficiently the transport, storage and utilization of these metals in cells and tissues and can also reduce the prospect of free radical toxicity arising from their redox catalytic action. Similarly, under the same conditions of metabolic control, the toxic byproducts of free radical reactions such as superoxide and hydrogen peroxide can be neutralized or eliminated efficiently by the antioxidant molecules, pathways and mechanisms, which usually involve low molecular weight antioxidants such as vitamin E, vitamin D, ascorbic acid and lipoic acid or proteins such as superoxide dismutase and catalase. Aerobic organisms continuously face the threat of generation of superoxide and hydrogen peroxide during the utilization of oxygen but the last two enzymes effectively eliminate them. Superoxide dismutase converts the superoxide radical to hydrogen peroxide and water and catalase the hydrogen peroxide to oxygen and water.

Malfunction or oversaturation of the antioxidant pathways and mechanisms as well as nutritional deficiencies of antioxidants reduce the prospects of controlled free radical production and increase the prospects of free radical imbalance and toxicity. Many factors can affect the antioxidant pathways and mechanisms, as well as the protection against toxic free radicals in each individual. Such factors include disease, infection, the state of the immune system, organ function, genetic manifestation in relation to antioxidant mechanisms and metabolic pathways, the age etc.

Metabolic imbalance and increase in free radical production and toxicity has been suggested in almost all the conditions of tissue damage including cardiomyopathies, stroke, acute kidney disease, liver disease, rheumatoid arthritis, cancer, Alzheimer's disease, Parkinson disease etc (1-5). Similarly, ionizing radiation, cigarette smoking, drugs and other xenobiotic substances from food, as well as natural compounds can also cause free radical toxicity and damage (FRD).

The catalytic role of iron and copper on free radical production and FRD is widely reported in many experimental and disease models at various levels including molecular, sub-cellular, cellular and tissue damage. With regards to molecular effects, the breakdown or structure modification of all organic biomolecules including sugars such as the breakdown of deoxyribose, lipid peroxidation, crosslinking of proteins etc are some of the characteristic effects of FRD. On the sub-cellular level free radicals can cause damage to many organelles resulting in structure modifications of cellular components and the malfunction of cellular processes. The mechanisms of cell apoptosis are also affected. Subsequently these FRD changes can overall lead to a wider tissue and organ damage.

Repair mechanisms against FRD are available at all the stages of molecular, cellular, tissue and organ levels, which are usually effective and the damage is reversed or minimized or in other cases the damaged cells are removed or replaced. However, in some cases the damage can increase progressively to a stage where it may not be reversible resulting in permanent damage.

In all the cases of FRD specific mechanisms and conditions apply, which have to be considered before antioxidant therapeutic strategies could be developed in order to protect the organism.

Food consumption for example is associated with increased production of free radicals when food is metabolized. Caloric restriction diets and antioxidant-rich diets can reduce the levels of free radical production and this may reduce the aging process and result in an increasing life span. Similarly, irradiation toxicity from X-rays, sunlight or radioactive substances can cause free radical mediated DNA damage, which may cause cancer. The level of intensity and the duration of radiation exposure are important parameters affecting this process.

In contrast, in some cases free radicals and FRD is required for the protection of the organism. Radiotherapy for example, which is used for the treatment of cancer patients, produces increased amounts of free radicals, which facilitate the destruction of tumors. However, in patients with agranulocytosis the reduced or absent production of neutrophils, which utilise free radicals during the process of phagocytosis for the killing of invading microbes may result in the development of infections. This may result in fatal infection outcomes for immunocompromised patients.

The timing and rate of FRD, as well as the physiological state of the organism are also important parameters, which can determine the overall effects of FRD. In some cases FRD may be the cause of a disease, in other cases FRD is considered as an after event of a toxicity progress and in ageing is a long progressive state of deterioration leading to an irreversible state, namely death.

4. IRON AND COPPER METABOLIC PATHWAYS AND ASSOCIATED DISEASES OF METAL METABOLIC IMBALANCE

The catalytic activity of iron and copper in the production of free radicals is one of the major metabolic processes utilized by many iron and copper containing enzymes, which are involved in a variety of biochemical pathways, essential for the normal function and development of the organism. In general, iron and copper are essential for humans and almost all other living organisms, with iron and copper containing proteins playing an important role in many physiological processes both in health and disease states. Many of these iron and copper containing proteins are involved in oxygen and electron transfer and the utilization or control of free radicals and other FRP. There are many metabolic controls associated with the absorption, distribution and excretions of these essential metals. However, iron and copper body distribution as well as associated metabolic processes could be affected by a variety of dietary, genetic, environmental, iatrogenic and other factors, resulting in abnormal metabolic effects and disease (7).

Under normal conditions body iron and copper levels and organ distribution are governed by homeostatic controls of iron and copper uptake, distribution and storage. These levels are mainly regulated by the gastrointestinal absorption of dietary copper and iron and sometimes other factors such as in the case of iron, the erythropoietic activity of the bone marrow. The absorption of both metals from the intestine is mostly regulated by metabolic pathways involving proteins. Several other factors could affect their absorption including their quantity present in the diet, the chemical form (eg haem iron, ferrous, ferric, cuprous or cupric), the chemical components forming metal complexes in the intestine (eg phosphates, phenols, and sugars), the presence of other metal ions such as zinc and the presence of other substances with metal binding properties eg drugs with metal binding ligands. Following their uptake from the gut lumen into the enterocytes, iron is transported in blood by serum transferrin and copper by ceuroplasmin, which then deliver them to the liver and other tissues (6, 7).

Normal adult humans contain 3-5 g of iron and much less copper. Copper is a component of many enzymes and is partly stored intracellularly in the protein metallothionein. Iron is mainly present in the form of haemoglobin (58%) in red blood cells, myoglobin (9%) in muscle tissue and as intracellular ferritin/haemosiderin (30%) mainly in the liver (5-7). Iron transport and distribution is tightly controlled. The intracellular uptake of iron from transferrin and its storage in the tissues is regulated by the iron regulatory proteins (IRPs) through the translational control of the synthesis of the transferrin receptor at the cell surface and that of intracellular ferritin. Cells differ on the number of transferrin receptors and intracellular iron. The iron released following the breakdown of effete red blood cells and of other cells of the body is transported in blood by transferrin, which maintains equilibrium between the sites of iron absorption, storage

and utilisation. Intracellular iron uptake is accomplished by the binding of two molecules of mono- or diferric transferrin to a transferrin receptor on the cell surface and subsequent incorporation in the cell within an endosome. Iron release from transferrin in the endosome is accomplished by acidification of the endosome from pH 7.4 to 5.6. Iron is released in a low molecular weight form in the "transit iron pool", which is then transported for incorporation inside the apoproteins for the formation of iron containing proteins or for storage into ferritin or haemosiderin. Under normal conditions transferrin is saturated 25-35% with iron, whereas in primary haemochromatosis or transfusional iron overload the saturation may exceed 100% and non-transferrin bound iron (NTBI) could be detected in the serum (6,7).

There are many iron and copper metabolic disorders affecting millions of people. The most serious copper imbalance diseases are congenital. For example copper deficiency is observed in Menkes disease in male infants, whereas copper overload in Wilson's disease. In the latter copper accumulation mainly in the liver and the brain causes severe toxicity including neurological disorders and hepatotoxicity (8).

Iron deficiency is estimated to affect one in four individuals during their life time. The main causes of iron deficiency anaemia are increased iron requirements for rapid growth during infancy and for the foetus during pregnancy, as well as blood loss and low levels or reduced bioavailability of dietary iron. Overall, the rate of iron absorption is insufficient to maintain iron balance and is lower than the rate of iron excretion or overall iron loss (7). In most cases body iron levels can be restored and reach normal ranges only if the level of iron absorption exceeds the level of iron excretion. This can be achieved by iron supplements.

In contrast to iron deficiency, iron overload is the most common metal toxicity condition. Iron overload could be caused by increased gastrointestinal iron absorption (primary haemochromatosis) or multiple red blood cell transfusions (secondary haemochromatosis) or combination of these two processes. Patients with refractory anaemias such as thalassaemia are regularly transfused with 1-3 units (1 unit= 200 mg of iron) of red blood cells every 2-4 weeks. The iron accumulated from transfusions is not excreted but is mostly stored intracellularly. The iron storage proteins ferritin and especially haemosiderin increase substantially in concentration in almost all the organs and in particular the liver and spleen of the transfused patients. Organ damage due to iron overload and toxicity is detectable when about 50-100 units of red blood cells have been transfused and is so extensive that in many cases is irreversible (9).

Iron overload caused by repeated red blood cell transfusions in refractory anaemias has the highest mortality and morbidity rate worldwide by comparison to any other iron or metal overloading condition. The most seriously affected group of transfused patients are those with thalassaemia, which is the commonest genetic disorder with estimated over 100 million asymptomatic heterozygote thalassaemia gene carriers worldwide (9).

Under normal physiological conditions both iron and copper are mostly found in their oxidised forms and are also almost insoluble at physiological pH. The reduced ferrous (Fe (II)) and cuprous (Cu (I)) ions are more soluble than their ferric (Fe (III)) and cupric (Cu (II)) counterparts and are more readily catalysing free radical reactions. Iron (III) forms oligonuclear and mostly polynuclear oxo-complexes at physiological pH, which cannot catalyse free radical reactions unless is solubilised and reduced. In iron overload iron accumulates at high concentrations in cells such as hepatocytes and similar polynuclear oxo-iron complexes are deposited intracellularly within ferritin and haemosiderin. Under these conditions ferritin arrays are formed mainly in primary lysosomes and haemosiderin aggregates accumulate in secondary lysosomes as previously shown by electron microscopy (10, 11). In heavy iron overload there are many iron-laden lysosomes, some of which are ruptured into the cell sap releasing hydrolytic enzymes and potentially toxic forms of iron, which can catalyse the production of free radicals and cause FRD. This damage could progressively lead to cellular, tissue and organ damage (9). Transfusional iron loaded thalassaemia patients usually die from iron overload related cardiomyopathy, whereas idiopathic haemochromatosis patients from iron overload related hepatocellular carcinoma. Localised iron accumulation and related FRD is also evident in other conditions such as the anaemia of chronic disease, where iron accumulates in the reticuloendothelial system, in Friedriech's Ataxia, where it accumulates in mitochondria, in Hallervorden-Spatz syndrome, where it accumulates in the brain etc (5).

In general any form of excess iron and copper are potentially toxic, especially if these metals are not bound or controlled by the proteins of iron and copper transport and storage. In non metal overloading conditions the same form of toxicity applies especially if localized non protein bound excess metal ions of copper or iron are released and under certain conditions catalyse free radical formation, which subsequently could cause localized organic biomolecular damage, cellular and tissue toxicity. Such mechanisms have been postulated in many conditions such as in the joints of rheumatoid arthritis patients, in acute kidney disease, in the formation of neoplasmic cells following DNA damage etc.

Iron chelating drugs are mainly used for the treatment of transfusional iron overloading conditions such as thalassaemia, mylelodysplasia and sickle cell disease. Copper chelating drugs are mainly used for the treatment of Wilson's disease. These drugs could also affect the treatment of other conditions related to iron and copper metabolic imbalance and free radical toxicity. In principle, specific iron and copper chelating drugs could be designed and targeted to prevent, modify or inhibit the free radical catalytic activity of these metals and delay, prevent or eliminate free radical toxicity and related damage. Accordingly these could be used in the treatment of free radical related toxicity conditions.

5. MECHANISMS OF IRON AND COPPER BINDING BY CHELATING DRUGS: MOLECULAR ASPECTS OF CHELATION THERAPY

Chelator is an organic compound which possesses at least two ligands with electron donor atoms such as N, O and S, which have affinity for binding metal ions. The complex or complexes formed between the chelator and the metal ion has different physicochemical, pharmacological and toxicological properties by comparison to the chelator or the metal involved in the complex. There are many organic biological molecules possessing electron donor atoms on ligands, which can be involved in metal complex formation. Such donor atoms could be present in ligands of acidic groups such as -COOH, -OH, -SH, -NOH, where the proton could be displaced by the metal ion or in Lewis bases such as -C=O, -NH2, -O-R, -S-R. These functional groups with chelating potential could have variable affinity for several metal ions such as Fe, Cu, Zn and Al. There are many drugs and organic biomolecules with chelating potential for iron and copper such as proteins, fatty acids, sugars, ATP, DNA and RNA (3, 7). The major chelating drugs used worldwide for the treatment of iron overload are deferoxamine (DF), deferiprone (L1) and deferasirox (DFRA) and for the treatment of copper overload is penicillamine and triethylenetriamine. Ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) are also used mainly for other metal toxicity conditions but also in some cases in iron toxicity (12).

Metal chelating proteins have been evolved for the mobilisation and transport of copper and iron in plasma. Transferrin, which is a specific protein for chelating and transporting iron in plasma, has binding sites with high affinity for iron but also affinity for some other metal ions such as Al, In, Ga etc. (13). Similarly, caeruloplasmin is a specific protein for chelating and transporting copper, whereas intracellularly metallothionein is utilised for the storage of copper, zinc and other xenobiotic metals. Chelating drugs have to compete with transferrin, caeruloplasmin and other such endogenous naturally occurring chelators for iron and copper respectively at all the stages of absorption, metabolism and excretion of the chelating drugs and their metal complexes. The competition between transferrin and caeruloplasmin with chelating drugs for iron and copper respectively, as well as the interaction between the chelating drugs for iron and copper is governed by thermodynamic and kinetic parameters. Deferiprone for example can exchange iron with transferrin under certain conditions, which mainly depends on the concentration of both and also on their saturation with iron (6). In contrast, iron exchange between transferrin and DF is less feasible due to kinetic restrictions imposed by the chemical structure of DF and its iron complex. Similar interactions have been shown between L1and DF and their iron complexes and also with other drugs, which have iron binding properties (14).

One method of assessment of the affinity of chelators for various metal ions is the determination of the metal stability constants (log beta (β)). Among the iron chelating drugs L1 appears to have the highest stability constant for iron (log β= 35) by comparison to DF (log β= 31) and DFRA (log β= 27) (9). For L1 the second and third most competing metal ions with iron appear to be Cu and Al respectively (14). DTPA is less specific for iron and during its clinical use in addition to an increase in iron excretion, the excretion of zinc, copper and magnesium also appear to increase (15). Minor increases in zinc excretion were also observed in a few iron loaded patients receiving iv DF or oral L1 (16, 17).

In vitro estimations of the affinity of chelating drugs for iron or copper, such as the stability constants or other physicochemical parameters cannot reflect the ability of the drugs to remove iron or copper in vivo. The toxicity, pharmacokinetic and metabolic properties of the chelating drug may not allow sufficient time and concentration of the active chelating molecule to bind and remove sufficient amounts of iron or copper, which may be present in various toxic forms in vivo. Other factors affecting the efficacy of chelating drugs is the number of metabolites and their chelating properties as well as the lipid/water partition and clearance of their iron or copper complexes, which may overall influence the efficacy of the drug in iron or copper removal and also the extent of their toxicity (9).

6. THE EFFECT OF NATURALLY OCCURRING LOW MOLECULAR WEIGHT IRON CHELATORS ON IRON METABOLISM

Many naturally occurring organic molecules possess ligands which can form complexes with iron and copper. The role of these naturally occurring metal binding molecules on iron and copper metabolism and free radical toxicity formation is not fully characterised (3). Although the major role in maintaining iron and copper homeostasis is assigned to the proteins of iron and copper transport, storage and utilisation, the mechanism of the transport of iron or copper into an intracellular iron or copper pool and subsequently in enzymes incorporating these metals, can only be envisaged through the context of a labile “transit” iron or copper low molecular weight (MWt) pool (6). The components of this pool are thought to be mainly low molecular weight iron or copper complexes, some of which can exchange their iron with apo-proteins (6). Many low molecular weight naturally occurring chelators found in cells eg ATP, ADP, citrate or some of which are absorbed from food eg ascorbate and phytic acid, as well as molecules containing iron binding ligands can all be considered to be involved in the formation of such an intracellular low molecular weight iron or copper pool. The same molecules can also be involved in the transfer of iron and copper and the formation of ternary metal complexes with apo-proteins or the incorporation of these metals into the metal domain of proteins. The iron or copper uptake and release processes that are involved intracellularly to and from the naturally occurring chelators and their complexes are governed by the same thermodynamic and kinetic parameters as for other chelators and their metal complexes (7). It is anticipated that citrate (0.01 M, in plasma) and other low molecular weight naturally occurring chelators may form low molecular weight iron

and copper complexes intracellularly and in plasma, similar but to a much smaller extent than the iron chelating drugs L1 and DF.

The low molecular weight iron pool in plasma (NTBI), which is usually observed when transferrin is fully saturated with iron, has been shown to be present in conditions of iron overload and also in non iron loading conditions eg during cancer chemotherapy (6). Similarly, some forms of iron are not available for transferrin binding even if transferrin is not fully saturated with iron. Low molecular weight oligonuclear iron cannot be incorporated into transferrin unless it becomes mononuclear. This form of iron and also of iron found bound to natural chelators, which are not exchanging their iron with transferrin, as shown with DF, can also be present in plasma and intracellularly and can potentially facilitate the catalytic formation of toxic free radicals.

Endogeneous or dietary metal binding molecules, which can increase the size of the low molecular weight iron pool, may play a role in the urinary elimination and overall excretion of iron or copper. Increased faecal iron or copper excretion may also be induced by low molecular naturally occurring chelators in a mechanism resembling the mode of iron removal activity of DFRA, which predominantly increases iron excretion in the faeces (9). In contrast, other molecules such as 8-hydroxyquinoline, which decrease the size of this low molecular weight iron pool by diverting iron or depositing iron to other tissues may minimise iron excretion and have the opposite effect ie increase the body iron load and intracellular free radical toxicity (18). Iron chelating drugs such as DF and L1, which cause an increase in the low molecular weight iron pool (as iron complexes) intracellularly and in plasma, have been shown to increase urinary iron excretion and also the elimination of iron and aluminium during the dialysis procedure in renal dialysis patients (19-21). Usually the presence of excess chelator, at much higher concentrations than the molar ratio of the chelator: iron or copper is required for the binding and removal of these metal ions in vivo.

The mode of action of naturally occurring chelating molecules can have variable effects on iron and copper metabolism and free radical formation and toxicity, similar to those observed during the use of the chelating drugs L1, DF, EDTA and DFRA. Some naturally occurring iron binding compounds such as phytates, tannins and phosphates decrease iron absorption. Similar effects have been observed during iron absorption studies with metal binding drugs such as tetracycline. In contrast, other compounds such as the naturally occurring lipophilic chelator maltol or the lipophilic synthetic chelators 8-hydroxyquinoline and omadine, have been shown to increase iron absorption (22).

There is a large number of other naturally occurring iron chelators of microbial and plant origin, which have variable affinity for iron and copper and which can play a role on iron and copper absorption and excretion, as well as the formation of free radicals, through their iron or copper complexes. Some examples of chelators of microbial origin are enterobactin, mycobactin, aspergillic acid and many other similar microbial siderophores including DF, which is isolated from strerptomyces. Chelators derived from plants, as well as some of which may be present in food have not yet been fully investigated for their iron and copper binding and ability to catalyse free radical production. Some examples of powerful iron and copper chelators of plant origin are catechols, mimosine, tropolone and maltol which are known to affect iron and copper metabolic pathways and to inhibit free radical formation when present in excess by comparison to the metal ion concentration (23).

Almost all plant antioxidants including polyphenols, caffeic acid, ascorbic acid etc have iron and copper binding affinities and can affect the metabolism as well as the catalytic activity of these metals in the formation of free radicals (23, 24). Ascorbate for example can cause reduction of ferric to ferrous iron, which can result in an increase of iron absorption from the gut but also under certain conditions it may increase the catalytic production of free radicals.

Competing metal ions may also affect the absorption, distribution and excretion of iron and copper as well as their potential for the catalytic formation of free radicals. Zinc acetate for example is used in the treatment of Wilson's disease by preventing copper absorption, whereas selenium is used as an antioxidant promoter and gold complexes as anti-inflammatory drugs in rheumatoid arthritis (3, 7, 8).

7. GENERAL PROPERTIES OF DEFERIPRONE

Deferiprone belongs to the α-ketohydroxypyridine (KHP) class of iron chelators, which were originally designed and tested in the period 1979-1981 (25-27). Deferiprone is a white needle like crystalline solid and is stable at room temperature for more than 5 years and stable in solutions of physiological and acidic pH. It is more soluble in acid, for example the stomach acidity than in alkaline or neutral pH. It is sparingly soluble in water at pH 7.4 (about 20 mg/ml, at 37 C) and forms red colour complexes with iron, green complexes with copper, blue complexes with U and colourless complexes with aluminium (28-31). Its affinity for iron is greater than copper, aluminium, zinc, indium, gallium and uranium and other metals at pH 7.4 (14). Deferiprone is a hydrophilic chelator (K par = 0.18) forming a more hydrophilic iron complex (K par = 0.01) at physiological pH. This ensures rapid excretion and no accumulation in lipids of both its native and iron complex forms. It is more lipophilic than DF and much more hydrophilic than DFRA (9).

Many factors affect the efficacy of drugs including individual variations in the absorption, distribution, metabolism, clearance and toxicity (32-38). Pharmacokinetic studies have shown that orally administered L1 is rapidly absorbed from the stomach and appears in blood within minutes with a half-life of absorption to the stage of peak serum concentration ranging from about 1 to 32 minutes. In a few cases, a lag period of

1-3 hours in the appearance of L1 in blood was observed. The half-life of clearance of L1 from blood ranges from about 47-134 minutes and in most cases almost all the L1 is cleared from blood within about 6h (32-34).

Deferiprone is metabolised through glucuronide conjugation at the 3-OH position, which blocks the iron binding site and metal chelating properties of the drug (32). Peak serum concentrations of the L1 gluguronide appear at about 2h after the peak serum concentration of L1 and its clearance from blood following its oral administration is about 8 hours. Deferiprone, its gluguronide metabolite, and its iron and other possible metal complexes are all excreted in the urine to almost 100% recovery (34). No detectable levels of L1, L1-glucuronide or increased iron excretion, were measured in the faeces of patients treated with L1, or in clinical metabolic studies using Fe radio-labeling (37). In general iron chelation appears to precede glucuronidation and the rate of iron excretion to depend mainly on the availability of chelatable iron rather than the extent of glucuronidation of the drug (39).

The level of iron excretion caused by L1 depends mainly on the dose, frequency of administration and the iron load of patients. Daily doses totaling 50-110 mg/kg subdivided into 15-50 mg/kg doses have been widely used in iron loaded patients (39). Iron excretion in normal individuals using doses of as much as 50 mg/kg is by comparison negligible (1-2 mg iron/day), suggesting that the level of iron excretion depends on the iron load of patients (32, 40). The highest level of iron excretion ever recorded by L1 was in an iron loaded thalassaemia patient who excreted 325 mg of iron following the administration of six divided doses to a total of 16g (about 250 mg/kg) within 24 hr. This high dose was well tolerated and urinary iron excretion was continuous (33). Deferiprone has been shown to cause negative iron balance in many groups of iron loaded thalassaemia patients who have been taking effective doses (more than 80 mg/kg) for periods of 0.5-1 year and also to cause a decrease in serum ferritin to near normal levels (41-42). Progressive depletion of iron deposited in the liver and the myocardium has also been observed using the MRI T2 and T2* techniques following the daily use of effective doses of L1 (80-110 mg/kg/day). This process could take a few months or years depending on the dose protocol used (43, 44). In contrast to facilitating iron excretion, L1 does not appear to cause an increase in iron absorption when administered in combination with iron (22, 45). Similarly, L1 does not appear to cause an increase in copper excretion in iron loaded patients but it may increase zinc excretion in some patients and aluminium excretion in aluminium loaded renal dialysis patients.

The high affinity of L1 for iron in vitro and in vivo is well documented, but its effect on copper especially in vivo needs further investigations. Copper is found at much lower concentrations than iron in the body and its metabolism is more tightly controlled than iron or zinc. The stability constant of L1 for copper (log β= 19.6) is sufficient theoretically to influence the metabolic pathways of copper metabolism and to increase copper excretion in copper overload such as in Wilson's disease. Deferiprone has also been shown to inhibit the activity of copper containing enzymes and the free radical catalytic activity of copper (46).

8. TOXIC SIDE EFFECTS OF DEFERIPRONE THERAPY AND SAFETY LEVELS OF ITS USE

Deferiprone is one of a few drugs in daily use in medicine, which has been administered daily for so many years at such high doses (50-100 mg/kg) with very low toxicity. The LD5O of L1 administered orally and intraperitoneally in rats has been estimated to be between 1-2 g/kg and 600-700 mg/kg respectively (26). There have been thousands of patients using L1 worldwide, which is approved in Europe, India, China and many other countries but not yet in the USA. The maximum dose ever used within 24 h was 250 mg/kg subdivided into six doses and the highest daily dose long term was 150 mg/kg/day for two years (33). With regards to long term safety there are patients, who have been taking L1 daily at 75-110 mg/kg for over 16 years with no reports of major toxicity. Overdose of L1 at 250 mg/kg/day for several months has been reported to cause neurological abnormalities in 2 iron loaded patients. Deferiprone has been reported to cause maternal, embryo and teratogenic toxicity in animals (26). L1 appears to enter most organs and in addition to blood it has also been detected in the saliva of patients (47).

The toxic side effects of L1 during clinical use in thousands of patients in the last 16 years at doses of 50-150 mg/kg/day are mainly: a) transient agranulocytosis in 0.6 %, b) neutropenia in about 6% c) transient musculoskeletal and joint pains in about 15% d) gastric intolerance in about 6% and e) zinc deficiency in about 1% (48-52). All the toxic side effects of L1 are considered reversible, controllable and manageable but their cause is not known. Some of these are considered to be idiosyncratic and to be related to a combination of factors (27).

The incidence of agranulocytosis is monitored using mandatory weekly or fortnightly blood counts. The recovery of patients following L1 induced agranulocytosis is usually within 1-7 weeks and treatment may involve the use of growth colony stimulating factors (G-CSF). A few fatal cases of agranulocytosis have been reported involving patients not adhering to the mandatory blood counts (53). Patients who develop agranulocytosis or patients with prolonged neutropenia are not usually allowed to continue with the L1 treatment. In patients with musculoskeletal and joint toxicity the pains may subside despite continuation of L1 therapy, or following reduction of the dose or treatment with non-steroidal anti-inflammatory drugs.

Therapeutic effects in FRD can only be achieved if L1 or other antioxidants can reach the target protein, cell or tissue at sufficient concentrations. However, the therapeutic concentration of L1 or other antioxidants may also cause toxicity. For example, maximum iron removal effects from iron containing proteins eg from transferrin could be accomplished at the maximum concentration which could be reached in vivo e.g. up to 0.45 mM in serum by L1

administered at doses of 50 mg/kg in thalassaemia patients. However, at low concentrations e.g. 10 μM, L1 could deliver iron to apotransferrin and possibly other iron apoproteins. The "dilution effect" of bidentate chelators such as L1 at low concentrations e.g. 10 μM, which forms very weak iron and copper complexes is much in evidence than that of hexadentate chelators such as DF, which form relatively strong iron complexes under the same conditions. In contrast, at low concentrations of L1 the anticipated formation of labile iron and copper complexes could result in the inability of L1 to inhibit free radical formation and this may increase relevant toxicities, such as the promotion of the growth of some microbial species (54). In addition to toxicity, the metabolic transformation and clearance of the drug, organ function abnormalities and other factors could also influence the access of L1 or other antioxidants to iron or copper targets of FRD and overall influence possible therapeutic effects. Overall, the toxic side effects of L1 and other antioxidants should be monitored, especially during long term antioxidant therapies.

9. MODE OF ACTION OF DEFERIPRONE AND OTHER CHELATORS AGAINST FREE RADICAL DAMAGE AND OTHER CHELATOR INTERACTIONS AFFECTING THIS PROCESS

The ability of L1 to inhibit the redox catalytic activity of iron and copper in the formation of free radical cascades is the basis of its antioxidant properties. The metabolic pathways involving iron and copper catalysis in relation to free radical formation and toxicity are many and variable with distinguished characteristics and impact on FRD in each case. The intervention by L1 in each case has its specific characteristics and specific considerations apply such as the timing of intervention, the duration of administration and the selection of the appropriate dose for achieving a therapeutic effect.

There are many forms of molecular interactions of L1 and other chelators with iron or copper containing enzymes in addition to metal removal. These include metal donation, metal exchange, ternary complex formation between the chelator and the protein through the metal, allosteric interactions of a side chain of the protein with the chelator or metal chelator complex, redox changes and catalytic oxidation/reduction of biomolecules. Almost all these forms of interactions could result in the modification, including loss, of enzymatic activity and could have different effects on free radical formation and FRD.

Chelating drug induced redox changes are some of the most important interactions leading to free radical formation and usually involve low molecular weight iron or copper and proteins containing these metals. At physiological pH the chelating drugs L1, DF and other chelators with similar iron binding sites convert aqueous ferrous iron into ferric iron and form ferric iron complexes within minutes. This process is similar to the mode of action of plasma transferrin, which removes toxic low molecular weight iron and also oxidizes ferrous to ferric iron and forms ferric but not ferrous iron complexes. The same mechanism of potential prevention of free radical damage can also take place in the presence of lactoferrin in bodily secretions and in neutrophils. Similarly, the oxidation of cuprous to cupric ion and formation of a cupric complex by L1 is the mechanism of prevention of free radical damage by low molecular weight copper. Under physiological conditions the iron and copper complexes of L1 are redox inert, especially when L1 is in excess over iron and copper. Even in cases of copper or iron overload, the concentration of chelating drugs is sufficiently high for removing low molecular weight iron and copper complexes provided the administration of the chelating drugs is continuous and the doses are effective.

Iron and copper chelators such as L1 can inhibit free radical toxicity not only by binding and forming free radical inactive metal complexes but also through other pathways affecting redox changes such as the reduction of tetravalent iron in haem into ferric iron (55). Haem containing tetravalent iron is considered a more toxic species and source of redox catalytic iron involved in the formation of toxic free radicals by comparison to low molecular weight iron. Similarly, some chelating agents such as 2,3-dihydroxybenzoic acid can cause oxidation of iron in iron containing proteins, for example of cytochrome C (56). Other chelators such as DF can have other oxidizing effects, for example they can increase the rate of oxidation of haemoglobin to methaemoglobin (57). Metal complexes of chelators can also affect free radical formation or inhibition by several mechanisms, for example iron complexes of L1 and plant products containing the catechol iron binding site can act as superoxide radical scavengers (58). For each of the above cases the inhibitory effects and other interactions by chelators on free radical formation and FRD are concentration depended.

Free radical formation and FRD by iron and copper could be initiated both intracellularly and extracellularly. The targets of chelating drugs in non iron or copper loading conditions are mostly low molecular weight metal complexes or intracellular iron or copper containing enzymes. Chelators can have direct access to these enzymes provided they can cross the cell membrane. However, the turnover of intracellular iron or copper containing enzymes can also be affected indirectly by the mobilisation of intracellular low molecular weight iron or copper, which is “in transit” before their incorporation into the apoproteins. Indirect effect on the turnover of these enzymes can also be mediated through chelator interactions with the metal transporting proteins ie transferrin and caeruloplasmin, which are directly related to the turnover of iron and copper pools “in transit” intracellularly. Similar effects can also be mediated through interactions with the receptors of these transporting proteins. Within this context, there are many iron and copper containing enzymes and related metabolic pathways, which could be effected. Allosteric interactions of chelators with these enzymes and other proteins may also influence their activity. For example, circular dichroism studies have indicated a conformational change of hemoglobin in the presence of L1, with the helicity of hemoglobin being reduced in the presence of increasing concentrations of L1 (59).

The various forms of chelator interactions with iron and copper could be used to target free radical toxicity arising from the catalytic centers containing these metals. The targeting of iron and copper containing enzymes is of particular interest, as it has been used for new strategies in the design of new pharmaceuticals for a variety of conditions including cancer and inflammatory diseases.

10. FREE RADICAL INHIBITORY EFFECTS OF DEFERIPRONE IN *IN VITRO* MODELS OF FREE RADICAL DAMAGE

The free radical inhibitory effects of L1 and a number of other natural and synthetic chelators have been identified and characterised as long ago as 1986 in three different models of free radical toxicity (23). Many of these chelators, including L1 and DF have been shown to inhibit FRD in a variety of experimental models including the iron induced free radical damage on deoxyribose, the sugar component of DNA. However, some chelators have been shown to have the opposite effect and to increase FRD in some of these experimental models. EDTA for example, which is less specific chelator for iron than L1 and DF and forms semi co-ordinated iron complexes has been shown to increase the iron induced free radical damage on deoxyribose in the same study (23). The inhibitory effects of L1 and other chelators like DF were dose dependent with almost complete inhibition being achieved between 0.1-0.2 mM of L1. In another model of free radical damage using UV irradiation on IgG, which resembles the formation of immunocomplexes of IgG in inflammation eg in the joints of rheumatoid arthritis patients, the inhibitory effect of L1 (0.05-0.1 mM) was moderate, whereas that of catechol and 2,3-dihydroxybenzoic acid was more substantial. It was anticipated that the hydroxyl groups on the catechol ring in the last two chelators were used not only for iron chelation but also as a scavenger of hydroxyl radicals converting the hydroxyl group to an orthoquinone. In the third model of free radical damage, resembling tissue damage observed in many conditions such as muscular dystrophy, thalassaemia, cancer and rheumatoid arthritis, the effect of chelators on lipid peroxidation in mouse skeletal muscle homogenates was studied at 0.5 mM. Complete inhibition has been achieved by L1 in this model, suggesting that L1 could play a therapeutic role in conditions of tissue damage (23). Following these original findings on the use of L1 in the prevention of free radical toxicity at concentrations that can be achieved in patients, many more studies in different models have confirmed the antioxidant effects of L1. Some examples are the inhibition by L1 (0.4 mM) of microsomal lipid peroxidation induced by iron (III) / ADP and NADPH, inhibition of LDL oxidation caused by copper and iron and protection of human umbilical vein endothelial from the cytotoxic effects of oxidized LDL in a concentration-dependent manner (19,46,60). L1 has also been shown to inhibit increased generation of EPR-detected radicals, NF-kappaB activation and TNFalpha induction in hepatic macrophages of an animal model of alcohol liver disease (61). Protection by L1 (0.050 mM) of rat cardiac tissue injury during ischemia and reperfusion has been shown by monitoring contractile function and the release of lactate dehydrogenase (62). L1 was also shown to reverse the doxorubicin induced myocyte cytotoxicity, which is thought to be caused by the ability of doxorubicin to induce oxidative stress on the heart muscle, both through reductive activation to its semiquinone form, as well as by the production of hydroxyl radicals mediated by its complex with iron (63).

Inhibition of the catalytic formation of free radicals by chelators is generally based on their physicochemical properties, lipid/water partition, the stereo-chemical specificity and redox potential of their iron and copper complexes as well as other factors (3, 6, 7). The targeting of proteins involved in the production of free radicals and other FRP is an important parameter in the mode of antioxidant action of chelators. Usually as a result of the physicochemical and other property differences of chelators, the targeting process involves specific parts of the metal metabolic pathways of these proteins and each protein plays a distinct role in each disease state associated with free radicals.

There are many examples of protein targeting such as the iron containing enzyme ribonucleotide reductase, which is involved in DNA synthesis. This enzyme has been targeted for the development of anticancer therapeutics and has been shown to be inhibited by either iron removal, e.g. by 8-hydroxyquinoline or the inhibition of its free radical by the anticancer chelating drug hydroxyurea or the depletion of the intracellular iron pool by chelators such as L1 (27). Similarly, L1 and other chelators have also been shown to inhibit one of the hydroxylase iron containing enzymes involved in collagen synthesis and may have a use in the treatment of fibroproliferative disorders (64).

Inhibition of cyclooxygenase, which may be of therapeutic value in the treatment of inflammatory conditions, has been shown to take place using several chelators including L1, other α-ketohydroxypyridines and DF (65). The inhibition, which was thought to be through the reduction of pro-inflammatory prostanoid synthesis and possibly other toxic free radicals, was reversible when iron and aluminium were introduced or when the chelators were washed off the incubation medium. It was also shown that the relative potency of inhibition was related to the hydrophilicity of the chelators in the order DF> L1> L1NEt> L1NPr (where L1NEt is the 1-ethyl- and L1NPr is the 1-propyl– 2-methyl-3-hydroxypyrid-4-one analogues of L1), suggesting that cyclooxygenase inhibition was facilitated through a hydrophilic compartment in cells. The IC50 by L1 and DF for cyclooxygenase in vitro was 0.8-2.0 mM. Some inhibition of about 2-10% can be expected in vivo at the maximum concentrations achieved in humans e.g. by L1 (0.45 mM).

Similar studies have also been carried out using the iron containing protein lipoxygenase as a target. The inhibition of lipoxygenase, including platelet aggregation and thromboxane A2 synthesis has been shown to take place in a dose depended manner by L1, other α-ketohydroxypyridines and DF suggesting that chelators may have a possible therapeutic use in thrombotic, atherosclerotic and inflammatory diseases (66). These

findings suggest that high concentrations and long-term administration of chelators such as L1 will be required in order to produce inhibitory effects on intracellular iron containing enzymes such as cyclooxygenase and lipoxygenase. However, the targeting of these proteins and the inhibitory activity by L1 and other more specific chelating drugs in the future could be limited by other factors, such as cell permeability and intracellular access to the iron containing compartments.

The design of bifunctional drugs containing characteristics of the established anti-inflammatory drugs with chelating side chains as well as combinations of chelators with established anti-inflammatory drugs may improve the targeting for these two proteins. The ability of L1 to inhibit free radical formation and FRD has been shown in several other in vitro models as well as in animal models.

11. FREE RADICAL INHIBITORY EFFECTS OF DEFERIPRONE IN *IN VIVO* MODELS OF FREE RADICAL DAMAGE

The effect of L1 on free radical formation and FRD has been studied in several animal models. Some examples to illustrate the mode of action and effects of L1 in vivo, which seem to confirm the in vitro findings have been summarised below.

Deferiprone (75 mg/kg/day for 30 days) has been shown to inhibit lipid peroxidation in a model of alcoholic liver disease in rats, where excess non haem iron is thought to be released causing increased production of lipid peroxidation (67). Deferiprone was also found to be effective in eliminating the reduction of the rat phrenic nerve-daphragm, which is considered to be caused by reactive oxygen species formed intracellularly (68). Another model of free radical toxicity and FRD is cerebral vasospasm, which occurs after subarachnoid hemorrhage through the iron catalyzed generation of free radicals. Deferiprone was found to be effective in attenuating experimental cerebral vasospasm in rabbits, providing further evidence of its ability to penetrate the blood-brain barrier and inhibit free radical toxicity (69).

The anti-inflammatory activity of L1in experimental colitis and gastritis was examined in rats. The free radical inhibitory activity of L1 appeared to have been accompanied by significant decrease in colonic and gastric myeloperoxidase (MPO) and nitric oxide synthase (NOS) activities and also of colonic prostaglandin E2 (PGE2) generation (70).

Inhibition of other iron or copper enzymes has also been detected using L1 (100 mg/kg ip) in rats. Concomitant inhibition of catechol-O-methyltransferase (COMT), tyrosine and tryptophan hydroxylase was observed with similar time-courses. COMT was inhibited with a threshold dose of about 1 mg/kg ip and a median effective dose of about 10 mg/kg ip. While COMT inhibition by L1 is probably related to the structural similarity of L1 with catechol related compounds which are substrates of the enzyme tyrosine and tryptophan hydroxylase, inhibition is more likely due to coordination of iron and copper bound to these enzymes, or the metal ion pools required for the turnover of these enzymes. Under the same conditions DF (100 mg/kg ip) has not shown comparable effects (71).

In an iron loaded mouse model both L1 and DF were shown to inhibit lipid peroxidation and to increase glutathione (GSH) levels in the liver. Both DF and L1 caused a decrease in glutathione peroxidase (GSH-Px) activity in this model, despite that neither GSH-Px nor catalase activity were affected by iron loading (72).

The antioxidant effects of L1 were also confirmed in a rat model where a reversal of the toxicity of acute 2 hour exposal to a 60-Hz sinusoidal magnetic field at intensities of 0.1-0.5 millitesla (mT) showed increases in DNA single- and double-strand breaks in brain cells. Iron release in the brain cells of the rats exposed to the magnetic fields was thought to be the mechanism of the toxicity. Melatonin and other antioxidants were also effective in attenuating this damage (73).

The ability of L1 to protect mitochondrial function and structure from damage induced by doxorubicin has been demonstrated in spontaneously-beating isolated atria from rats. In this model, pretreatment with L1 resulted in reversal of the reduction of the contractility caused by doxorubicin as well as reversal of other structural damage to mitochondria, increase in copper, zinc superoxide dismutase (Cu, Zn-SOD) activity and decrease in malondialdehyde (MDA) production levels (74).

A mouse model of multiple sclerosis (MS), where experimental autoimmune encephalomyelitis (EAE) was induced is another model where L1 has been shown to be able to reduce disease activity. Deferiprone (150 mg/kg) treated mice had significantly less disease activity and lower levels of inflammatory cell infiltrates than EAE mice used as controls (75).

Some effects of L1 related to free radical toxicity can also be seen in animal models of other diseases. For example porphyria cutanea tarda, which is a liver disease characterized by elevated hepatic iron and excessive production and accumulation of uroporphyrin (URO) can be treated using phlebotomy. Hepatic URO accumulation was also completely prevented in a mouse model of porphyria cutanea tarda at low doses of L1, which partially depleted hepatic nonheme iron but not Kupffer cell iron. In the same study L1 treatment had no effect on levels of hepatic cytochrome P4501A2 (76). Although FRD related byproduct was not measured, the presence of excess iron was suggested as the mechanism of FRD.

In an in vitro and an in vivo model of atherosclerosis in rabbits, L1 has been shown to prevent LDL oxidation. In vitro, L1 prevented oxidation of LDL and protected human umbilical vein endothelial cells. In vivo, L1 reduced thoracic aorta cholesterol content and also significantly decreased total plasma cholesterol, very-low-density lipoprotein cholesterol and LDL cholesterol by

comparison to control animals. In this study it was concluded that L1 possesses antioxidant activity in vitro and may reduce atherogenesis in vivo (77).

Overall, the in vitro and in vivo results in a variety of models are overwhelming with respect to the potent antioxidant properties of L1. The inhibition of FRD by L1 has been shown in almost all the levels of molecular, protein, cellular and tissue damage, under different conditions. Similar results have been obtained in clinical studies.

12. THE ANTIOXIDANT EFFECTS OF DEFERIPRONE AND OTHER CHELATING DRUGS IN CLINICAL CONDITIONS INVOLVING FREE RADICAL TOXICITY AND TISSUE DAMAGE

Deferoxamine, L1 and many other chelators have been shown to have inhibitory effects on many models of free radical formation and toxicity both in vitro and in vivo. The possibility of using L1 in many clinical conditions, not only related to iron and other metal overload or imbalance, but also in conditions related to free radical toxicity in tissue damage has also been suggested more than 20 years ago (23). The principle mechanism of the antioxidant effects of L1 is its ability to inhibit free radical reactions and cascades initiated by iron and copper catalytic centers, which are thought to be involved at different stages of tissue damage in relation to increased oxidative stress.

There are many conditions described in the literature, where increased free radical formation and oxidative stress have been implicated in tissue damage, such as rheumatoid arthritis and other inflammatory conditions, atherogenesis, cancer, ischaemia reperfusion injury, radiation injury, ageing, iron and copper overload or decompartmentalisation toxicity, radioactive metal (eg Pu and U) toxicity etc (27).

In each condition there are many variable parameters related to the cause, duration and extent of free radical toxicity and tissue damage, all of which have to be identified and considered for designing protocols and for increasing the prospects of therapeutic application of chelating or antioxidant drugs in relation to oxidative stress and FRD. These include the identification of the cause(s) and mechanism(s) of the toxicity, the accessibility of the target tissue, the level and rate of the oxidative stress, the therapeutic level of the chelating and or antioxidant drug(s), the pharmacology, pharmacokinetic and toxicity properties of the antioxidant etc. Within this context no direct studies have yet been carried out to determine all these parameters, but a number of clinical findings have been reported providing encouraging evidence and increasing prospects of wider applications of L1 and other chelating drugs in antioxidant therapies in different clinical conditions.

Despite that L1 was primarily designed for the removal of iron in the treatment of thalassaemia and other transfusional iron loaded patients, many other categories of non-iron loaded patients have been studied in clinical trial settings and also in normal volunteers. These include patients with renal dialysis, malaria, rheumatoid arthritis with the anaemia of chronic disease, Friedreich ataxia, acute kidney failure, diabetic nephropathy etc (5).

There is a large amount of information from the clinical studies involving L1 in iron loaded and non-iron loaded patients, all of which have a bearing in the design of dose protocols for its use in antioxidant therapies. Within this context the selection of the appropriate dose protocols may cause different effects. Doses as small as 10 mg/kg have for example been shown to be sufficient in causing a net increase in urinary iron excretion in iron loaded myelodysplasia patients, suggesting that L1 competes effectively with endogeneous chelators at this dose and also that low levels of labile, potentially toxic iron can be removed at such low doses (78). In other studies, iron overload induced cardiac failure has been shown to be reversed and excess cardiac iron to be normalised in thalassaemia patients treated with L1 long term (about a year) at effective doses of more than 85 mg/kg/day (79). Furthermore, it was also observed in regularly transfused iron loaded thalassaemia patients that at such doses iron accumulation in the heart, liver and other organs could be prevented through the removal of transferrin and non-transferrin bound iron, which is present in excess in such patients (6, 80).

In contrast to iron loaded patients, studies in non iron loaded patients have also shown that intracellular iron is mobilised and exchanged with transferrin resulting in a temporary and reversible increase in transferrin iron saturation and possibly iron redistribution and normalisation (40). The latter has also been observed for example in patients with localised excess iron in the reticuloendothelial system in the anaemia of chronic disease of rheumatoid arthritis patients and in the mitochondria in Friedreich ataxia patients (81-83). It would appear that there is a potential role of a balancing mode of action by L1, which overrides pathological mechanisms in the abnormal distribution and toxicity of iron and which causes the redistribution of localised excess intracellular toxic iron to non toxic metabolic forms intracellularly or extracellularly to other tissues (5, 6).

Another important parameter in the possible application of L1 and other chelators in antioxidant therapies is safety, especially during long term administrations. In addition to the short term administration safety results of L1 in renal dialysis, malaria and rheumatoid arthritis patients, there are also further encouraging results during the long term use of L1 in acute kidney failure patients, Friedreich ataxia and thalassaemia patients with normal iron stores, which suggest that L1 is non toxic during long term administration in non iron loaded patients (5, 83-85). It may be envisaged that iron deficiency anaemia can be caused during the long term administration of L1. However, since the increase in urinary iron excretion in normal individuals taking L1 is about 1-2 mg/day, this amount of iron can easily be replaced from dietary iron and iron deficiency anaemia be prevented during long term treatments (32,40).

In addition to the wide variation in the selection of therapeutic doses in antioxidant therapies, there is also wide difference with regards to the cause and ultrastructural changes in the tissue damage observed in each of the conditions described above, as well as the mode of the repair mechanisms caused by L1 and other chelators. This can be illustrated in the following examples of clinical conditions.

Iron overload toxicity is usually caused from increased gastrointestinal iron absorption (primary haemochromatosis) or red blood cell transfusions (secondary haemochromatosis) or combination of both. In primary haemochromatosis excess iron is accumulated in the hepatocytes of the liver causing progressive damage and increased mortality mainly due to hepatocellular carcinoma (9, 86). Mitochondrial, lysosomal and DNA structural and functional damages as well as increased oxidative stress and reduction of antioxidants appear to be associated with the progressive damage of the liver in this condition (86). Similar ultrastructural pathological changes are observed in the liver, the heart and possibly other organs in iron overload toxicity in secondary haemochromatosis (10, 11, 87).

In transfusional iron overload in thalassaemia patients, excess iron from transfused senescent red blood cells is accumulated in the Kuppfer cells and the hepatocytes of the liver and in other organs such as the pancreas and the heart, causing progressive damage and increased mortality mainly due to congestive cardiac failure. Ultrastructural pathological examination of cardiac biopsies of thalassaemia patients, who suffered congestive cardiac failure, revealed that there is variable distribution of excess storage iron in myocytes, with extensive iron deposition mainly in the form of haemosiderin in large secondary lysosomes and in the form of ferritin ring structures located inside small primary lysosomes. Damaging effects of excess iron include the disruption of lysosomes, substantial loss of myofilaments, the presence of large cytoplasmic vacuoles, increased amounts of heterochromatin and swollen mitochondria with no iron deposits but with loss of their cristae (87). Similarly, in several other studies it has also been shown that excess iron seems to be associated with many fold increases in the oxidative stress in the peripheral blood of the patients (88-90). The clearance of excess cardiac iron following treatment with L1 has been shown to be associated with the reversal of the cardiomyopathy (87). Similarly, the combination therapy of DF and L1 was shown to reduce excess liver iron and to improve glucose metabolic disturbances and diabetes mellitus (91).

Increasing number of clinical studies have been undertaken to examine the effects and possible applications of L1 in many other conditions related to iron and copper overload or abnormal metabolism, as well as many conditions associated with free radical toxicity and FRD in patients with normal iron and copper iron stores.

In the condition Friedreich ataxia increased iron accumulation in the mitochondria and oxidative damage is observed as a result of abnormalities in the metabolic pathway of iron-sulphur and heme iron formation. This abnormality affects mainly sensory neurons, the myocardium and the endocrine glands. Some improvement of the condition has been shown using antioxidant therapy (92). The possibility of using L1 to remove and redistribute excess localised toxic iron in the brain and the heart as well as other organs in Friedreich ataxia patients was suggested as long ago as 2003 (5). A clinical study involving nine Friedreich ataxia patients, who were treated for 6 months with a dose of 20-30 mg/kg/day of L1 has shown that L1 can reduce excess toxic iron in the brain, reduce ataxic gait and neuropathy in general (86). No toxic side effects were observed during the study. Similar improvement results are expected from the use of L1 in patients with the Hallervorden-Spatz syndrome, where excess toxic iron is present in the brain (93).

Major advances in the use of L1 in acute kidney disease and other related conditions have also taken place in the last few years. Increased toxic iron and oxidative damage has been detected in patients with glomerulonephritis, diabetic nephropathy and healthy volunteers who received radiocontrast agents. In all these cases L1 seems to ameliorate the toxic effects of labile toxic iron and to improve the condition of the affected patients.

In 14 patients with primary glomerulonephritis the treatment with L1 at 50-75 mg/kg/day for 6 months led to consistent reduction in proteinuria (about 50%) regardless of aetiology (84). Similarly, 37 patients with diabetic nephropathy who were treated with L1 at a mean dose of 50 mg/kg/day for 9 months have shown significant reduction in albumin to creatinine ratio at the end of the study period. These findings suggest that L1 can be used to halt the progression of kidney disease and other related renal and cardiovascular symptoms (85).

There are many other clinical findings reporting substantial improvements in the prognosis of patients treated with L1, which may be related to the reduction of oxidative stress such as the reduction of requirements of red blood cell transfusions in regularly transfused patients in thalassaemia and other conditions, reversal of bone marrow failure in Fanconi anaemia etc (94, 95). In these and many other cases L1 may inhibit free radical damage and reduce oxidative stress, which is caused by endogeneous or exogeneous cytotoxic factors, such as labile low molecular weight iron.

13. FUTURE PROSPECTS OF THE USE OF DEFERIPRONE AND OTHER CHELATORS AS ANTIOXIDANT PHARMACEUTICALS

There is a wide spectrum of conditions and increase prospects of the clinical use of antioxidant therapies including the use of chelating drugs, especially L1. In each case the therapeutic approach is different and depends on the cause of the toxicity, the underlying disease, the target tissue, the interaction with other forms of treatments, the therapeutic index of the antioxidant, the length of treatment

etc. Some of the major questions in the ongoing battle against oxidative stress and the use of antioxidants in different conditions are mainly related to the timing of administration of the antioxidant, the method of administration and the dose to be used.

Although the prophylactic use of antioxidants is widely advertised and promoted, more appropriate antioxidant therapies could be established by identifying and measuring an oxidative stress byproduct or indicator or the use of a radical probe or method for measuring excess toxic free radical production in each case and for each FRD related condition. Some methods, probes and byproducts are used for determining excess oxidative stress but these are not standardized for each condition nor are these widely available for use in patients.

Another parameter to be considered in antioxidant therapies is the duration of administration. The prophylactic long term administration of antioxidants could be considered in many conditions such as ageing, cardiomyopathy, liver disease, protection of the skin from excess sunlight toxic radiation during the summer, anti-wrinkle creams for the skin, conditions with increased oxidative stress eg iron and copper overload etc. Long term use of antioxidants could also be considered in chronic conditions with increased oxidative stress and tissue damage such as rheumatoid arthritis, atherosclerosis, cancer, Alzheimer's and Parkinson's disease, radiation accidents with radioactive metal (eg Pu, U) in the nuclear industry etc. Short term use of antioxidant therapies could be used in burns, radiotherapy, stroke, drug toxicities etc.

In all the above and many other conditions the cause of FRD should be identified and the appropriate antioxidant targeted therapy introduced. Such therapies have also to be considered within the context of other therapies of the underlying condition and possible interactions with other drugs. Antioxidant therapies involving a combination of lipid and water soluble natural antioxidants or antioxidant chelating drugs, as well as combinations of natural antioxidants with antioxidant chelating drugs may be more effective than antioxidant monotherapies. These combinations may also offer more potent antioxidant protection especially when the oxidative stress cannot be controlled and free radical toxicity and tissue damage is rapidly progressing. Similar combination therapies of L1 and DF have been used for the treatment of transfusional iron overload in thalassaemia and other conditions with very promising results. Selective dose protocols, especially the International Committee on Chelation (ICOC) protocol of L1 at 80-110 mg/kg/day and DF at 40-60 mg/kg at least 3 days per week, were sufficient to normalize the iron stores of chronically transfused iron loaded thalassaemia patients. It is envisaged that similar dose protocols could be used as potent antioxidant chelation therapies with or without combinations with other antioxidants (96).

14. CONCLUSIONS

There is increasing evidence from in vitro, in vivo and clinical results that L1 is one of the most potent antioxidant drugs, which can be used as a monotherapy or in combination therapies with other chelating drugs and also with classical antioxidants for the treatment of oxidative stress related tissue damage in many conditions. The antioxidant effects, high therapeutic index, oral administration and long term experience in iron loaded and non-iron loaded patients with L1, increases the prospects of its wider application as an antioxidant pharmaceutical. Its ability to reach high therapeutic concentrations in extracellular and intracellular compartments of many tissues with increased oxidative stress, including the heart and the brain, as well as its ability to inhibit both iron and copper catalysed free radical reactions, could be considered as some of the most important parameters for its therapeutic antioxidant mode of action.

There is wide variation in the conditions affected by oxidative stress and no established diagnostic methods are yet available for its measurement or methods for targeting the tissue affected. Within this context the criteria for selecting antioxidant therapeutic protocols can not yet be fully established. Despite these limitations, as well as the safety considerations, increasing number of patients with various conditions have been receiving L1 with encouraging results. This wider application process and the establishment of new antioxidant therapies, which will include L1 is likely to continue mainly because of its high safety profile and unique properties. Very few drugs are known to be administered at such high doses as L1 and to have so few toxic side effects. Within this context a new era in antioxidant therapies is emerging with increasing prospects of limiting oxidative stress and tissue damage in many clinical conditions using chelating drugs and especially L1.

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Abbreviations: FRD: Free radical damage; FRP: Free radical products; deferiprone: L1; deferoxamine: DF; deferasirox: DFRA; MWt: molecular weight ; ip: intraperitoneally; ig: intragastrically

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