ANTIHISTAMINES AS NEUROPROTECTIVE AGENTS

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ABSTRACT

Biomolecules are continuously exposed to potentially harmful oxidative stress as a consequence of free radical formation. Increased free radical generation has been associated with aging as well as neurodegenerative disorders. Antioxidants affect processes associated with oxidative stress by quenching free radicals and acting as oxygen scavengers. Parkinson's disease results from a deficiency in the neurotransmitter, dopamine and it is also evident that damage caused by free radicals play an important role in the progression of this neurodegenerative disorder. The histamine H_1 antagonist, diphenhydramine is used to treat mild cases of Parkinson's disease and cimetidine can scavenge hydroxyl radicals. The histamine H_3 antagonists are known to promote the release of dopamine. Together with a free radical scavenging activity, these compounds might have a dual therapeutic effect in the reduction of the progression of Parkinson's disease. The aim of this study was thus to determine whether histamine antagonists could act as free radical scavengers and to compare the results with aspirin, a known free radical scavenger. Ibuprofen was included to compare the free radical scavenging activities of aspirin to another non-steroidal anti-inflammatory drug.

The free radical scavenging activities of the following compounds were determined and compared: diphenhydramine; cimetidine; roxatidine; clobenpropit; impentamine; thioperamide; aspirin and ibuprofen. The ORAC assay was used to determine whether the test compounds were able to scavenge peroxyl radicals and the FRAP assay was used to determine the reducing abilities of the compounds. No meaningful results were obtained from these two assays, suggesting that the compounds were not able to act as direct antioxidants or as peroxyl radical scavengers. The comet assay was used to determine whether the compounds were able to reduce oxidative damage after MPTP administration. No damage was however obtained after a single dose of MPTP and it is suggested that one year old mice and chronic rather than acute treatment is used. Using a cyanide model to induce neurotoxic effects in rat brain homogenates, the neuroprotective properties of the histamine antagonists were examined and compared to aspirin. Superoxide anion levels and malondialdehyde concentrations were assessed using the nitroblue-tetrazolium and lipid peroxidation assays. Clobenpropit and thioperamide significantly reduced superoxide anion generation and lipid peroxidation. At a concentration of 1mM, these two histamine H_3 antagonists reduced lipid peroxidation to values lower than that of the control. Impentamine reduced lipid peroxidation at all concentrations used and superoxide anion generation at a concentration of 1mM. Diphenhydramine (0.25 and 0.5mM) significantly reduced both variables at lower concentrations. Cimetidine (1mM) was able to reduce superoxide anion generation and significantly reduced lipid peroxidation at all concentrations

used. Roxatidine (0.5mM and 1mM) significantly reduced the rise in superoxide anion generation and significantly reduced malondialdehyde concentration in a dose dependent manner. Ibuprofen significantly decreased superoxide anions in a dose dependant manner and lipid peroxidation at a concentration of 1mM. In the lipid peroxidation assay, all the drugs compared favourably to aspirin. The *in vivo* free radical scavenging effects of the selected compounds were also examined with the nitroblue tetrazolium and lipid peroxidation assays. MPP^+ was used to induce a Parkinsonian like condition. Diphenhydramine, ibuprofen, thioperamide and clobenpropit significantly reduced free radical generation in both assays. Thioperamide and clobenpropit were able to reduce lipid peroxidation and superoxide anion generation to values lower than that of the control, suggesting that these two compounds could be able to attenuate normal free radical processes in the brain. Cimetidine did not have the expected *in vivo* scavenging effects and it is suggested that blood-brain barrier permeability might play a role. All the compounds, except diphenhydramine had significantly lower *in vivo* values than aspirin.

The superoxide anion plays an important role in the formation of further free radicals. It leads to the formation of peroxyl radicals during the inititation step of lipid peroxidation and also leads to the generation of hydroxyl radicals, where transition metals like iron, is a key factor. Diphenhydramine at lower concentrations, and the newly discovered histamine H_3 antagonists, clobenpropit and thioperamide significantly reduced superoxide anion generation and lipid peroxidation. Although the compounds did not have meaningful effects in the FRAP and ORAC assay, their significant ability to reduce lipid peroxidation and superoxide levels make them promising tools to attenuate oxidative damage.

This study demonstrates the potential of these agents to be neuroprotective with a dual therpeutic effect by exerting a scavenging effect on superoxide anions and increasing dopamine levels.

OPSOMMING

Die liggaam word voortdurend blootgestel aan vry radikale. Hierdie radikale veroorsaak oksidatiewe stres wat beskadiging en apoptose van selle tot gevolg het en moontlik 'n rol speel by neurodegeneratiewe siektes soos Parkinsonisme. Antioksidante verminder die effekte van vry radikale deur dit op te ruim en te vernietig. Parkinsonisme word gekenmerk deur 'n gebrek aan dopamien in die brein asook die teenwoordigheid van vry radikale. Difenhidramien, 'n histamien H_1-antagonis, word gebruik om minder ernstige gevalle van Parkinsonisme te behandel en simetidien is bekend vir die vermoë om hidroksielradikale op te ruim. Die histamien H_3-antagoniste bevorder die vrystelling van dopamien. Indien die verbindings vry radikale kan opruim en dopamien vrystelling kan bevorder, sal 'n dubbele beskermings effek moontlik teen Parkinsonisme verkry word. Die doel van hierdie studie was om te bepaal of antagoniste van die histamienreseptor as vryradikaalopruimers kan optree en om die resultate met aspirien, 'n bekende vryradikaalopruimer, te vergelyk. Ibuprofeen, 'n nie-steroïed anti-inflammatoriese middel, is in die studie ingesluit om die vryradikaalopruimingseffek daarvan met aspirien te vergelyk.

Die vermoë van die volgende verbindings om vry radikale op te ruim is bepaal: difenhidramien; simetidien; roksatidien; klobenpropit; impentamien; tioperamied; aspirien en ibuprofeen. Die moontlikheid dat hierdie verbindings as antioksidante kan optree is geëvalueer deur middel van twee *in vitro* toetse wat die volgende ingesluit het: die vermoë om yster te reduseer (FRAP) en die vermoë van die toetsverbindings om suurstofradikale op te ruim (ORAC). Die resultate van die eksperimente dui daarop dat die verbindings nie direkte antioksidante is nie en ook nie peroksielradikale opruim nie. Met die doel om 'n *in vivo* model daar te stel vir die evaluering van antioksidante is die neurotoksien MPTP aan muise toegedien en DNA skade is met behulp van die komeetanalise bepaal. Geen DNA skade is egter opgemerk nadat MPTP eenmalig subkutaneus toegedien is nie. 'n Sianiedmodel in rotbreinhomogenate is ook gebruik om die meganisme van neurobeskerming van die histamienantagoniste te bepaal en te vergelyk met die van aspirien, 'n bekende vryradikaalopruimer. Die afname in superoksiedanioon vlakke en maloondialdehiedkonsentrasies is bepaal met die nitrobloutetrasolium- en lipiedperoksidasie-analises. Die superoksiedanioon konsentrasie en lipiedperoksidasie-aktiwiteit is betekenisvol deur klobenpropit en tioperamied verminder. Beide die verbindings het waardes laer as die kontrole gehad by 'n konsentrasie van 1 mM. Impentamien het lipiedperoksidasie verminder by alle konsentrasies wat gebruik is, maar superoksiedanione is net betekenisvol verminder by 1 mM. Difenhidramien was net effektief by lae konsentrasies (0.5 en 1 mM). Roksatidien (0.5 en 1 mM) en simetidien (1 mM) het die superoksiedanioon, asook lipiedperoksidasie by al die

konsentrasies wat getoets is verlaag. Die verbindings se vermoë om maloondialdehied-konsentrasies te verlaag het goed ooreengestem met die van aspirien. Die nitrobloutetrasolium- en lipiedperoksidasieanalises is ook gebruik om die *in vivo* effekte van die verbindings te bepaal. MPP^+, 'n metaboliet van MPTP, is gebruik om oksidatiewe skade in rotte te induseer. Die geïnduseerde vry radikale is betekenisvol verminder deur difenhidramien, ibuprofen, tioperamied en klobenpropit. Met albei analises het klobenpropit en tioperamied waardes beter as die kontrole gehad. Dit dui daarop dat hierdie verbindings in staat is om nie net geïnduseerde radikale te verminder nie, maar ook die radikale wat vrygestel word deur normale metaboliese prosesse in die brein, op te ruim. Simetidien het nie die verwagte verlaging in oksidatiewe skade tot gevolg gehad nie, moontlik omdat die konsentrasie in die brein te laag was. Al die verbindings wat *in vivo* getoets is, het betekenisvol beter waardes as aspirien, die bekende vryradikaalopruimer gehad en was dus meer effektief in die MPP^+ rot model.

Die superoksiedanioon speel 'n belangrike rol tydens die vorming van vry radikale. Tydens lipiedperoksidasie veroorsaak superoksiedanione die vorming van peroksielradikale. Superoksiedanione lei ook tot die vorming van hidroksielradikale wat op hulle beurt weer 'n sleutelfaktor is by oksidatiewe skade deur metale soos yster veroorsaak. Superoksiedanione en lipiedperoksidasie is betekenisvol verlaag deur difenhidramien, klobenpropit en tioperamied. Alhoewel die verbindings nie as direkte antioksidante optree en ook nie peroksielradikale opruim nie, het hulle uitstekende superoksiedanioonopruiming tot gevolg gehad. Hierdie vermoë van die toetsverbindings kan 'n belangrike rol speel in die verlaging van oksidatiewe skade.

Die resultate van hierdie studie dui dus daarop dat histamienantagoniste ook as superoksiedanioonopruimers kan optree en tesame met 'n verhoging in dopamien vlakke, in neurodegeneratiewe toestande aangewend kan word.

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BHT	Butylated hydroxytoluene
CAT	Catalase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
Cu	Copper
DAT	Dopamine transporter
DHA	Semi-dehydroascorbate radical
FE	Iron
FRAP	Ferric reducing antioxidant power
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
4-HNE	Trans-4-hydroxy-2-hexenal
H_2O	Water
H_2O_2	Hydrogen peroxide
HMPA	High melting point agarose
HOCL	Hypochlorous acid
KCN	Potassium cyanide
LMPA	Low melting point agarose
LPx	Lipid peroxidation
MAO-B	Monoamine oxidase B
MDA	Malondialdehyde
Mn	Manganese
MPP^+	1-methyl-phenylpyridinium ion
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NBD	Nitroblue diformazan
NBT	Nitroblue tetrazolium
NO	Nitric oxide
NSAIDS	Non-steroidal anti-inflammatory drugs
O_2	Oxygen
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anions
$OH^{\cdot}$	Hydroxyl radicals
8-OhdG	8-hydroxy-2'deoxyguanosine

$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbing capacity
PBS	Phosphate buffered saline
$RO^·$	Alkoxyl radical
$ROO^·$	Peroxyl radicals
ROS	Reactive oxygen species
SC	Subcutaneous
SCGE	Single cell gel electrophoresis
SN	Substantia nigra
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TBHP	*tert*-Butyl hydroperoxide
TCA	Trichloroacetic acid
TPTZ	2,4,6-tripyridyl-s-triazine
Vit A	Vitamin A
Vit E	Vitamin E
Zn	Zinc

CHAPTER 1:

Introduction

1.1. Introduction

Over-production of free radicals is implicated in aging and in various degenerative disorders (Kim *et al.*, 2002). Reduction of oxygen by the transfer of a single electron produces the superoxide anion, which is not in itself a particularly damaging species, but is a source of hydrogen peroxide and the toxicity thereof lies in its ability to inhibit certain enzymes (Cheeseman *et al.*, 1993; McCord, 2000). Another easily accessible target of free radicals are the presence of membrane phospholipids at sites where these damaging species are formed. Peroxidation of lipids in fatty acids may lead to a radical chain reaction known as lipid peroxidation (De Zwart *et al.*, 1999). A hydrogen atom is abstracted from a polyunsaturated fatty acid chain, leaving behind a carbon-centered radical in the membrane. This leads to the conversion of membrane lipids to lipid hydroperoxides and to the formation of malondialdehyde, a marker of oxidative damage (Halliwell, 1992). Antioxidants affect these processes by quenching free radicals and acting as oxygen scavengers (Gülcin *et al.*, 2002).

Parkinson's disease is one of the most common neurodegenerative diseases in humans. The exact cause of the disease remains largely unknown, but it is attributed to a variety of actions including depletion of dopamine, oxidative stress and alterations in antioxidant systems (Schapira *et al.*, 1993). Current treatment of Parkinson's disease can alleviate some of the symptoms in patients, but it does not alter the progressive course of the disease. The major symptoms of this disease are related to the loss of the midbrain dopamine neurons within the substantia nigra which causes profound dopamine depletion in the striatum. This is responsible for most of the motor symptomatology of the disease (Speciale, 2002).

It was found that the H_3 receptor acts as a heteroreceptor, regulating the release of neurotransmitters such as acetylcholine, serotonin, noradrenaline and dopamine. Antagonists of this receptor could thus have a therapeutical effect in neurological disorders.

Parkinson's disease is primarily characterised by disorders of movement resulting from a deficiency in the neurotransmitter, dopamine, in the basal ganglia of the brain due to destruction of dopaminergic neurons. A persistant increase in free radical generation is believed to be the

cause of neuronal death in this case. Previous studies have shown that induced denervation of dopaminergic neurons resulted in a marked increase in the density of histamine H_3 receptors in the striatum and substantia nigra of the rat suggesting that these receptors are under tonic dopaminergic influence (Ryu *et al.*, 1994). Reducing the H_3 mediated inhibition of dopamine release with histamine H_3 receptor antagonists and determing whether these antihistamines are able to reduce free radicals, could thus have therapeutic benefits in Parkinson's disease.

Diphenhydramine, a histamine H_1 antagonist, is used to treat mild cases of Parkinson's and therefore it is important to determine the mode of action of this compound (Kenneth *et al.*, 1996). If diphenhydramine is able to scavenge free radicals, a dual therapeutic effect will be obtained and more effective application of the drug might be possible.

Studies have also indicated that cimetidine is able to scavenge hydroxyl radicals, significantly *in vitro*. The drug may be effectively used as a hydroxyl scavenger *in vivo* suggesting that some therapeutical effects may be related to its antioxidant capacity (Lapenna *et al.*, 1994). I included roxatidine, another histamine H_2 antagonist, to determine whether this compound is also able to scavenge free radicals. If the histamine H_2 antagonists are able to effectively inhibit free radical generation *in vivo* and reduce oxidative damage, these drugs might be useful in diseases associated with oxidative stress.

It is believed that aspirin is an effective antioxidant and effectively scavenges free radicals (Carrasco & Werner, 2002). Therefore we used aspirin as a positive control to determine the free radical scavenging effects of ibuprofen and of histamine receptor antagonists. I included ibuprofen in our study to determine whether another anti-inflammatory compound is also able to scavenge free radicals.

When taking the above information into consideration, it is thus imperative to determine whether antagonists of the histamine receptor can act as free radical scavengers and have a dual therapeutic effect, increasing dopamine levels and protecting dopamine neurons against further degradation.

In this study the ability of selected compounds to reduce induced free radical generation is measured to determine whether these compounds might be useful in neurodegenerative diseases such as Parkinson's disease.

1.2. Aim of this study

The aim of this study was to investigate the possible free radical scavenging effects of histamine antagonists. *In vitro* and *in vivo* methods were used to assure reliable results.

Different methods are used to determine whether a compound is able to scavenge free radicals. As screening methods, we used the ferric reducing antioxidant power (FRAP) and the oxygen radical absorbance capacity (ORAC) assays to determine the oxidizing/reducing ability of the selected compounds. Compounds that are able to reduce free radical generation, will reduce oxidative stress and also oxidative damage. The single cell gel electrophoresis assay is a simple and reliable method to measure oxidative damage. N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin, was used to induce dopamine depletion and the ability of the compounds to reduce oxidative damage was determined.

To induce neurotoxic effects, a cyanide model was used *in vitro* and a Parkinsonian model *in vivo*. The neuroprotective properties of histamine receptor antagonists and ibuprofen were examined by looking at the ability of the compounds to reduce superoxide anion and malondialdehyde levels. Superoxide anion levels and malondialdehyde concentration were assessed using the nitroblue-tetrazolium and lipid peroxidation assays. The results were compared to a known free radical scavenger, aspirin.

To achieve the aim of this study the following objectives were set:

- Screening method to determine *in vitro* antioxidative activity using the FRAP assay.
- Determination of *in vitro* peroxyl scavenging activity using the ORAC assay.
- Determination of the abilities of the compounds to reduce oxidative damage using the single cell gel electrophoresis assay.
- Determination of the ability of compounds to reduce superoxide anions *in vitro* and *in vivo*, using the nitro blue tetrazolium assay.
- Determination of the ability of compounds to reduce malondialdehyde concentration *in vitro* and *in vivo* using the lipid peroxidation assay.
- Comparison of the free radical scavenging activity of the compounds to a well known free radical scavenger, aspirin.
- Determination of the free radical scavenging activity of another anti-inflammatory agent, ibuprofen.

CHAPTER 2

Free radicals, oxidative stress, antioxidants, and neurodegenerative diseases

2.1 Free radicals

During the course of their lifetime, biological structures are continuously exposed to potentially harmful oxidative stress as a consequence of free radical formation (Pryor *et al.*, 1983). Free radicals are any chemical species capable of independent existence that contains an unpaired electron. Free radicals are unstable and highly reactive, pooling electrons from surrounding molecules (Girotti *et al.*, 2002).

The fact that free radicals are highly reactive means that they have low chemical specificity and can react with most molecules in their vicinity. To gain stability, an electron is captured and the original state is lost when reacting with surrounding molecules.

A free radical is formed when a covalent bond between entities is broken and one electron remains with each newly formed entity. The newly formed free radical starts a chain reaction and this leads to a cascade finally resulting in the disruption of a living cell. Free radicals are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis.

In biological systems free radical species can be formed in one of three ways. These include haemolytic cleavage, heterolytic fission and electron transfer reactions. They are generally produced in cells by electron transfer reactions. The major source of free radicals in human cells is through electron leakage from the electron transport chain located in the endoplasmic reticulum and mitochondria (Cheeseman *et al.*, 1993). Vital biological structures such as DNA, proteins and lipids can be damaged and cellular functions disrupted by free radical reactions (Girotti *et al.*, 2002; Chen *et al.*, 1994; Mecocci *et al.*, 1993; Sohal *et al.*, 1995; Dean *et al.*, 1997).

Table 2.1: Biologically significant free radicals (Faoud, 2003)

Reactive oxygen species	
O_2^-	Superoxide anion
•OH	Hydroxyl radical
ROO•	Peroxyl radical
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen
NO	Nitric oxide
$ONOO^-$	Peroxynitrite
HOCL	Hypochlorous acid

The main source of free radicals is molecular oxygen. The univalent reduction of molecular oxygen results in reactive oxygen species such as superoxide anions, hydroxyl radicals, nitric oxide and peroxyl radicals. Hydrogen peroxide can lead to the formation of free radicals through various chemical reactions. Thus hydrogen peroxide, in the presence of a reduced metal, forms the highly reactive hydroxyl radical via the Fenton reaction (table 2.1; figure 2.2) (Cadenas, 1995).

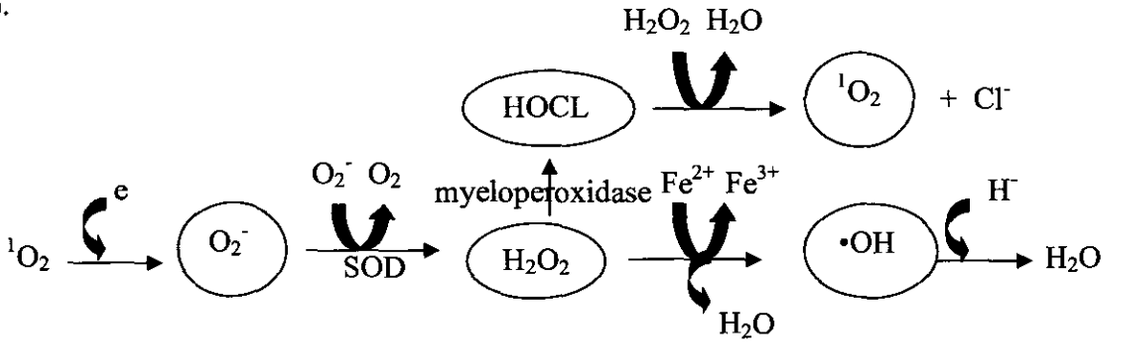

Figure 2.1: Generation of reactive oxygen species.

The generation of reactive oxygen species is induced by the oxidation of singlet oxygen leading to the formation of superoxide anions. Myeloperoxidase leads to the formation of hypochlorous acid (HOCL) from hydrogen peroxide and the hydroxyl radical is generated through the Fenton reaction. Hypochlorous acid is converted to singlet oxygen and a chloride anion (figure 2.1) (Fouad, 2003).

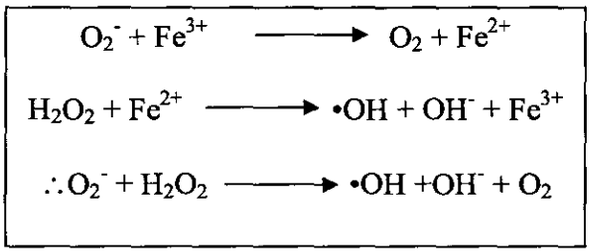

Figure 2.2: The Fenton reaction (Cadenas, 1995)

Reactive oxygen species are increased under stressed conditions. These cytotoxic species cause oxidative damage in the cell. Products of free radical oxidative damage are formed from amino acids, nucleic acids, lipid peroxides and directly from reactive oxygen species (Figure 2.3) (De Zwart *et al.*, 1999).

Free radicals may react with different cellular macromolecules such as DNA, cell membranes and proteins which may lead to many different products (figure 2.3) (De Zwart *et al.*, 1999).

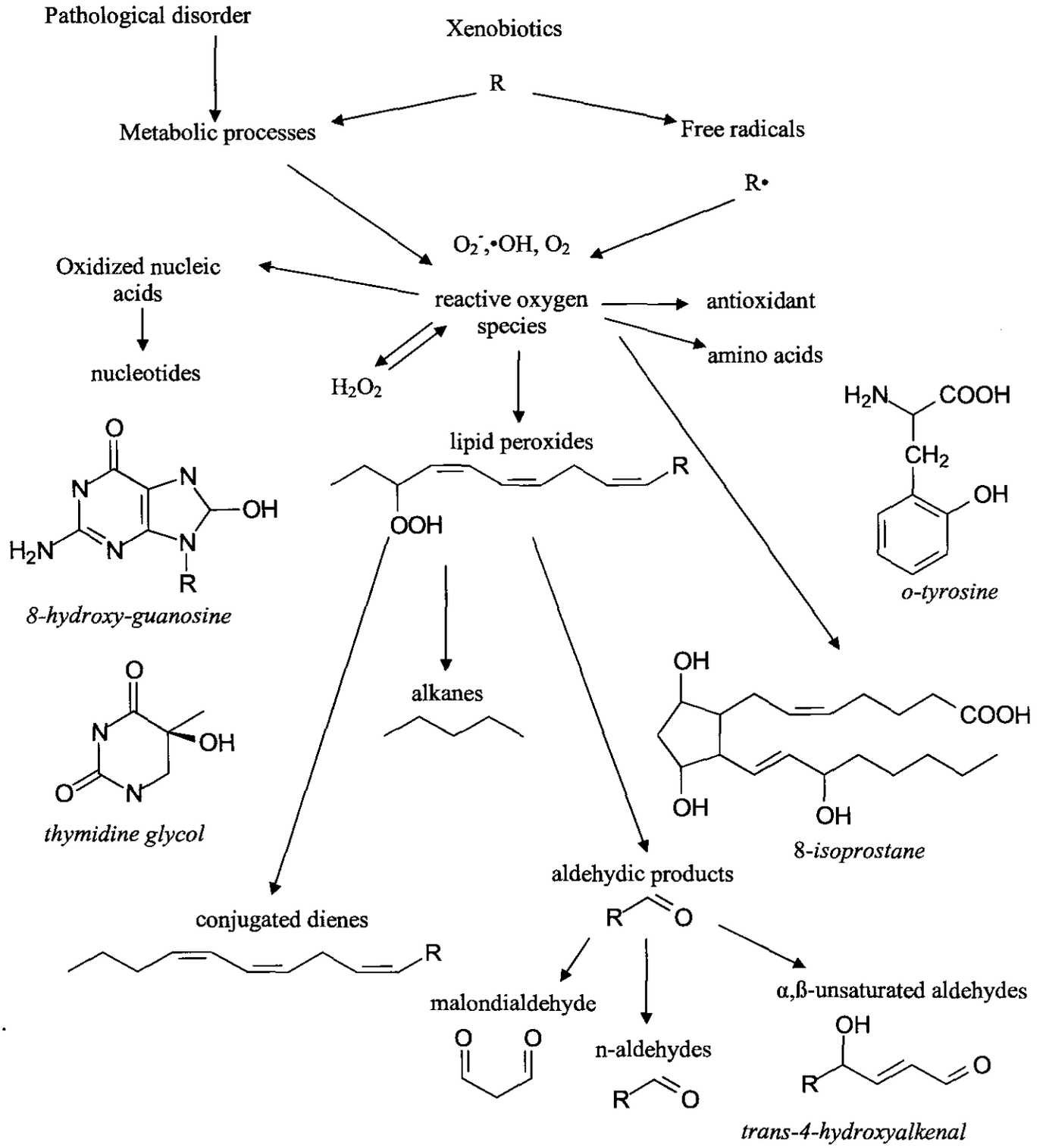

Figure 2.3: Products of free radical damage (De Zwart *et al.*, 1999).

Ageing can be viewed as a process of irreversible injuries associated with oxidative changes (Inal *et al.*, 2001). Reactive oxygen species are involved in the pathogenesis of various diseases

including cancer, rheumatoid arthritis, heart and arterial diseases, as well as in degenerative processes leading to neurological disorders and degeneration, such as Parkinson's and Alzheimer's diseases (Gaboriau *et al.*, 2002; Sagar *et al.*, 1992; Ames *et al.*, 1993).

2.1.1 Types of free radicals

2.1.1.1 The superoxide anion

The toxicity of the superoxide anion is seen in its ability to inhibit certain enzymes, its effects on major classes of biological molecules and the formation of hydrogen peroxide (McCord, 2000).

Although the superoxide anion is a free radical, it is not a particularly damaging species, but is often at the start of the oxidative stress cascade. It is mostly reductive in nature and its main significance is probably as a source of hydrogen peroxide and as a reductant of transition metal ions (Cheeseman *et al.*, 1993). The superoxide anion is generated enzymatically by a number of oxidases, including xanthine oxidase and cytochrome oxidase (Reiter *et al.*, 1995) as well as non enzymatically. Reduction of oxygen by the transfer of a single electron will produce the superoxide free radical anion (figure 2.4).

$$O_2 + e^- \rightarrow O_2^-$$

Figure 2.4: Generation of the superoxide anion.

The mitochondrium is one of the main producers of superoxide anions (McCord, 1985). During the production of ATP via the electron transport chain, the oxygen molecule can be reduced to the superoxide anion (Dawson *et al.*, 1996). The body protects against this by kinetically restricting these reactions. Nevertheless high-energy electrons come in close proximity to oxygen resulting in the formation of superoxide anions (Coyle. *et al.*, 1993).

Superoxide radicals can also be generated chemically by auto-oxidative reactions with catecholamines, tetrahydrofolates and reduced flavins. This can lead to a free radical chain

reaction as the production of the superoxide anion leads to the autocatilisation of more superoxide anions.

It has also been demonstrated that the superoxide anion is produced by the metabolism of arachidonic acid by lipoxygenases and cyclooxygenases to form eiconasoids (Cunha, *et al.*, 1997).

Superoxide anion generation and the efficiency of antioxidants to reduce the generation thereof can be determined with the nitroblue tetrazolium assay (section 4.4).

2.1.1.2 Hydrogen peroxide

Hydrogen peroxide is often generated in biological systems through the production of superoxide. Two superoxide molecules react to form hydrogen peroxide and oxygen (figure 2.5).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Figure 2.5: Generation of hydrogen peroxide.

Hydrogen peroxide crosses cell membranes and can inactivate cell enzymes (Beal, 1995). Although hydrogen peroxide is not a free radical, it can be toxic to cells at high concentrations. Hydrogen peroxide is unique, because it can be converted to the highly damaging hydroxyl radical or be catalysed and excreted as water. Glutathione peroxidase is essential for the conversion of glutathione to oxidised glutathione, during which hydrogen peroxide is changed to water (Allessio *et al.*, 1997). If hydrogen peroxide is not converted into water, singlet oxygen is formed. Singlet oxygen can transfer energy to a new molecule and act as a catalyst for free radical formation (Reiter *et al.*, 1995).

2.1.1.3 The hydroxyl radical

The hydroxyl radical is the most reactive free radical as it will react with almost all molecules in living cells. It is so reactive that no enzyme system utilising it as a substrate exist (Delibas *et al.*, 2002). The hydroxyl radical is generated from oxygen and hydrogen peroxide via the Fenton reaction (figure 2.2; figure 2.6).

The hydroxyl radical reacts with a number of molecules in living cells, including DNA, which causes changes in the composition of deoxyribose and of the purine and pyrimidine bases. The cytotoxicity of hydroxyl radicals in human cells seems to involve DNA damage (Buettner *et al.*, 1978).

$$O_2^- + H_2O_2 \rightarrow O_2 + \bullet OH + \bullet OH$$

Figure 2.6: Generation of the hydroxyl radical.

This molecule is very reactive and has an extremely short half-life, but is capable of causing great damage within a small radius of production. Most of the hydroxyl radicals generated are formed when transition metals (Oliver *et al.*, 1990) or biologically active chelators, such as porphyrins and flavins are present simultaneously with an oxidant, such as hydrogen peroxide (Poeggeler *et al.*, 1994).

2.1.1.4 The peroxyl radical

Another type of one-electron transfer reaction that contributes to the formation of oxyradicals involves the quenching of carbon centered radicals by molecular oxygen. This reaction leads to the formation of peroxyl radicals (figure 2.7).

$$R\bullet + O_2 \rightarrow ROO\bullet$$

Figure 2.7: Formation of peroxyl radicals.

The generation of electrophilic peroxyl radicals can be measured by a variety of experimental models such as the lipoxygenase reaction. The lipoxygenase-catalysed breakdown of peroxidised fatty acids initially produces alkoxyl radicals, which reacts with peroxides to yield peroxyl radicals.
Peroxyl radicals react with unsaturated fatty acids, thus leading to lipid peroxidation (Cadenas, 1995).

2.1.1.5 Singlet oxygen

Singlet oxygen is known as a nonradical reactive oxygen species, often associated with free radicals, that has strong oxidising activity. Singlet oxygen is an electronically excited and mutagenic form of oxygen. It is generated by input of energy, for example radiation, enzymatically, through thermo-decomposition of dioxetanes, during the respiratory burst of phagocytes and when they are illuminated in the presence of oxygen (Fouad, 2003). Preferential targets for chemical reactions are double bonds, for example in polyunsaturated fatty acids or guanine bases in DNA (Stahl *et al.*, 2000).

2.1.1.6 Nitric oxide

Nitric oxide is a common gaseous free radical recognised to play a role in vascular physiology and is also known as an endothelium derived relaxing factor. Vascular endothelium produces nitric oxide from arginine with the use of the enzyme nitric oxide synthetase (Fouad, 2003).

2.1.1.7 Hypochlorous acid

Hypochlorous acid (HOCl) is generated by the action of myeloperoxidase on chloride ions in the presence of hydrogen peroxide (figure 2.8).

$$H_2O_2 + Cl^- \rightarrow HOCl + \bullet OH$$

Figure 2.8: Generation of HOCl.

Hypochlorous acid can cross cell membranes and generate hydroxyl radicals in the presence of transitional metal ions. It has been shown to be capable of initiating lipid peroxidation, combining with hydrogen peroxide to damage DNA and DNA repair processes and altering intracellular free calcium (Ca^{2+}) (Fouad, 2003).

2.1.2 Sources of free radicals

2.1.2.1 Endogenous sources

Autoxidation

Autoxidation is a by-product of the aerobic internal milieu. Autoxidation of any molecule in a reaction results in the reduction of the oxygen diradical and the formation of reactive oxygen species. Superoxide is the primary radical formed by this process (Fouad, 2002).

Enzymatic oxidation

A variety of enzyme systems are capable of generating significant amounts of free radicals, including xanthine oxidase, prostaglandin synthase, lipoxygenase, aldehyde oxidase and amino acid oxidase (Halliwell *et al.*, 1995).

Respiratory burst

Respiratory burst describes the process by which phagocytic cells consume large amounts of oxygen during phagocytosis. The phagocytic cells possess a NADPH-oxidase system that initiates a respiratory burst at the cell membrane leading to superoxide production (Fouad, 2003).

Sub cellular organelles

Organelles such as mitochondria, chloroplasts, microsomes, peroxisomes and nuclei have been shown to generate superoxide anions. The mitochondrium is the main cellular organelle for cellular oxidation reactions and the main source of reduced oxygen species in the cell. The leaks in the mitochondrial electron transport system allow oxygen to accept a single electron, forming the superoxide anion (Halliwell, 1995).

Transition metals ions

Iron and copper play a major role in the generation of free radical injury and the facilitation of lipid peroxidation. Transition metal ions participate in the reaction that generates hydroxyl radicals from the superoxide anion and hydrogen peroxide (Fouad, 2003).

Ischemia reperfusion injury

During ischemia two processes occur, first the production of xanthine and xanthine oxidase are greatly enhanced. Secondly, there is a loss of both antioxidant superoxide dismutase and glutathione peroxidase. The molecular oxygen supplied on reperfusion serves as an electron acceptor and cofactor for xanthine oxidase causing the generation of the superoxide anion and hydrogen peroxide (Fouad, 2003).

2.1.2.2 Exogenous sources

Drugs

A number of drugs can increase the production of free radicals in the presence of increased oxygen stress. These agents appear to act additively with hyperoxia to accelerate the rate of damage. These drugs include antibiotics that depend on quinoid groups or bound metals for activity, antineoplastic agents and methotrexate (Fouad, 2003).

Radiation

Radiotherapy may cause tissue injury that is caused by free radicals. Primary radicals are formed which can undergo secondary reactions with oxygen or cellular solutes (Fouad, 2003).

Tobacco smoking

Oxidants in tobacco exist in sufficient amounts to suggest that they play a major role in injuring the respiratory tract (Fouad, 2003).

Inorganic particles

Inhalation of inorganic particles, also known as mineral dust, can lead to lung injury that seems in part to be mediated by free radical production (Fouad, 2003).

Gases

Ozone is not a free radical, but a very powerful oxidising agent that degrades under physiological conditions to hydroxyl radicals. Free radicals are thus formed when ozone reacts with biological substrates (Fouad, 2003).

2.1.3 Molecular targets and markers of oxidative damage

Proteins, lipids, and nucleic acids can be modified by reactive oxygen species. A wide variety of products may be generated from oxidative damage, but their relevance in biological settings is limited by the specificity and sensitivity of the methods that can be used for their detection.

Amino acids can also be the object of oxidative damage and aromatic amino acids, histidine, methionine and cysteine are particularly sensitive. The detection of protein carbonyls has been widely used as an index of protein oxidation (section 2.1.3.3).

Fatty acids, especially when polyunsaturated, are damaged by reactive oxygen species through the abstraction of hydrogen atoms from methylene groups resulting in lipid peroxidation.

Glutathione, a cellular thiol and endogenous antioxidant, is a very important compound in the defence system against reactive oxygen species. Glutathione reacts directly with radicals in nonenzymatic reactions and is an electron donor in the reduction of peroxides catalysed by glutathione peroxidase (figure 2.15). When oxidative stress occurs, the levels of reduced glutathione are significantly decreased and can be measured, indicating an important biomarker of oxidative damage (Dringen, 2000).

2.1.3.1 Lipid peroxidation

Lipid peroxidation is probably the most extensively investigated process induced by free radicals (Mahadik *et al.*, 1999). The presence of membrane phospholipids at sites where radicals, specifically reactive oxygen species are formed render them easily accessible endogenous targets, rapidly affected by free radicals. This group of polyunsaturated fatty acids is highly susceptible to reactions with free radicals. Peroxidation of lipids in fatty acids may lead to a radical chain reaction with three main features (figure 2.9).

Initiation	ROS• + RH ⟶ ROSH + R•	Reaction 2.9a
Reaction	R• + Oxygen ⟶ ROO•	Reaction 2.9b
	ROO• + RH ⟶ R• + ROOH	Reaction 2.9c
Termination	2 ROO• ⟶ Non-radical products	Reaction 2.9d

Figure 2.9: Chain sequence for free radical auto-oxidation (Pieri *et al.*, 1994).

During the initiation step (reaction 2.9a) polyunsaturated fatty acids are attacked by reactive oxygen species (ROS•), resulting in the removal of a hydrogen atom from the polyunsaturated fatty acid (RH) and the formation of a lipid-derived radical (R•). The propagation step (reaction 2.9b) normally begins with the rapid addition of molecular oxygen to R•, to form the lipid peroxyl radical. This radical can attack other lipids, to generate a further lipid radical and a lipid hydroperoxide (ROOH). A self perpetuating autocatalytic reaction then follows with reactions 2.9b and 2.9c undergoing a number of cycles (Krinsky, 1992). The final step is a termination step, which results when two peroxyl radicals react together to form a non-radical product (reaction 2.9d) (Burton *et al.*, 1989).

One substrate radical (R•) may result in the formation of many equivalents of lipid peroxides (LOOH). These degenerative propagation reactions in lipid membranes are usually accompanied by the formation of a variety of products, including alkanes and carbonyl compounds. Two products of lipid peroxidation have been extensively studied namely trans-4-hydroxy-2-hexenal (4-HNE) an unsaturated aldehyde because of its toxicity and malondialdehyde (MDA). Measured with the thiobarbituric acid-reactive substances (TBARS) assay, malondialdehyde is widely used as an index of lipid peroxidation (section 4.5) (Giasson. *et al.*, 2002; Delibas *et al.*, 2002). Plasma malondialdehyde levels increase with age, indicating increased lipid peroxidation (Rodriguez-Martinez *et al.*, 1998).

The removal of reactive oxygen species by various antioxidant systems is therefore essential to limit lipid peroxidation occurring in cells.

2.1.3.2 Free radical damage to DNA

Reactive oxygen species can attack almost any cellular structure or molecule. However, in aging, DNA is considered a major target. Reactive oxygen species may cause DNA-protein cross-link, damage to the deoxyribose-phosphate backbone as well as specific chemical modifications of purine and pyrimidine bases.

Oxidative base modifications may result in mutations, whereas oxidation of deoxyribose modulation may induce base release or DNA strand breaks. Hydroxyl radicals generate multiple products and singlet oxygen preferentially modifies guanine. *In vivo*, damaged DNA is repaired by endonucleases and glycosylases, liberating deoxynucleotides and bases respectively. The bases are directly excreted in urine whilst the deoxynucleotides are further metabolised to mono-nucleosides and then excreted into the urine. Thymine glycol and thymidine glycol are two DNA hydroxylation products, but the most interest has been focused on measuring 8-hydroxy-2'-deoxyguanosine. The analytical method involves solid phase extraction and HPLC with electrochemical detection. (De Zwart *et al.*, 1999).

Many methods have been proposed to measure DNA damage. A reliable and quick method is the single cell gel electrophoresis assay (Collins *et al.*, 1997). This assay has a lot of advantages over other DNA damage methods because of its high sensitivity and the fact that DNA strand breaks are determined in individual cells (section 4.3) (Lee *et al.*, 2003).

2.1.3.3 Free radical damage to proteins

Protein oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino acid side chains, reactive oxygen-mediated peptide cleavage and from reactions with lipid and carbohydrate oxidation products. It is now becoming clear that the presence of carbonyl groups in proteins may indicate that the proteins have been subjected to oxidative free radical damage. An increase in protein carbonyl content of tissues is associated with pathological disorders such as arthritis, Alzheimer's disease, arteriosclerosis and Parkinson's disease.

Products of protein oxidation are ortho-tyrosine, dityrosine, gamma-glytamyl semialdehyde and 2-amino-adipic semialdehyde. These products may serve as biomarkers for oxidative free radical damage to proteins (De Zwart *et al.*, 1999).

2.2 Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidant protection mechanisms (Sies, 2000). Oxidative stress increases with age, leading to oxidative DNA damage, lipid peroxidation products and oxidative damaged proteins (Chevion *et al.*, 2000; Onorato *et al.*, 1998; Tahara *et al.*, 2001). Oxidative stress appears to serve as an initiator of cell death in neurons of the substantia nigra and any factor which would enhance oxidative species generation from dopamine, would likely hasten the onset of cell injury and subsequent death (Cadet *et al.*, 2000; Naoi *et al.*, 1999; Jones *et al.*, 2000; Rabinovic *et al.*, 1998). It is now believed that oxidative stress makes a significant contribution to all inflammatory diseases, ischemic heart disease, emphysema, gastric ulcers, Parkinsons and Alzheimer's diseases and many others (McCord, 2000; Ozben *et al.*, 1998; Rice-Evans *et al.*, 1992).

The reason that overproduction of free radicals is a feature of such a broad spectrum of diseases derives from the fact that oxidative metabolism is a necessary part of every cell's metabolism. If a cell is sick or injured in any way that results in mitochondrial injury, then increased production of superoxide is the likely result (McCord, 2000).

A pro-oxidant is a toxic substrate that can cause oxidative damage to lipids, proteins and nucleic acids, resulting in various pathologic events (Prior *et al.*, 1999). Cells have intact pro-oxidant/antioxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. When additional oxidative events occur, the pro-oxidant systems outbalance the antioxidant systems, potentially producing oxidative damage that can ultimately lead to cell death in severe oxidative stress.

Mild and chronic oxidative stress may alter the antioxidant systems by inducing or repressing proteins that participate in these systems and by depleting cellular stores of antioxidant materials such as glutathione and vitamin E.

A disturbance in pro-oxidant/antioxidant systems can result from a myriad of different sources;

- diminished antioxidants,
- depletions of dietary antioxidants and other essential dietary constituents,
- increased production of reactive oxygen species and
- excessive activation of natural reactive oxygen species systems (Halliwell *et al.*, 1999).

2.2.1 Consequences of oxidative stress

2.2.1.1 Adaptation

Cells can usually tolerate mild oxidative stress, which often results in up-regulation of the synthesis of antioxidant defence systems in an attempt to restore the oxidant/antioxidant balance. In some cases, mild oxidative stress can up-regulate defences so as to protect the cell against much more severe oxidative stress applied subsequently. Mechanisms of adaptations often involve changes in gene expression that result in elevated antioxidant defences. Oxidative stress can also decrease transcription of certain genes. However, adaptation to oxidative stress need not always involve increased antioxidant defences (Halliwell *et al.*, 1999).

2.2.1.2 Necrosis

Oxidative stress can cause damage to all types of biomolecules, including DNA, proteins and lipids. It is uncertain which target is the most important, since injury mechanisms overlap widely. The primary cellular target of oxidative stress can vary depending on the cell, the type of stress imposed and how severe the stress is. At some stage cellular injury may become irreversible, the cell passes a "point of no return" and dies. Necrosis is characterised by early cell and organelle swelling, loss of integrity of mitochondrial, peroxisomal, lysosomal and plasma membranes and eventual breakdown of the cell, leading to release of its contents into the surrounding area. Necrosis of cells affects the surrounding cells, due to the release of lysosomal enzymes and pro-oxidants like iron and copper (Halliwell *et al.*, 1999).

2.2.1.3 Apoptosis

In contrast, to necrosis, the earliest changes in apoptosis consist of cell shrinkage and condensation and fragmentation of chromatin. Other features of apoptosis are collapse of cytoskeletal structures, nuclear fragmentation and eventual break-up of the entire cell into apoptotic bodies, without rupture of mitochondrial or lysosomal membranes or release of cell contents. Apoptosis of cells is essential during embryonic development to eliminate thymocytes that recognise "self-antigens" and unwanted cells during tissue remodelling. The cell suicide mechanism appears to persist in all cells and can be triggered by certain stresses, or by the simple withdrawal of life signals, from the cellular environment. Among insults that trigger apoptosis are interference with energy metabolism, oxidative stress and the DNA-damaging effects of some drugs used in cancer chemotherapy. However, these agents can also cause necrosis, depending upon the cell type studied and the level of stress applied (Halliwell *et al*, 1999).

The mitochondrium is essential in controlling specific apoptosis pathways. Decreased efficiency of mitochondrial oxidative phosphorylation leads to inefficient energy production, which in turn leads to a change in the transmembrane potential that has a profound effect on the activity of the NMDA receptor. NMDA receptors then cause influx of calcium into the cytosol (Perlmutter, 1999). The rapid increase in mitochondrial calcium results in a greater increase in free radical production.

The mode of cell death in Parkinson's disease is uncertain, but the biochemical abnormalities present in Parkinsonian substantia nigra may precipitate apoptosis (Strijks *et al.*, 1997).

2.2.2 Oxidative stress and the brain

The brain is particularly vulnerable to oxidative stress because it is rich in non-heme iron, which is catalytically involved in the production of oxygen free radicals (Subbarao *et al.*, 1990). The brain contains relatively high levels of unsaturated fatty acids that are particularly good substrates for peroxidation reactions (figure 2.10) (Ogawa, 1994). It is particularly susceptible to free radical attack, because it generates more of these toxic substances per gram of tissue than

does any other organ (Reiter, 1995). Oxidant species in the brain are amongst others; hydrogen peroxide, hydroxyl radicals and superoxide anions. The targets of these species are proteins, lipids, nucleic acids and metabolites, thus leading to oxidation products (figure 2.10). Just as formation of oxidative damage products occur, processes involved in their repair or removal also occurs. The defence mechanisms the brain uses against oxidative stress is via enzymatic antioxidants (figure 2.10) (Arivazhagan *et al.*, 2002).

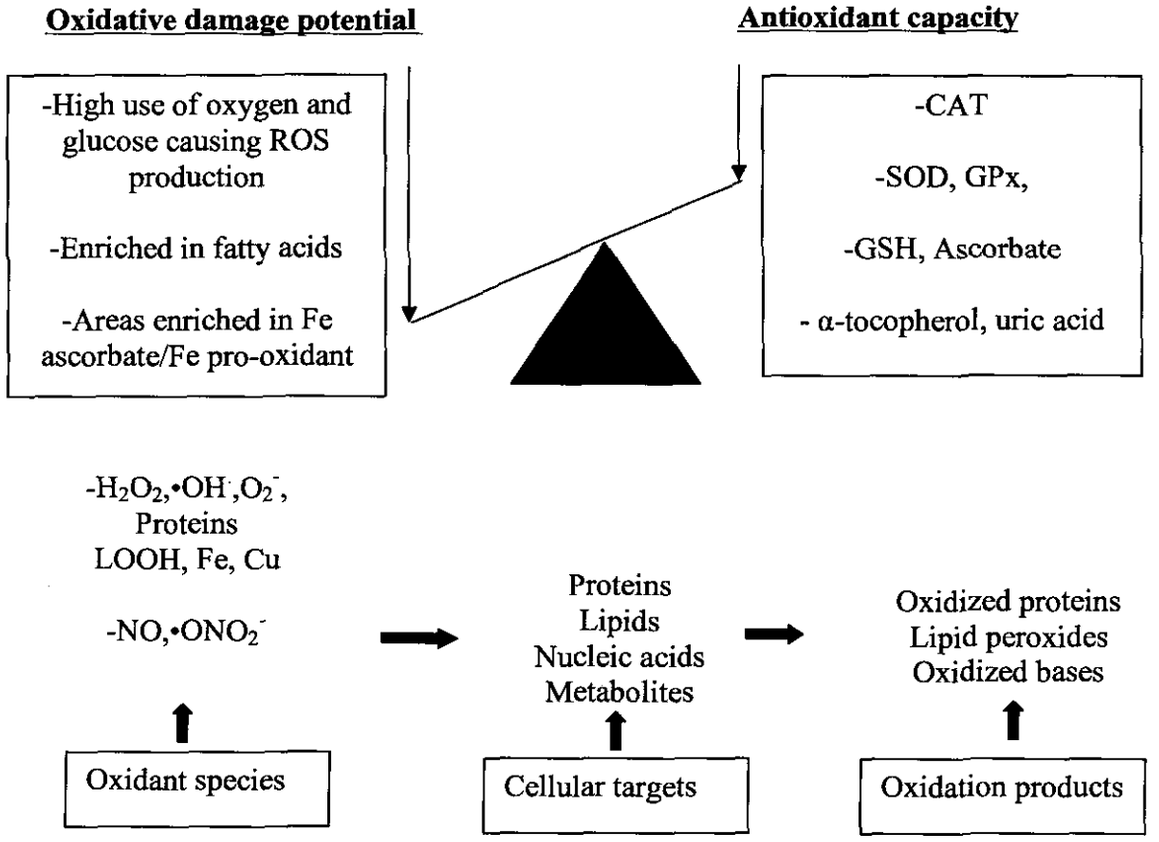

Figure 2.10: Brain at risk of oxidative damage.

The oxidant species are produced and interact with cellular targets producing unique oxidation products and they, in some cases, exert oxidative stress upon the tissue as well (figure 2.10).

Oxidative damage to DNA produces strand breaks as well as many oxidised bases. Typically, strand breaks are repaired and oxidised bases are removed by specific enzymes (Floyd *et al.*, 2002).

2.2.3 Compounds used to induce oxidative stress

2.2.3.1 N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical contaminant of synthetic heroin, was found to produce neuropathological changes and clinical features similar to idiopathic Parkinson's disease in human and nonhuman primates (Heikkila *et al.*, 1984). This environmental toxin has been extensively used in experimental models of this disease.

MPTP provides a model to understand the selective vulnerability of dopaminergic neurons. MPTP toxicity necessitates the conversion to the 1-methyl-phenylpyridinium ion (MPP+) by monoamine oxidase B (MAO-B) in the glial cells. The compound is actively transported into presynaptic dopaminergic nerve terminals by the dopamine transporter. MPTP-induced dopaminergic cell degeneration is dependent on the presence of the dopamine transporter and blocking this dopamine transporter may prevent the accumulation of MPP+ and reduce its toxicity (Pan *et al.*, 2003).

A primary effect of MPP+ is to inhibit complex I of the electron transport chain, thus leading to the production of reactive oxygen species, loss of the mitochondrial membrane potential and ATP production and causing dopaminergic cell injury (figure 2.11; Gluck, *et al.*, 1994; Cassarino *et al.*, 1999). MPP^+ also induces dopamine efflux, increasing dopamine autoxidation and oxidative damage (Chiueh *et al.*, 1993; Rollema *et al.*, 1988).

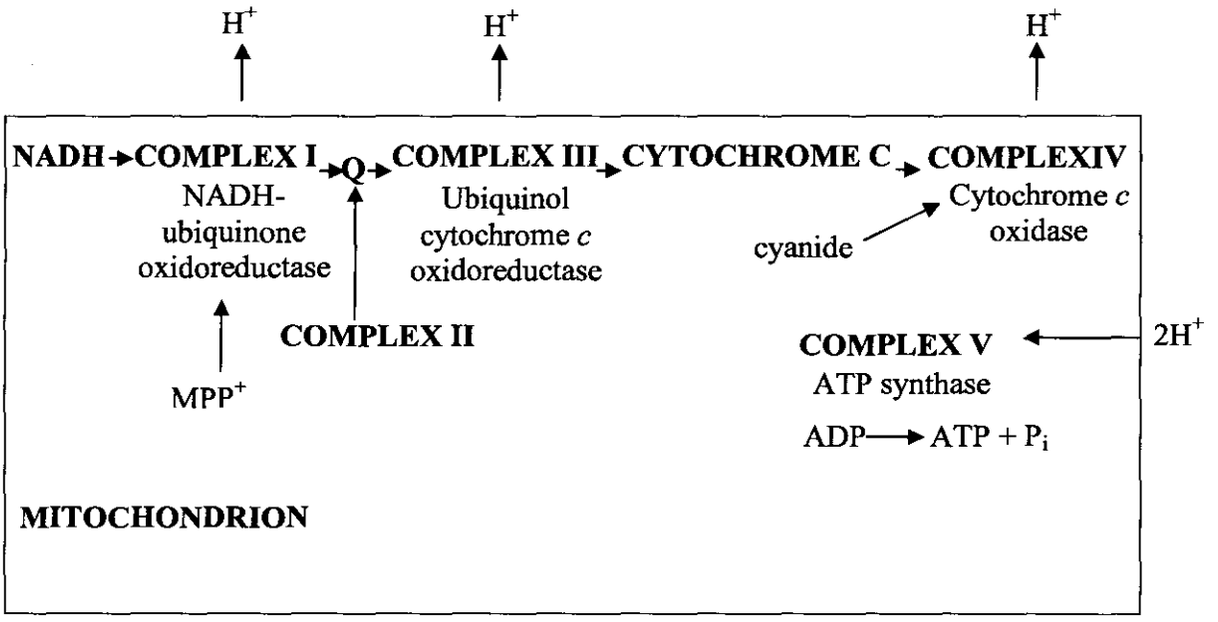

Figure 2.11: The electron transport chain with the site where MPP+ and cyanide act as inhibitors.

Inhibition of complex I may be considered as the explanation for the neurotoxicity of MPTP, but observations suggest that the mechanism is more complex and that oxidative damage plays an important role. The inhibition of complex I by MPP+ increases the generation of superoxide anions *in vitro* and induces free radical formation *in vivo* because of the displacement of dopamine, resulting in an increase in dopamine oxidation (Giasson *et al.*, 2002).

MPTP can be used *in vivo* in the single cell gell electrophoresis (comet) assay to induce neurotoxicity and to determine the effects of the selected compounds against oxidative damage (section 4.3).

2.2.3.2 Cyanide

The brain is the primary target for cyanide toxicity (Gunesekar *et al.*, 1996). Acute cyanide neurotoxicity has been attributed to production of cellular anoxia in the brain, production of tonic and clonic seizures, convulsions and a Parkinson-like condition as a post toxicity sequel (Yamamato *et al.*, 1996; Utti *et al.*, 1985). Cyanide also produces dopaminergic toxicity accompanied by impaired motor function (Gunesekar *et al.*, 1996). Due to a number of antioxidant enzymes being inhibited by cyanide, it is also believed that oxidative stress plays an

important role in cyanide induced neurotoxicity (Ardelt *et al.*, 1989). Increased intracellular calcium after cyanide treatment generates reactive oxygen species leading to peroxidation of lipids and subsequent neuronal damage (Johnson *et al.*, 1987).

Cyanide is a well known inhibitor of cytochrome oxidase (figure 2.11). Administration of potassium cyanide has been found to produce rigidity as well as bilateral lesions in the basal ganglia and white matter of primates and rats (Hurst, 1942).

In vitro studies have shown that cyanide induces calcium accumulation in neuronal cells (Maduh *et al.*, 1990). In cultured hippocampal neurons cyanide produces both calcium accumulation and cell death (Patel *et al.*, 1992). Cyanide administration also leads to a depletion of glutathione in hepatocytes and to lipid peroxidation in mouse brain consistent with oxidative stress (Mithofer *et al.*, 1992; Johnson *et al.*, 1987).

Potassium cyanide is used in the nitroblue tetrazolium assay and lipid peroxidation assay to induce oxidative stress and to determine free radical scavenging activity.

2.2.3.3 *tert*-Butyl hydroperoxide (TBHP)

tert-Butyl hydroperoxide, an organic hydroperoxide, is widely used in a prooxidant model to induce oxidative stress in various systems (Muralidhara, *et al.*, 1999). Two different mechanisms of cell damage by *tert*-butyl hydroperoxide have been reported. The first is a mechanism dependent on iron metabolism that plays a role in free radical formation inducing oxidative DNA damage. The second mechanism is dependent on the reduced form of glutathione, which can indirectly lead to DNA strand breaks. Thus, treatment with *tert*-butyl hydroperoxide induces the formation of DNA single strand breaks and oxidised bases, cell death, lipid peroxidation and redox state alteration (Lazzé *et al.*, 2003).

2.3 Antioxidants

Antioxidants (AO) are substances present at low concentrations compared with those of an oxidisable substrate that delay or prevent oxidation of that substrate (Halliwell, 1995). They are substances that protect other molecules of the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species (ROS) within the body, hence preventing the process of oxidation. Antioxidants change free radicals into less harmful molecules thereby preventing the formation of new free radical species (Paździoch-Czochra & Widenska, 2001). The formed molecules are easily removed from the organism (Koratkova *et al.*, 2002). Antioxidants are thus believed to be important in health maintenance through the modulation of oxidative processes in the body (Ozben, 1998).

Antioxidants interfere with the oxidation process by four specific mechanisms:

1. Chain breaking reactions, for example α-tocopherol acts in the lipid phase to trap reactive oxygen species.
2. Reduction of reactive oxygen species with glutathione (GSH).
3. Scavenging of initiating radicals by superoxide dismutase (SOD), which acts in an aqueous phase to trap superoxide free radicals.
4. Chelation of transition metals that are well-established pro-oxidants (Gülçin, *et al.*, 2002; Shahidi & Wanasundara, 1992; Sanchez-Moreno *et al.*, 1999).

During these reactions the antioxidant sacrifices itself by becoming oxidised. However, antioxidant supply is not unlimited as one antioxidant molecule can only react with a single free radical. Therefore, there is a constant need to replenish antioxidant resources, whether endogenously or through supplementation (Fouad, 2002).

The body has developed several endogenous antioxidant systems to deal with the production of reactive oxygen species. Cellular oxidant defences can be categorised into primary and secondary defence systems. The primary defences consist of non enzymatic compounds such as α-tocopherol, ascorbic acid, glutathione and β-carotene along with a variety of antioxidant enzymes, superoxide dismutase, glutathione peroxidase (GPx) and catalase (CAT) (table 2.2) (Little & O'Brien, 1968; Chance *et al.*, 1979; Cutler, 1991).

Table 2.2: Classification of antioxidants (Fouad, 2002).

Enzymes	Antioxidant	Role	Remarks
	SOD Mitochondrial Cytoplasmic Extracellular	Dismutates superoxide anions to hydrogen peroxide	Contains manganese (Mn.SOD) Contains copper and zinc (CuZnSOD) Contains copper (CuSOD)
	CAT	Dismutates hydrogen peroxide to water	Tetrameric hemoprotein in peroxisomes
	GPx	Breaks lipid peroxidation Lipid peroxide, superoxide anions and hydroxyl scavenger	Primarily in the cytosol Also in mitochondria Uses glutathione
Vitamins	α-tocopherol	Superoxide anion, peroxyl radical and hydroxyl scavenger Prevents vitamin A oxidation Binds to transition metals	Fat soluble vitamin
	ß-carotene	Direct superoxide anion, hydroxyl and hydrogen peroxide scavenger. Neutralises oxidants from neutrophils. Regenerates vitamin E.	Fat soluble vitamin
	Ascorbic acid		Water soluble vitamin

Vitamin E (vit E) has been described as the major chain-breaking antioxidant in humans. Because of its lipid solubility, vitamin E is located within cell membranes, where it interrupts lipid peroxidation and may play a role in modulating intracellular signalling pathways that rely on reactive oxygen species (Kagan *et al.*, 1990; Azzi *et al.*, 1993). Vitamin E can also directly quench reactive oxygen species, including superoxide anions (O_2^-), hydroxyl radicals (•OH) and singlet oxygen ($^1O_2$) (figure 2.11). Vitamin E, ascorbic acid and ß-carotene inhibit cellular damage induced by oxidative stress (OS) (Burtonb & Traber, 1990; Palozzo *et al.*, 1997; Simon-Schnass, 1992).

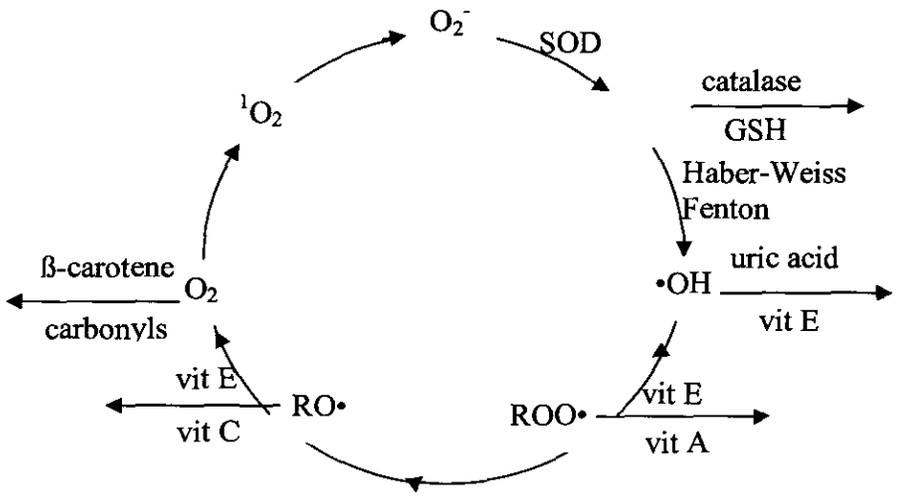

Figure 2.12: Generation sequence of reactive oxygen species following univalent reduction of oxygen and the various sites of action of the different endogenous antioxidants.

The superoxide anion is generated from singlet oxygen and superoxide dismutase converts superoxide anions to hydrogen peroxide (H_2O_2). Catalase and glutathione scavenge hydrogen peroxide. Hydroxyl radicals are formed through the Haber-Weiss and Fenton reaction. Uric acid is a powerful scavenger of hydroxyl radicals and vitamin E scavenges hydroxyl radicals, peroxyl radicals (ROO•) and alkoxyl radicals (RO•). Vitamin A (vit A) also scavenges peroxyl radicals. Vitamin C (vit C), alkoxyl radicals and ß-carotene and carbonyls quench excited oxygen (figure 2.12) (Fouad, 2003).

The enzymatic antioxidants include superoxide dismutase, which catalyses the conversion of superoxide anions to hydrogen peroxide and water (H_2O), catalase which then converts hydrogen peroxide to water and oxygen (O_2) and glutathione peroxidase (GSH) which reduces hydrogen peroxide to water (Fouad, 2002).

The enzymatic and nonenzymatic antioxidant systems are intimately linked and appear to interact with one another. Both ascorbic acid and glutathione have been implicated in the recycling of α-tocopherol radicals. Trace elements like selenium, manganese (Mn), copper (Cu) and zinc (Zn) also plays important roles as nutritional antioxidant cofactors. Selenium is a cofactor for the enzyme glutathione peroxidase, manganese, copper and zinc are cofactors for superoxide dismutase. Zinc acts as a stabilizer to the cellular metallothionein pool, which has

free radical quenching ability. The interactions of these different antioxidant systems are complex and may imply that therapeutic strategies will depend on combination therapy, rather than a single agent (Fouad, 2002).

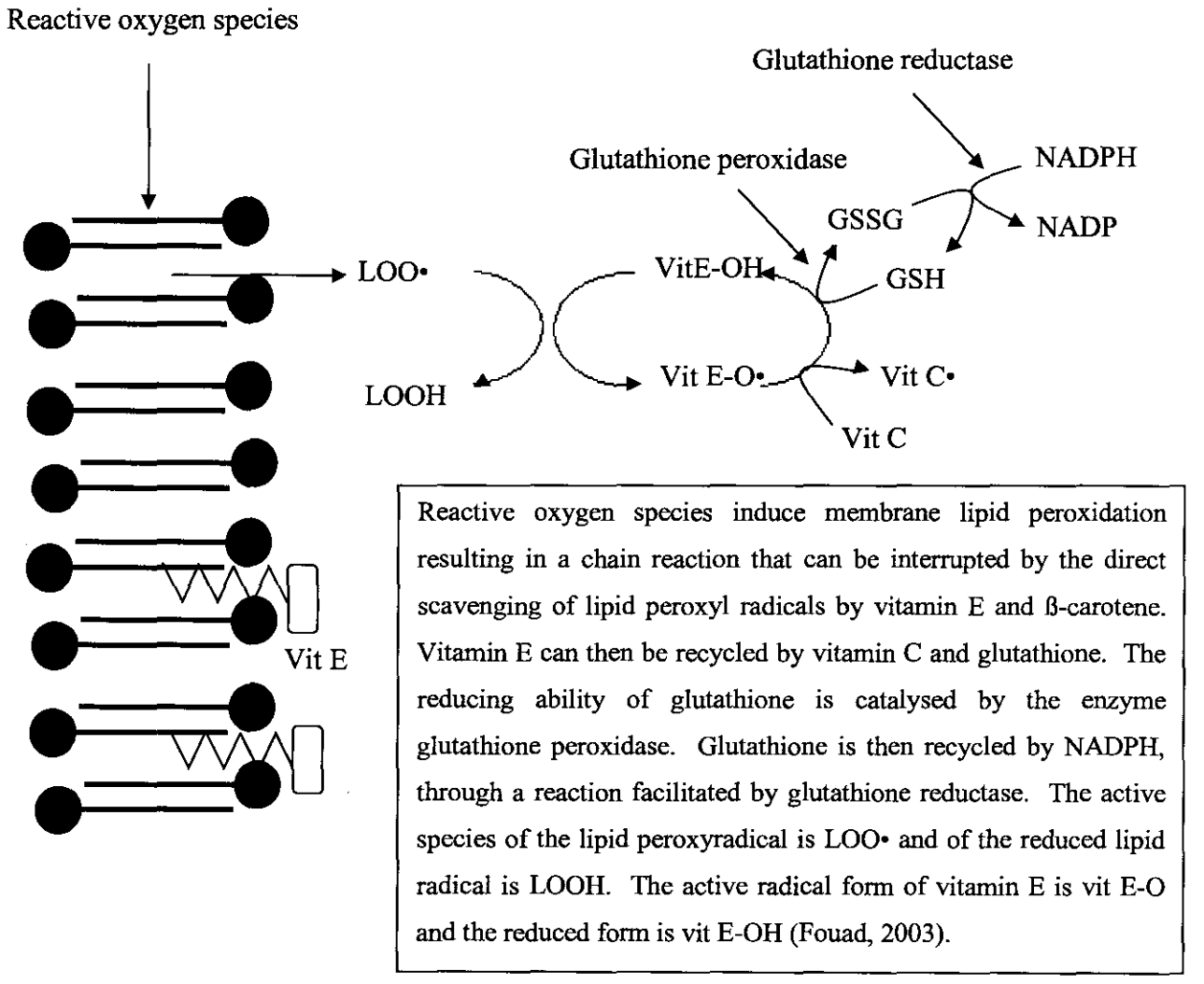

Figure 2.13: Interactions among antioxidants.

Secondary defences consist of a varied host of enzymes (phospholipase A_2, fatty acyl transferases, redoxynuclease and proteinase). They repair or eliminate cell components that were damaged by oxidants or free radical reactions that escaped the primary antioxidant defences. These enzymes repair membrane phospholipids, proteins and DNA (Chen, 1995).

2.3.1 Non-enzymatic antioxidant defences

2.3.1.1 Alpha-tocopherol (Vitamin E)

History

Vitamin E is the major lipid soluble antioxidant found in cells. In the early 1920s vegetable oil was discovered to restore fertility in rats and the unknown substance was designated vitamin E in 1924.

Vitamin E is a generic term that includes all entities that exhibit the biological activity of natural vitamin E, namely d-alpha-tocopherol. Eight substances have been found to have vitamin E activity of which d-alpha tocopherol has the highest bio potency and its activity is the standard against which all the others are compared. It is the predominant isomer of vitamin E in plasma (Fouad, 2002).

Source and nature

Vitamin E is an essential nutrient that functions as an antioxidant in the human body. The body cannot manufacture its own vitamin E and thus supplements and food must provide it.

Tocopherols are present in oils, nuts, seeds, wheat germ and grains. Absorption is associated with intestinal fat absorption and approximately 40% of ingested tocopherols are absorbed. α-Tocopherol tends to concentrate in phospholipids of the mitochondria, the endoplasmic reticulum and plasma membranes (Fouad, 2002).

Mechanism of action

Vitamin E is more appropriately described as an antioxidant than a vitamin because it does not act as a co-factor for enzymatic reactions.

The main function of vitamin E is to prevent peroxidation of membrane phospholipids and avoid cell membrane damage through its antioxidant action. The lipophilic character of tocopherol enables it to locate in the interior of the cell membrane bilayers.

Reduced tocopherol can transfer a hydrogen atom with a single electron to a free radical, thus removing the radical before it can interact with cell membrane proteins or generate lipid peroxidation (Fouad, 2002).

2.3.1.2 Beta-carotene

Source and nature

Carotenoids are micronutrients present in fruits and vegetables, are precursors of vitamin A and have antioxidative effects. Over 600 carotenoids have been found with ß-carotene the most widely studied (Fouad, 2002).

Mechanism of action

The antioxidant function is due to its ability to quench singlet oxygen, scavenge free radicals and protect the cell membrane lipids from the harmful effects of oxidative degradation. Quenching involves a physical reaction in which the energy of the excited oxygen is transferred to the carotenoid, forming an excited state molecule.

The ability of ß-carotene as quencher is limited because of the fact that the carotenoid itself can be oxidised during the process (autoxidation). Carotenoids are also thought to quench other oxygen free radicals and it is suggested that ß-carotene might react directly with peroxyl radicals (Fouad, 2002).

2.3.1.3 Ascorbic acid (Vitamin C)

Source and nature

Ascorbic acid is a water-soluble antioxidant, present in citrus fruits, potatoes, tomatoes and green leafy vegetables. Humans are unable to synthesise ascorbic acid and must therefore obtain the compound from dietary sources (Fouad, 2002).

Mechanism of action

Ascorbic acid acts as a chain breaking antioxidant that scavenges free radicals and reactive oxygen species that are produced during metabolic pathways of detoxification. The structure is reminiscent of glucose, from which it is derived in the majority of mammals.

An important ability of the compound is to act as a reducing agent, thus reducing molecular oxygen, nitrate and cytochromes a and c.

Donation of one electron gives the semi-dehydroascorbate radical. Ascorbate reacts with superoxide anions and hydroxyl radicals to generate semi-dehydroascorbate radicals, a source of vitamin C (Fouad, 2002).

2.3.2 Enzymatic antioxidant defences

The first line antioxidant system includes enzymes that can protect against oxidative injury (Kim *et al.*, 2002). The most efficient way to eliminate undesirable toxic species is by means of catalysis (McCord, 2000). Families of antioxidant enzymes have evolved for this purpose. Superoxide dismutase catalyses superoxide anions to hydrogen peroxide. Catalase converts hydrogen peroxide to molecular oxygen and water, thereby preventing the formation of hydroxyl radicals. Glutathione peroxidase catalyses the degradation of hydrogen peroxide and hydro peroxides originating from unsaturated fatty acids (Delibas *et al.*, 2002).

These enzymes require trace metal cofactors for maximal efficiency (selenium for glutathione peroxidase, copper, zinc or manganese for superoxide dismutase and iron for catalase) (Halliwell, 1995).

2.3.2.1 Superoxide dismutase

Source and nature

Superoxide dismutase is an endogenously produced intracellular enzyme present in essentially every cell in the body. Humans have three genes encoding superoxide dismutase, which localise in the mitochondria (Mn-SOD), the cytosol (Cu-Zn SOD) and the extra cellular spaces (CuSOD). These genes are derived from two ancestral genes. One gene gives rise to the copper and zinc containing enzymes, the other to the manganese or iron containing enzymes and can be traced back to the most primitive organisms (Fouad, 2002).

Mechanism of action

Superoxide dismutase is considered fundamental in the process of eliminating reactive oxygen species by reducing superoxide to form hydrogen peroxide (figure 2.14):

$$O_2^- + O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Figure 2.14: Formation of hydrogen peroxide by superoxide dismutase.

This dismutation reaction makes use of the fact that superoxide is both an oxidant and a reductant, eager to get rid of its extra electron or to take on another. The enzyme uses one superoxide radical to oxidise another (McCord, 2000).

The respective enzymes, glutathione peroxidase, superoxide dismutase and catalase that interact with superoxide and hydrogen peroxide are tightly regulated through a feedback system. Excessive superoxide anions inhibit glutathione peroxidase and catalase to modulate the equation

from hydrogen peroxide to water. Catalase and glutathione peroxidase conserve superoxide dismutase by reducing hydrogen peroxide and reducing superoxide anions. Superoxide dismutase conserves catalase and glutathione peroxidase. A balance in low levels of antioxidant enzymes, hydrogen peroxide and the superoxide anion is obtained through this feedback system that keeps the entire system in a fully functioning state.

Superoxide dismutase also exhibits antioxidant activity by reducing superoxide anions that would otherwise lead to the reduction of Fe^{3+} and thereby promote the formation of hydroxyl radicals.

The antioxidant enzymes function as a tightly balanced system and any disruption of this system would lead to promotion of oxidation (Fouad, 2002)

2.3.2.2 Glutathione peroxidase enzyme

The glutathione redox cycle is central to the reduction of intracellular hydroperoxides (figure 2.13 and 2.15).

Glutathione peroxidase is a tetrameric protein that has four atoms of selenium bound as seleno-cysteine moieties that confers the catalytic activity. An essential requirement is glutathione as a co substrate. It has 4 atoms of selenium bound as seleno-cysteine moieties.

Glutathione peroxidase reduces hydrogen peroxide to water by oxidising glutathione and forming glutathione disulfide (GSSG).

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

Figure 2.15: Formation of GSSG induced by glutathione peroxidase

Rereduction of the oxidized form of glutathione is then catalysed by glutathione reductase (figure 2.16).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Figure 2.16: Formation of glutathione induced by glutathione reductase

2.3.2.3 Catalase enzyme

Catalase is a protein enzyme present in most aerobic cells in animal tissues. Catalase is present in all body organs and is especially concentrated in the liver and erythrocytes.

Catalase and glutathione peroxidase seek out hydrogen peroxide and convert it to water and diatomic oxygen (figure 2.17).

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

Figure 2.17: The conversion of hydrogen peroxide

An increase in the production of superoxide dismutase without an elevation of catalase and glutathione peroxidase leads to the accumulation of hydrogen peroxide and conversion thereof to the hydroxyl radical. Even though catalase and glutathione peroxidase share the same substrate, hydrogen peroxide, catalase is more significant at protecting against severe oxidative stress, whereas the glutathione redox cycle is a major source of protection against low levels of oxidative stress (Fouad, 2002).

2.3.3 The enzymatic system and aging

The enzymatic system can reduce lipid peroxides as well as hydrogen peroxide and is very important in the prevention of lipid peroxidation to maintain the structure and function of biologic membranes (McCord, 2000).

According to the free radical theory of aging, one might expect the activity of antioxidant enzymes to be altered. In the aging process, the activity of these enzymes either decrease or increase leading to changes in hydrogen peroxide levels. According to studies, there are some mechanisms in senescent tissues that are generated at a higher rate during aging to provide an efficient antioxidant defence against free radicals (Fouad, 2002).

Superoxide dismutase activity decreases with age. It is irreversibly inactivated by its product, hydrogen peroxide (Salo *et al.*, 1988). Superoxide dismutase also includes copper and zinc, therefore a decrease in superoxide dismutase activity may reflect copper or zinc deficiency (Bertger *et al.*, 1979; Wasowics *et al.*, 1989). An increase in hydrogen peroxide as a result of aging, leads to an increase in glutathione peroxidase and catalase activity (Inal *et al.*, 2001).

Defects and/or changes in these enzymatic defences are associated with oxidative stress and oxidative damage (OD) (Schwemmer *et al.*, 2000).

2.3.4 Mode of action of antioxidants

2.3.4.1 Antioxidants as reducing agents

Many compounds with antioxidant activity are readily oxidisable materials. This property allows them both to intercept primary oxidants such as transition metal ions, molecular oxygen, hydroxyl radicals or hydrogen peroxide, and also to compete with chain-carrying free radicals to terminate auto oxidation processes. The effectiveness of the compound towards an oxidising species, such as an electrophilic free radical, lies in its ease of oxidation by an electron transfer process (Larson, 1995).

2.3.4.2 Antioxidants as radical quenchers

A general ability to enter into rapid reactions with free radicals is a great advantage for a potential antioxidant compound. Many synthetic antioxidants are specifically designed to react

with oxygen radicals and to form sterically hindered or inactive radical products that effectively terminate radical chains (Larson, 1995).

2.3.4.3 Antioxidants as singlet oxygen quenchers

Singlet oxygen-induced damage in tissues could be minimised by quenchers either by preventing the singlet oxygen from forming or reacting rapidly with singlet oxygen once it is formed. Many antioxidants are potent quenchers of singlet oxygen using two mechanisms:

- Quenching of an energetic molecule occurs with the transfer of the excess energy to the quencher. The energy is then dissipated by a variety of mechanisms.
- Singlet oxygen quenching occurs by the reaction thereof with an acceptor with the incorporation of singlet oxygen into the quencher molecule. The initially formed oxidation product decomposes spontaneously into new, more stable products (Larson, 1995).

2.3.4.4 Antioxidants as metal ion complexing agents

Several oxidised transition metal ions such as iron(III) and copper(II) have accessible reduced states and are present in high enough concentrations in many tissues to make them reactants for one-electron oxidations or reductions that could generate reactive free radicals. Antioxidants that bind reactive transition metal cations by complexation decrease their biological effects dramatically (Larson, 1995).

Antioxidant strategies are:

- The use of antioxidants including vitamins and nutrients.
- Modifications of endogenous antioxidant enzymes (Khaldy, 2003; Kitani *et al.*, 1999).

2.4 Neurodegenerative diseases

The term neurodegenerative diseases imply that there is no exact knowledge of the cause or pathogenesis of these diseases. The classification of these illnesses has therefore relied on a description of their pathologic anatomy and clinical features. A characteristic of the

degenerative diseases is that they are insidious in onset and run a gradually progressive inexorable course, which can continue over many years.

The neuropathologic features of these illnesses tend to be symmetric and involve degeneration of circumscribed groups of neurons, which may be functionally or neuroanatomically connected. There is an increased incidence of pathologic Parkinson's disease in Alzheimer patients and of dementia with pathologic features of Alzheimer's disease in Parkinson's disease patients (Beal, 1995).

2.4.1 Parkinson's disease

Parkinson's disease is a progressive neurodegenerative disease of unknown aetiology, which produces resting tremor, akinesia and rigidity. The major symptoms of this disease are related to the loss of the midbrain dopamine neurons within the substantia nigra, which causes profound dopamine depletion in the striatum (Speciale, 2002; Blandinni *et al.*, 2000). This is responsible for most of the motor symptomatology of the disease.

The cause of the cellular destruction in Parkinson's disease remains unclear, but it is attributed to a variety of causes:

- The action of endogenous neurotoxins or xenobiotics which act on the mitochondrial respiratory chain by depleting the production of ATP.
- The oxidative stress caused by an excessive production of free radicals.
- An alteration in protective antioxidant systems.

Several studies have indicated certain biochemical changes in the brain of Parkinson's disease patients which appear to be a consequence of oxidative stress (Schapira *et al.*, 1993; Youdim *et al.*, 2001). However it is not yet clear whether oxidative stress is actually an etiological factor in Parkinson's disease or a secondary factor which occur subsequent to an alternate process (Soto-Otero *et al.*, 2002). The vulnerability of dopaminergic neurons is because of their intrinsic predisposition to generate reactive species. The normal enzymatic metabolism of dopamine results in the generation of hydrogen peroxide and the nonenzymatic autoxidation of dopamine

results in the formation of reactive quinones and semiquinones that react to generate hydrogen peroxide, superoxide anions and hydroxyl radicals. Markers of oxidative stress, such as products of lipid peroxidation and oxidation of mitochondrial DNA and cytoplasmic RNA, are increased in dopamine neurons of Parkinson's disease brains (Giasson *et al.*, 2002).

Recent studies have demonstrated reduced activity of complex I of the electron transport chain in brain and platelets from patients with Parkinson's disease (figure 2.11). This defect is not due to debilitation or the effect of medication for Parkinson's disease and may be the result of processes that cause death of the nigral neurons (Parker *et al.*, 1989; Schapira *et al.*, 1990). The high occurence of the disease in the aging society make it imperative to investigate possible new treatments.

2.4.1.1 Possible treatment of Parkinson's disease

The histamine H_3-receptor regulates the release of dopamine and antagonists thereof could thus reduce the receptor's mediated inhibition of dopamine. If antagonists of the histamine H_3-receptor can act as free radical scavengers and have a dual therapeutic effect, increasing dopamine levels and protecting dopamine neurons against further degradation, the compounds will be tremendously effective in reducing the insidence of Parkinson's disease.

CHAPTER 3

Test compounds

In this study I examined the free radical scavenging abilities of histamine antagonists and compared the results to the well known free radical scavenger, aspirin. The newly discovered histamine H_3 antagonists, impentamine, thioperamide and clobenpropit, were the compounds of interest. These compounds are able to increase dopamine levels and a dual therapeutic effect might be obtained if they can act as free radical scavengers (Ryu *et al.*, 1994). The histamine H_1 and H_2 antagonists were included to compare the different groups of antihistamines and ibuprofen to compare the free radical scavenging activity with aspirin.

3.1. Histamine receptor antagonists

3.1.1. The histamine H_3 receptor

The histamine H_3 receptor was discovered as an auto receptor, presynaptically localised on histaminergic nerve terminals (Arrang *et al.*, 1983; Arrang *et al.*, 1987) and inhibits synthesis and release of histamine upon activation. It also interacts as a heteroreceptor with a variety of different neurotransmitter systems (Clapham & Kilpatrick, 1992). Functional studies provided evidence of inhibitory histamine H_3 receptors not only on histaminergic nerve terminals, but also on noradrenergic, serotoninergic, dopaminergic, glutamatergic and peptidergic neurons (Schwartz *et al.*, 2000).

The discovery of the third histamine receptor and its potent and selective ligands has clarified various aspects of histaminergic neurotransmission in the brain. The widespread distribution of these receptors in the CNS with an extensive modulatory role is indicative of their vast potential in varied functions of the mammalian brain (Stark *et al.*, 2000; Meier *et al.*, 2001).

It was found that induced denervation of dopaminergic neurons resulted in a marked increase in the density of histamine H_3 receptors in the striatum and substantia nigra of the rat (Ryu *et al.*, 1994). These results suggest that histamine H_3 receptors in these areas are under tonic dopaminergic influence. If histamine H_3 receptor modulation of dopaminergic activity is also

found in humans, reducing the histamine H_3 mediated inhibition of dopamine release by using histamine H_3 antagonists could have therapeutic benefits in Parkinson's disease (Rodrigues, 1996).

3.1.1.1. Histamine H_3 receptor antagonists

Various structural developments of histamine H_3 receptor antagonists have been reported (thioperamide, clobenpropit, ciproxifan, impentamine etc.), but they have not been introduced into therapy yet (Stark *et al.*, 2000; Meier *et al.*, 2001).

Selective ligands of this receptor hold great promise for therapeutic applications in many neuropsychiatric disorders. Recent neuropharmacological studies have shown a possibility of the clinical utility of selective histamine H_3 receptor antagonists in varied CNS disorders including cognitive/vigilance deficits, sleep disorders, epilepsy, obesity and also neurodegenerative diseases (Vohora *et al.*, 2001; Rodrigues, 1996).

Histamine H_3 receptor agonists increase deep, slow-wave sleeps and decrease wakefulness in several animal species. Conversely histamine H_3 receptor antagonists, which facilitate histamine release, increase arousal. Perhaps as a consequence of the role played by histaminergic neurons in arousal, administration of histamine H_3 antagonists enhanced the learning ability of rodents in a variety of paradigms, particularly when this ability was impaired by accelerated senescence (Schwartz *et al.*, 2000)

Thioperamide

Early histamine H_3 antagonists (impromidine and burimamide) had mixed effects since they were also agonists of the histamine H_2 receptor. Thioperamide was the first specific histamine H_3 antagonist experimentally available. As a prototypical histamine H_3 antagonist, thioperamide can potentiate the release of histamine and reverse the action of histamine H_3 autoreceptor agonists (Kenneth & Serafin, 1996).

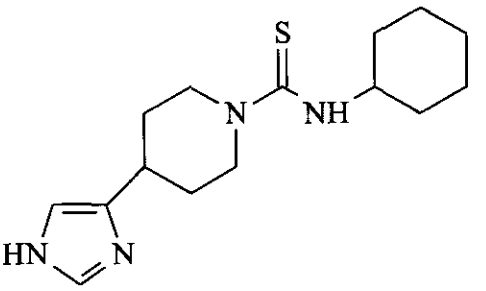

Figure 3.1: Structure of thioperamide

Induced slow-wave sleep was prevented by thioperamide due to the histamine releasing effect of the drug. The effect of thioperamide is blocked by a histamine H_1 receptor antagonist, thus suggesting that the wakeful effect is related to activation of histamine H_1 receptors in the CNS (Lin *et al.*, 1990). The release of endogenous neuronal histamine induced by thioperamide leads to stimulation of histamine H_1- and H_2 receptors, which in turn leads to anxiety (Imaizumi *et al.*, 1993).

Locomotor activity is increased by thioperamde with a decreased effect of histamine concentration in the whole-brain, suggesting that thioperamide activates the histaminergic neuronal system and causes hyperactivity via histamine released from the terminals (Sakai *et al.*, 1993). Food intake decreases in rats after administration of thioperamide and the drug antagonises the effect of histamine H_1 receptor agonist, imitet (Sakata *et al.*, 1991; Merali & Banks, 1994). This suggests an application for histamine H_3 receptor antagonists in weight disorders (Sakata *et al.*, 1991).

The stimulation of both histamine H_1 and H_2 receptors via thioperamide reduces the effect of memory loss (Miyazaki *et al.*, 1995). In correlation, it was found that certain brain histamine levels in Alzheimer's patients are significantly decreased, suggesting a disruption in brain histamine regulation (Mazurkiewicz-Kwilecki & Nsonwah, 1989). Other indications for the usage of thioperamide are for symptomatic treatment of peripheral vertigo and central vestibular disorders, inhibition of amygdaloidal kindled seizures, epilepsy and depression (Kamei & Okuma, 2001).

Clobenpropit

Clobenpropit results from an elongation of the side chain between the imidazole group and the cationic nitrogen of imetit and is one of the most potent histamine H_3 antagonists (Leurs *et al.*, 1995).

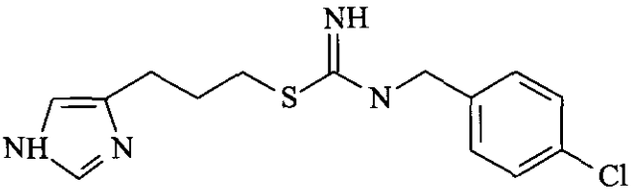

Figure 3.2: Structure of clobenpropit

Recent studies have shown that clobenpropit significantly decreased the duration of electrically induced convulsions through activation of the histamine H_1 receptor (Yokoyama *et al.*, 1993; Yokoyame *et al.*, 1994). When applied centrally, clobenpropit is approximately 10 times more potent than thioperamide, which makes it a promising research tool to investigate neuropathology (Rodrigues, 1996).

Another indication for the usage of clobenpropit is related to motion sickness and vertigo and it is suggested that histamine H_3 antagonists are powerful tools for symptomatic treatment thereof (Yabe *et al.*, 1993). Furthermore it is suggested that histamine H_3 antagonists enhance noradrenalin release and therefore, clobenpropit could be useful as an antidepressant agent (Rodrigues, 1996).

Impentamine

Impentamine is one of the most highly potent histamine H_3 antagonists. Structurally, the compound is a histamine homologue that does not possess the (iso)thiourea moiety of thioperamide and clobenpropit and therefore might have fewer side effects. This fact makes impentamine an attractive compound for future therapeutic development (Rodrigues, 1996).

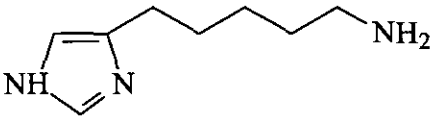

Figure 3.3: Structure of impentamine

Impentamine has excellent histamine receptor selectivity and behaves as a partial agonist on histamine H_3 responses in brain slices. Previous studies have shown that impentamine has high antagonist affinity on the histamine H_3 mediated bioassay. Thus, impentamine has the characteristics of a highly selective histamine H_3 receptor agent (Vollinga *et al.*, 1995; Leurs *et al.*, 1996).

3.1.2. The histamine H_1 receptor

The histamine H_1 receptor is distributed in the brain, most smooth muscle cells, endothelial cells, the adrenal medulla and the heart. The histamine H_1 receptor mediates smooth muscle contraction, stimulation of nitric oxide formation, endothelial cell contraction and increased vascular permeability, all of which are involved in allergic conditions. The receptor also mediates various excitatory responses in the brain. Various intracellular responses are associated with the stimulation of this receptor like inositol phosphate release, increase in Ca^{2+} fluxes, cyclic AMP or cyclic GMP accumulation and arachidonic acid release (Schwartz *et al.*, 2000).

3.1.2.1. Histamine H_1 receptor antagonists

All the histamine H_1 receptor antagonists (diphenhydramine, pyrilamine, chlorpheniramine etc.) are reversible, competitive inhibitors of the interaction of histamine with histamine H_1 receptors. Histamine H_1 antagonists inhibit responses of the smooth muscle to histamine, block formation of edema and wheal and decrease hypersensitivity reactions. First generation histamine H_1 antagonists (diphenhydramine, pyrilamine) can stimulate and depress the central nervous system (CNS) after appreciably crossing the blood-brain barrier (Kenneth & Serafin, 1996).

Diphenhydramine

Diphenhydramine is an ethylenediamine H_1 antagonist that does not prevent the release of histamine, but rather competes with free histamine for binding at histamine H_1 receptor sites.

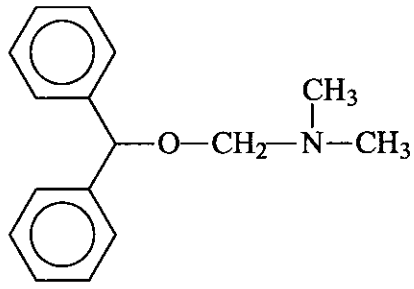

Figure 3.4: Structure of diphenhydramine

Diphenhydramine competitively antagonises the effects of histamine on H_1 receptors in the gastrointestinal tract, uterus, large blood vessels and bronchial muscle. Blockade of histamine H_1 receptors also suppresses the formation of edema, flare and pruritus that result from histaminic activity.

This antihistamine relieves irritated eyes, sneezing and runny nose caused by hay fever, allergies and the common cold. It also relieves itching and skin irritation and is used to prevent and treat motion sickness, induces sleep, treats mild cases of Parkinson's disease and relieves coughs resulting from minor throat irritations. Diphenhydramine also possess anticholinergic properties, which probably accounts for the antidyskinetic action and the use against drug-induced extra pyramidal symptoms.

The side effects caused by diphenhydramine are mostly anticholinergic, such as a dry mouth, drowsiness, upset stomach, chest congestion and headaches. Other symptoms that can occur are vision problems, muscle weakness and nervousness (Kenneth & Serafin, 1996).

3.1.3. The histamine H_2 receptor

Histamine H_2 receptors are located in acid secreting cells of the stomach, in the heart and are also widely distributed in the brain and spinal cord. Particularly high histamine H_2 receptor densities are found in the basal ganglia, parts of the limbic system and the superficial layers of

the cerebral cortex. This receptor type is linked to transduction systems involved in activation of adenylyl cyclase and increased production of cyclic AMP (Ramirez, 2002). It is involved in several physiological processes such as gastric secretion in peripheral tissues and synaptic transmission and plasticity in the central nervous system (Brown *et al.*, 1995).

Histamine H_2 receptors can inhibit a variety of functions within the immune system and there is also evidence that it can inhibit antibody synthesis, T-cell proliferation, cell-mediated cytolysis and cytokine production (Plaut & Lichtenstein, 1982).

3.1.3.1. Histamine H_2 receptor antagonists

Histamine H_2 receptor antagonists are highly selective and are used to inhibit gastric acid secretion and in the treatment of peptic ulcer diseases (Kenneth & Serafin, 1996).

Histamine H_2 receptor antagonists like cimetidine, famotidine and ranitidine have been shown to significantly scavenge hydroxyl radicals *in vitro*. The drugs may also be effectively used as hydroxyl scavengers *in vivo*. This suggests that some therapeutical effects of histamine H_2 receptor antagonists may be related to their antioxidant capacity and that these drugs might be useful in diseases associated with oxidative stress (Lapenna *et al.*, 1994).

Cimetidine

Cimetidine was developed from metiamide, by the replacement of a thiourea with a cyanoguanidine moiety (Brimblecombe *et al.*, 1978).

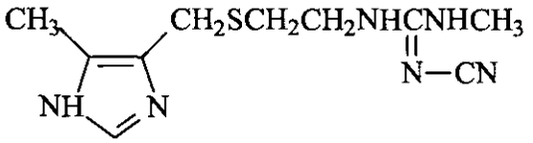

Figure 3.5: Structure of cimetidine

Cimetidine administration results in a significant reduction in gastric acid secretion stimulated by food, histamine, insulin, pentagastric infusion and caffeine (Brimblecombe *et al.*, 1975; Moberg *et al.*, 1977). Cimetidine is useful for the treatment of acute gastrointestinal haemorrhage in patients with fulminant hepatic failure and to treat gastrointestinal haemorrhage associated with gastric erosions (MacDougall *et al.*, 1977). There are also reports that cimetidine, due to its effects on the immune system and as a histamine H_2 receptor antagonist, can inhibit *in vitro* growth of human colon cancer cell lines (Adams *et al.*, 1994).

Side effects that may occur when cimetidine is used are hyperglycaemia and hyperosmolar nonketotic comas (Jeffreys & Vale, 1978). Cimetidine has been implicated in the development of a number of cases of agranulocytosis, pancytopaenia and thrombocytopaenia. It has also been reported that cimetidine has been known to cause psoriasis, giant urticaria and Stevens-Johnson syndrome (Hadfield, 1979). Following abrupt discontinuation of cimetidine, perforation of duodenal, oesophageal and gastric ulcers have been reported as well as acute pancreatitis, paralytic ileus and severe cases of diarrhoea (Gill & Saunders, 1977; Gazala & Hatum, 1978).

Previous studies have indicated that cimetidine effectively scavenges hydroxyl radicals *in vitro*, suggesting it might have further antioxidative properties (Lapenna *et al.*, 1994).

Roxatidine

Roxatidine is a histamine H_2 receptor antagonist with strong anti ulcerative activity. In contrast to the earlier histamine H_2 antagonists that possess a thiobutyl connecting chain and an urea-like moiety, roxatidine has a propyloxy connecting chain and an acetamide moiety. Studies also show differences in conformation and binding to the histamine H_2 receptor in comparison with cimetidine (Mathias *et al.*, 2003).

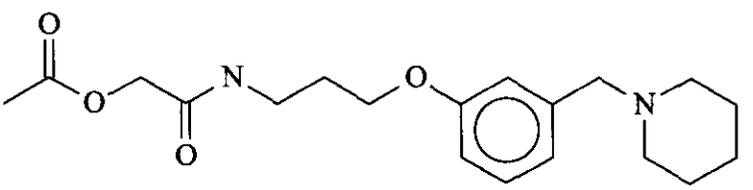

Figure 3.6: Structure of roxatidine

Roxatidine suppresses the effect of histamine on the parietal cells of the stomach and has a potent and selective inhibition of basal and stimulated gastric acid secretion through competitive blockade of histamine H_2 receptors. Total pepsin secretion is reduced and there is an independent mucosal protection action. This histamine H_2 antagonist is used for duodenal and benign gastric ulcers, reflux oesophagitis, prophylaxis of recurrent ulcers and for the prevention of acid aspiration during general anaesthesia.

Roxatidine may cause headaches, diarrhoea, constipation, nausea, vomiting and other gastro-intestinal complications. Further observed side effects are dizziness, troubled sleep, restlessness, drowsiness and visual disturbances (Brunton, 1996).

3.2. Non-steroidal anti-inflammatory drugs

The anti-inflammatory, analgesic and antipyretic drugs are a heterogeneous group of compounds, known as non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs share certain actions and side effects and their principal uses are the control of moderate pain of various musculoskeletal disorders, menstrual cramps and other mainly self-limiting conditions, including moderate postoperative discomfort (McPhee & Schroeder, 1999).

The activity of NSAIDs is mediated through inhibition of the biosynthesis of prostaglandins. These drugs inhibit cyclooxygenase 1 (COX-1) and/or cyclooxygenase 2 (COX-2) activity and thereby the synthesis of prostaglandins and thromboxane. All the NSAIDs, in varying degrees, inhibit platelet aggregation and may cause gastric irritation (Carrasco & Werner, 2002).

Although the primary action of NSAIDs is the inhibition of arachidonate cyclooxygenase and the subsequent decrease in the production of prostaglandins and tromboxanes, other mechanisms of action of these drugs, e.g. effects on free radical processes and on the defence enzyme systems should not be ruled out (Smith & Willis, 1971; Roth & Majerus, 1975).

Aspirin

One of the best-known aromatic acetates is aspirin, or acetylsalicylic acid, which is prepared by the esterification of the phenolic hydroxyl group of salicylic acid (figure 3.7).

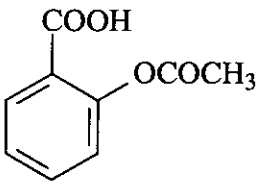

Figure 3.7: Structure of aspirin.

Aspirin, the prototype representative of the NSAIDs, is widely used for treatment of many diseases, of which the pathogenesis of some is associated with the formation of active oxygen species and enhancement of free radical processes (McCord, 1974; McCord & Roy, 1982; Halliwell & Gutteridge, 1985).

Aspirin possesses a number of properties that make it the most often recommended drug. It is an analgesic and effective pain reliever, an anti-inflammatory agent in providing some relief from the swelling associated with arthritis and minor injuries and it is also an antipyretic compound used for fever. The anti-inflammatory properties of aspirin are attributed to its ability to irreversibly inhibit cyclooxygenase activity and reduce the synthesis of pro-inflammatory prostaglandins (Vane *et al.*, 1998).

Although the use of aspirin is widespread and is readily available, it is not so innocuous a drug as one might imagine. Repeated use may cause gastrointestinal bleeding and large doses can provoke a host of reactions including vomiting, diarrhoea, vertigo and hallucinations (Insal, 1996).

Several mechanisms have been invoked to explain the neuroprotective effects of aspirin in brain tissue and it has been postulated that aspirin might inhibit oxidative stress (Anasuma *et al.*, 2001; De Cristobal *et al.*, 2002; Sagone & Husney, 1987). In previous studies it was shown that aspirin partially reduces the cytotoxicity of hydrogen peroxide in endothelial cells, suggesting that it can function as an antioxidant (Podhaisky *et al.*, 1997). There is also evidence that aspirin reacts with highly reactive hydroxyl radicals *in vitro* (Hiller & Wilson, 1983; Grootveld & Halliwell,

1986) and that the metabolite of aspirin, salicylic acid, protects against nigrostriatal dopamine toxicity (Mohanakumar *et al.*, 2000; Sairam *et al.*, 2003). However, the possibility that the protective mechanisms are unrelated to free radical scavenging and are rather because of COX-inhibition cannot be ignored (Carrasco & Werner, 2002).

Ibuprofen

Ibuprofen, an arylpropionic acid derivate, is an effective, useful group of NSAIDs. The drug inhibits the enzyme cyclooxygenase which catalyses the transformation of unsaturated fatty acids to prostaglandins.

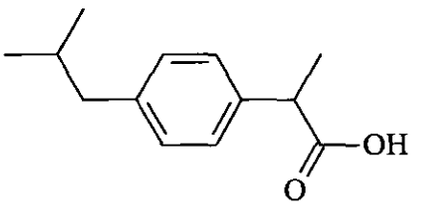

Figure 3.8: Structure of ibuprofen

Ibuprofen exists in two enantiomers, with the S-isomer best suited for the treatment of pain, in particular pain related to the musculoskeletal system, but also headaches, influenza, muscle aches, postoperative pain and dysmenorrhoea. It is also used as an anti-inflammatory agent via the decrease in vasodilator prostaglandins and as an antipyretic agent (Insal, 1996).

Previous studies have shown that ibuprofen protects dopaminergic neurons against glutamate toxicity *in vitro*. The proposed mechanisms for glutamate excitotoxicity include oxidative stress so that it might be possible that ibuprofen can scavenge free radicals and reduce oxidative stress (Casper *et al.*, 2000).

Ibuprofen predominantly causes gastrointestinal problems like dyspepsia, nausea, vomiting and epigastric pain. The drug offers significant advantages over aspirin and is better tolerated. Nevertheless, ibuprofen shares all of the detrimental features of the entire class of NSAIDs (Insal, 1996).

3.3. Free radical scavenging abilities

It is evident that the test compounds may have several beneficial activities in the brain. The histamine H_3 antagonists promote the release of dopamine and it is imperative to determine whether these compounds can also act as free radical scavengers and thus have a dual therapeutic effect in the treatment of Parkinson's disease. Diphenhydramine is used to treat mild cases of Parkinson's disease and cimetidine effectively scavenges hydroxyl radicals *in vitro*. Therefore, if these compounds are able to scavenge free radicals, therapeutic application in diseases associated with oxidative stress might be possible. Different screening tests as well as *in vitro* and *in vivo* assays were used to determine the free radical scavenging abilities of the compounds.

CHAPTER 4:

Methods and results

In vitro and *in vivo* tests were done to determine the free radical scavenging activity of the test compounds. *In vitro* tests included the FRAP and ORAC assays to determine whether the antihistamines and NSAIDs were able to act as direct antioxidants and to reduce peroxyl radicals. The comet assay was used to determine whether the test compounds were able to reduce oxidative damage to DNA and the free radical scavenging activities were measured with the nitroblue tetrazolium and lipid peroxidation assays. The results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $P< 0.05$ (Zar, 1974).

4.1. The ferric reducing antioxidant power (FRAP) assay

The FRAP assay is a recently developed direct test of total antioxidant power. The assay uses antioxidants as reductants in a redox-linked colorimetric method (Benzie & Strain, 1996). At a low pH, reduction of a ferric tripyridyltriazine (Fe^{111}-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, in that any half-reaction that has a lower redox potential under the same reaction conditions as that of the ferric/ferrous half-reaction, will drive the ferric to ferrous reaction. The change in absorbance, therefore, is directly related to the combined or "total" reducing power of the electron donating antioxidant present in the reaction mixture.

4.1.1. Experimental

Glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), ascorbic acid and $FeCl_3 \cdot 6H_2O$ were purchased from Sigma Chemical Co., St Louis, MO. All other chemicals used were purchased from Sigma and were of the highest chemical purity.

300mM acetate buffer, pH 3.6, plus 16 ml glacial acetic acid was diluted to 1ℓ with distilled water and mixed with 10mM TPTZ and 20mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1 to give the

working FRAP reagent. Aqueous solutions of freshly prepared ascorbic acid (pure antioxidant) were used for callibration of the FRAP assay.

An ascorbic acid standard of 1000μM is equivalent to 2000μM of antioxidant power expressed as FRAP. Solutions of 100, 250, 500 and 1000μM ascorbic acid were freshly prepared and used as quality control samples. The concentration of the test compounds was 1000μM.

The BioTek Fl 600 plate reader was used to determine accurate readings at 593 nm. The 0 to 4-minute reaction time window was used for data capture of total antioxidant power. Absorbance change was translated into a FRAP value by relating the absorbance of the test sample to that of a standard solution of a known FRAP value (e.g., 1000μM ascorbic acid) using equation 4.1.

$$\frac{\text{0 to 4-minute Absorbance}_{593\ nm}\text{ test sample}}{\text{0 to 4-minute Absorbance}_{593\ nm}\text{ standard}} \times \text{FRAP value of standard (μM)} \quad (4.1)$$

4.1.2. Results

The results obtained from the FRAP assay are presented in table 4.1 and figure 4.1. The assay was repeated several times and exactly the same values were obtained with each analysis, therefore SEM values are not applicable.

Table 4.1: FRAP absorbance values

Sample	Absorbance value
Baseline	0.305
Ascorbic acid	
0μM	0.286
100μM	0.375
250μM	0.472
500μM	0.937
1000μM	1.731
Diphenhydramine	0.300
Cimetidine	0.290
Roxatidine	0.298
Clobenpropit	0.277
Impentamine	0.310
Thioperamide	0.306
Aspirin	0.320
Ibuprofen	0.254

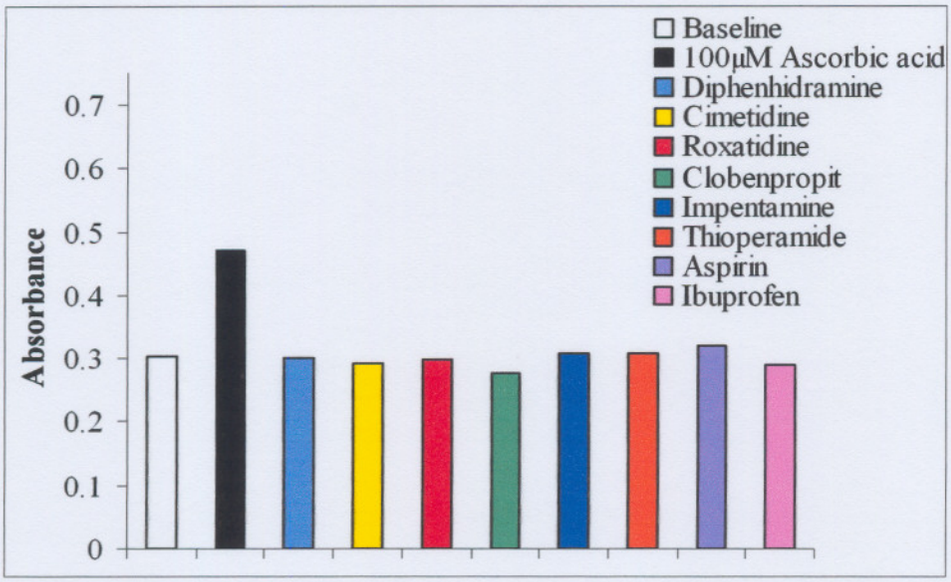

Figure 4.1: Total in vitro reduction power of selected compounds compared to 100µM ascorbic acid.

The results from the FRAP assay showed that the test compounds did not have values above the baseline, suggesting a lack of FRAP (table 4.1; figure 4.1).

4.2. The oxygen radical absorbance capacity (ORAC) assay

This assay is based on the procedure described by Cao *et al.* (1993) and utilizes ß-phycoerythrin as an indicator protein and 2,2'-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator. It is based on the inhibition of the peroxyl radical induced oxidation initiated by thermal decomposition of azo-compounds, such as 2,2'-azobis(2-amidinopropane) dihydrochloride. The delay in the end-point of a peroxyl radical reaction is directly proportional to the total peroxyl radical trapping antioxidant parameter and is measured in terms of the area under the curve. The value is usually expressed as micromoles of peroxyl radicals trapped by 1ℓ of sample. The antioxidant capacity is assessed relative to the ability of Trolox, the antioxidant standard, to trap peroxyl radicals and delay the end-point. Trolox has a known capacity to quench 2 mol peroxyl radicals per 1 mol Trolox.

In this method, all reactive components of the antioxidative defense are oxidised. Reactive species initiated by thermal decomposition is inhibited by the fast reaction of antioxidants and protects ß-phycoerythrin from attack (Prior & Cao, 2003). The kinetics of the fluorescence loss

of ß-phycoerythrin provides information on the rate of oxidative damage and the peroxyl scavenging efficiency of antioxidants (Rice-Evans, 2000).

4.2.1. Experimental

Trolox, 2,2'-azobis dihydrochloride(2-amidinopropane), fluorescein and perchloric acid were purchased from Sigma Chemical Co., St Louis, MO.

Trolox (500µM) was used as a standard. An opaque microlitre plate was used and wells containing appropriate aliquots of phosphate buffer and standard solution were prepared with a final volume of 20 µl. A calibration curve was generated by measuring the absorbance at 5µM increments of concentration (figure 4.2). The fluorescence, excitation at 485 nm and emission at 520 nm, was measured every 5 minutes for 2 hours, using a BioTek FI 600 plate reader.

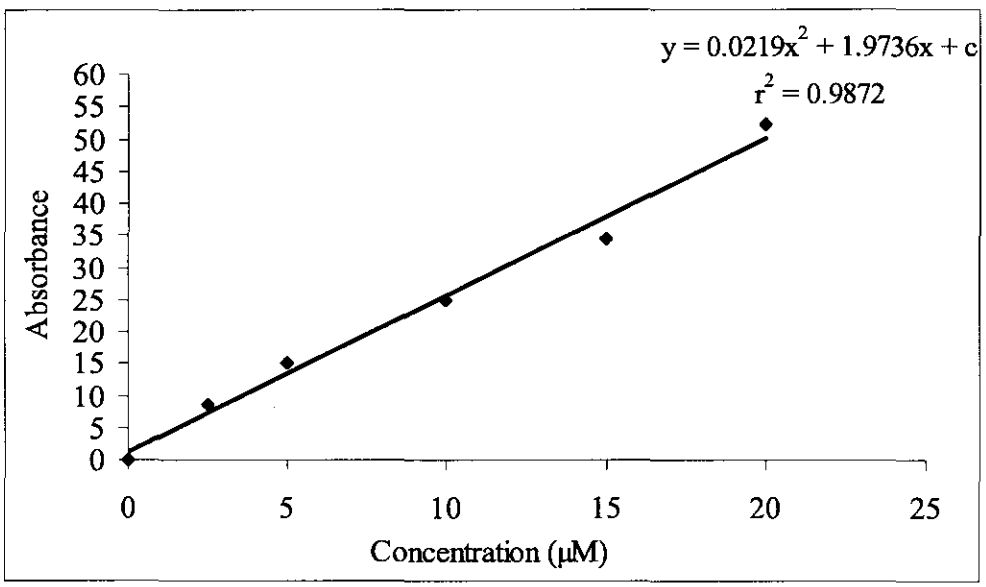

Figure 4.2: Trolox standard curve

Reaction mixtures in a 96-well opaque plate consisted of 120 µl of test compound (500µM), 160 µl of fluorescein (56nM), 20 µl 2,2'-azobis(2-amidinopropane) dihydrochloride (240mM) and 20 µl of phosphate buffer (75mM, pH 7.0). All reagents were prepared in phosphate buffer, pH 7.0 and the concentration of ß-phycoerythrin solutions was determined spectroscopically using a molar extinction coefficient of 2.41 x 10^6 $mol^{-1}.cm^{-1}$ at 545 nm. Once 2,2'-azobis(2-amidinopropane) dihydrochloride was added, fluorescence was measured every 5 minutes with

excitation and emission wavelengths of 485 and 520 nm, respectively, using a BioTek Fl 600 plate reader. The ORAC units were calculated based on the area under the fluorescence curve of ß-phycoerythrin in the presence of the test compound compared to the area generated by 500µM Trolox. The net area under the curve and ORAC values were calculated with the formulas described by Cao *et al.* (1993) (equation 4.2, 4.3 and 4.4).

- $y = ax^2 + bx + c$ (4.2)
- Use standards to draw graph and determine *a*, *b* and *c*.
- $$x = -b + \sqrt{\frac{b^2 - 4a(c - y)}{2a}}$$ (4.3)
- $S = (0.5 + fi/f0 + fii/f0 + ...fviii/f0) \times 5$ (4.4)

  (where f0 is initial fluorescence and fi is fluorescence at different time intervals)

  $y = S$

4.2.2. Results

The results obtained from the ORAC assay are presented in table 4.2 and figure 4.3. The assay was repeated several times and exactly the same values were obtained with each analysis, therefore SEM values are not applicable.

Table 4.2: ORAC values (µM trolox units/µM sample units)

Compounds	µM trolox units/µM sample units
Diphenhydramine	0.223
Cimetidine	0.371
Roxatidine	0.048
Clobenpropit	0.211
Impentamine	0.456
Thioperamide	0.374
Aspirin	0.096
Ibuprofen	0.029

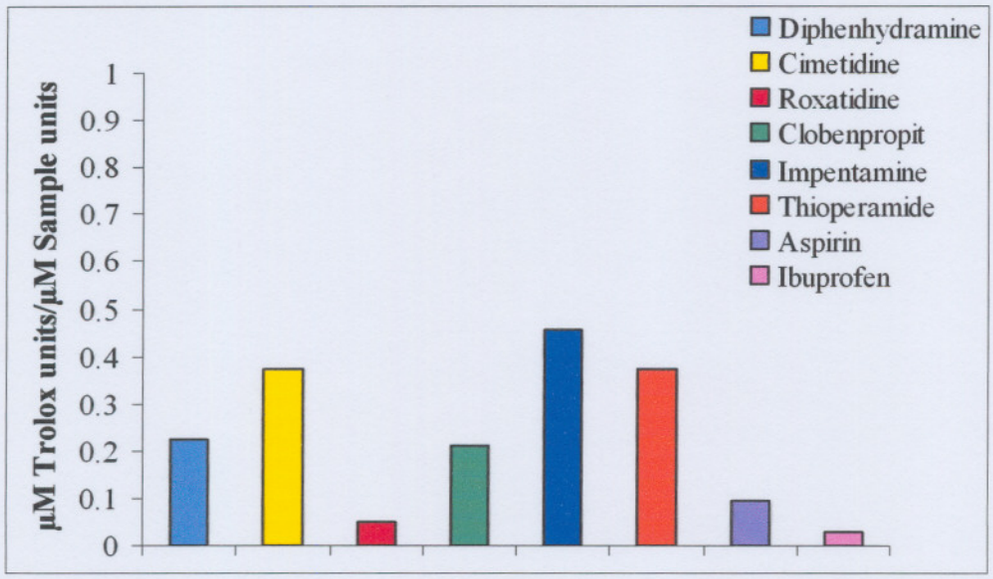

Figure 4.3: In vitro ORAC values of selected compounds.

As evident from the results shown, when compared to Trolox, none of the test compounds showed meaningful activities in the ORAC assay (table 4.2; figure 4.3). The values obtained are µM Trolox units per µM sample units and none of the tested compounds had values higher than 0.5, suggesting a very low peroxyl radical absorbing ability.

4.3. Single cell gel electrophoresis (SCGE) assay:

The single cell gel electrophoresis (SCGE or comet assay) is a quick, simple, sensitive and reliable way of measuring DNA damage (Collins, *et al.*, 1997). One of the most important advantages of the technique is that DNA lesions can be measured in the absence of mitotic activity. Genotoxicity in any mammalian organ can be detected (Sekihashi *et al.*, 2002).

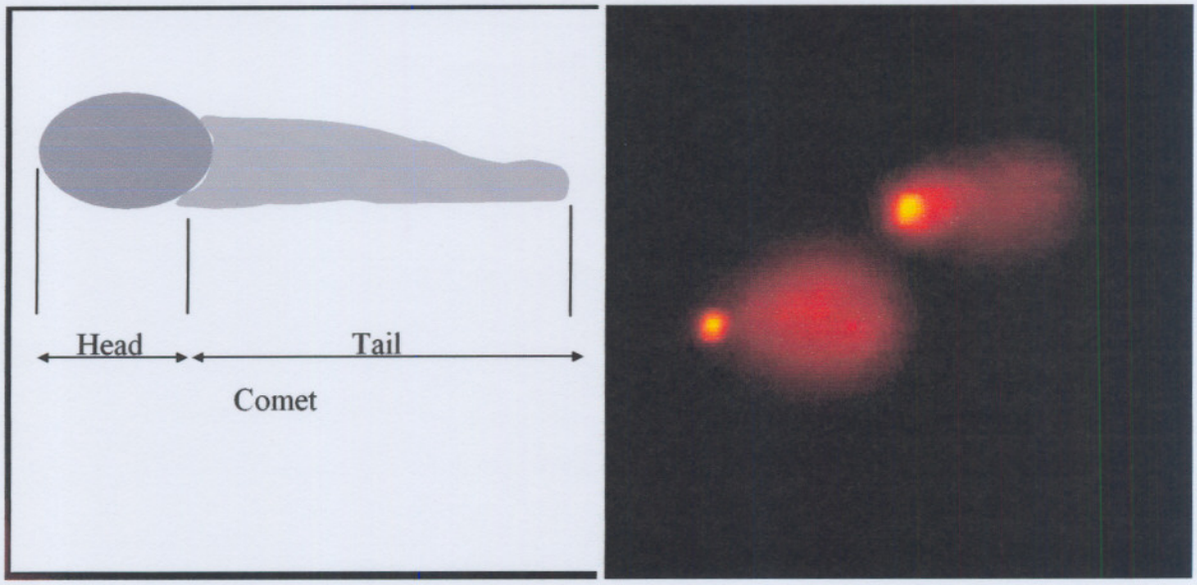

Figure 4.4: A diagram of a typical comet showing distribution of DNA in the tail and the head (Lee Steinert, 2003).

The nature of the genotoxic agent as well as the scatter in tail length, % migrated DNA and tail moment depend on the target cell population (Ejchart & Sadlej-Sosnowska, 2003). The results of the assay show the frequency of cells as a function of the tail moment (tail length x with DNA amount in the tail).

The SCGE assay was used to detect DNA damage in mice striatum after administration of MPTP (mechanism of toxicity described in section 2.2.3.1) in conjunction with test compounds.

4.3.1. Experimental

Dimethylsulfoxide (DMSO), disodium salt (EDTA), ethidium bromide, potassiumchloride, sodium chloride, sodium hydroxide, high melting point agarose, low melting point agarose, triton X-100 and tris HCl were purchased from Sigma Chemical Co., St Louis, MO.

An Olympus IX - 70 fluorescent microscope with sandblasted microscope slides (76 × 26mm) and a Bio-rad model 200/2 was used as the power supply unit.

Female C57 BL/6J mice were used for the experiment. The animals were housed in a windowless, well-ventilated constant environment room (CER) under a diurnal lightning cycle:

12 hours light; 12 hours darkness. Artificial lighting was provided by standard cool-white fluorescent bulbs (75 W) with an intensity of illumination of approximately 300 $\mu W.cm^{-2}$. Ambient temperature of the animal room was maintained between 22°C and 25°C. Cage cleaning and feeding were performed randomly and only in the photo phase, to avoid induction of secondary exogenous rhythms. Every effort was made to minimize stress to the animals, including experimental time. The protocol for the experiment was approved by the North-West University Animal Ethics Committee.

The animals were separated into ten groups of five animals each. One group was used as a toxin group, one as a control group and eight groups as test groups to determine the effects of the selected compounds.

MPTP (30 mg/kg) was used to induce neurotoxic effects. It was dissolved in normal saline and administered (s.c.) 30 minutes prior to the test compounds. The test compounds were dissolved in 0.9% saline and administered (s.c.) at a dose of 100 mg/kg.

On day four when maximum MPTP toxicity was reached, animals were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal structure, from the foramen magnum close to the orbit. The calvarium was removed, exposing the brain which was easily removed for use in experiments. The striatum was rapidly dissected and homogenised in 200 µl ice cold phosphate buffered saline (PBS) in a plastic homogeniser.

Low melting point agarose (LMPA; 0.8%) and high melting point agarose (HMPA; 1%) in 0.1M EDTA were prepared. The mixture was heated until the agarose dissolved and the LMPA container was placed in a 42°C water bath to stabilise the temperature. 100 µl heated HMPA was spread over the slide and allowed to cool. The slides were prepared prior to the commencement of the experiment on ice and kept at room temperature until needed.

LMPA (800 µl) was mixed with 50 µl of the striatum homogenate and spread on to the individual brackets. The slides were cooled on ice until the agarose gel became solid. It was then lowered into 0.1M EDTA, the lysing solution, and refrigerated for one hour. Slides may be stored for at least four weeks in the solution and must be kept under a red or yellow light to prevent further DNA damage.

The electrophoresis buffer consisted of 20 ml 0.05 M EDTA added to 500 ml of 0.6 M NaOH. The volume of the solution was adjusted to 1000 ml with the addition of 480 ml of ddH_2O. The slides were gently removed from the lysing solution and placed in electrophoresis buffer (pH > 13) for 30 minutes. The buffer allowed DNA to unwind and alkali-labile damage to be expressed. The slides were then electrophorised for 40 minutes at 28 V and 300 A. Following electrophoresis, the slides were drained for 15 minutes in Tris HCl buffer and then rinsed with ddH_2O. The cells were stained with ethidium bromide at 4°C for 1 hour and then scored. The computer program CASP was used to score the comets and the tailmoment was calculated as follows: tail length x with DNA amount in the tail.

4.3.2. Results

The results obtained in the comet assay are presented in table 4.3 and figure 4.5. The values obtained with the administration of the compounds alone are not included as the drugs on their own did not have any effect on the tailmoment.

Table 4.3: Tailmoment of the selected compounds after administration with MPTP.

Compounds	**Tailmoment ± SEM (n = 4)**
Saline	1.80 ± 0.12
MPTP	2.25 ± 0.17
Diphenhydramine + MPTP	0.82 ± 0.32
Cimetidine + MPTP	4.40 ± 0.14
Roxatidine + MPTP	0.64 ± 0.15
Clobenpropit + MPTP	0.97 ± 0.23
Impentamine + MPTP	0.73 ± 0.20
Thioperamide + MPTP	0.35 ± 0.10
Aspirin + MPTP	3.74 ± 0.18
Ibuprofen + MPTP	1.61 ± 0.16

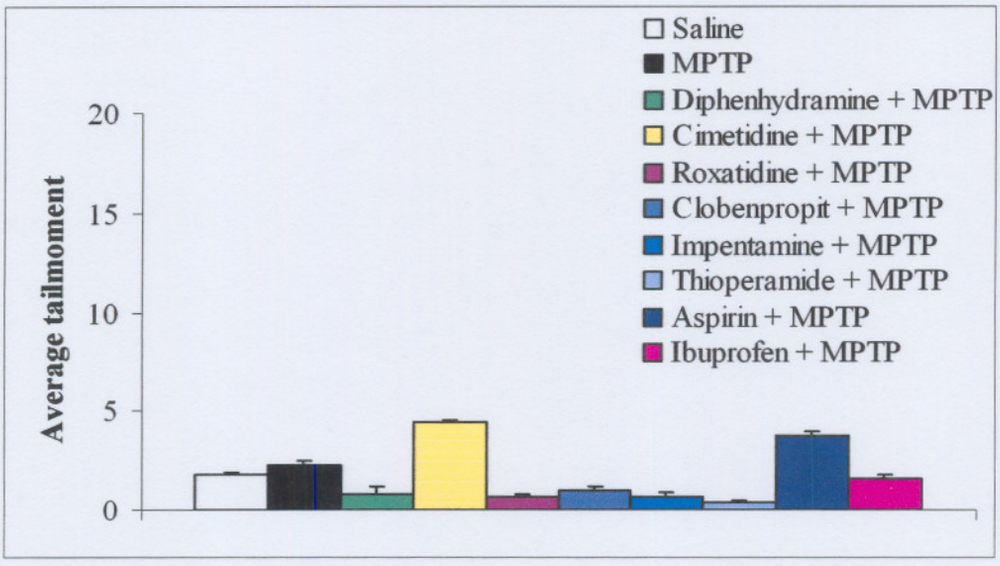

Figure 4.5: Tailmoment of selected compounds after administration with MPTP.

The tailmoment is calculated from the tail length and the amount of DNA in the tail. It is evident that the variables had very low values and therefore, as can be seen from the results, one dose of MPTP did not cause meaningful damage to the striatum and the protectective effect of the test compounds could not be assessed (table 4.3; fig.4.5).

4.4. Nitroblue tetrazolium assay

This method is generally accepted as a simple and reliable method for assaying the ability of compounds to scavenge superoxide free radicals (Ottino & Duncan, 1997). The principle of the assay is based on the ability of free radicals to reduce nitroblue tetrazolium (NBT) to insoluble nitroblue diformazan (NBD) which can be extracted with glacial acetic acid. The relative absorbance values of the diformazan were measured at 560 nm using an ultraviolet-visible spectrophotometer. The amount of NBD formed was determined from the standard curve and final results were expressed as µM diformazan produced/mg tissue. KCN was used to induce neurotoxic effects (mechanism of toxicity described in section 2.2.3.2).

4.4.1. Experimental:

Nitroblue tetrazolium, nitroblue diformazan, potassium cyanide and MPP^+ were purchased from Sigma Chemical Co., St Louis, MO and glacial acetic acid was purchased from SAARCHEM

(PTY) Ltd., Krugersdorp, South Africa. All other chemicals used were purchased from Merck , Darmstadt, Germany and were of the highest chemical purity.

Nitroblue diformazan (NBD) was used as a standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in acetic acid was prepared. A standard curve was generated by measuring the absorbance at 100μM increments of concentration (fig.4.6). The absorbance was read at 560 m using an ultraviolet-visible spectrophotometer.

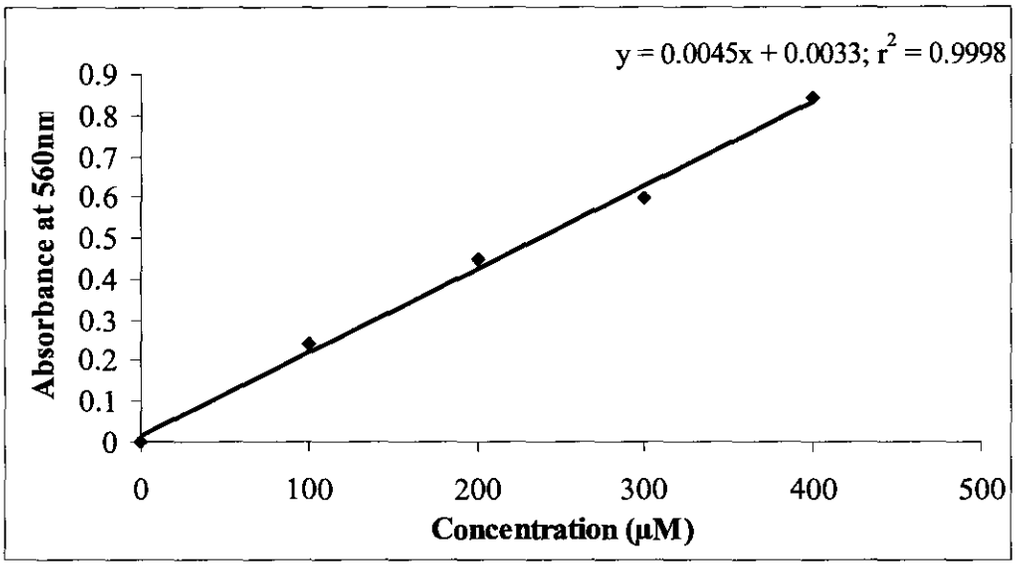

Figure 4.6: Nitroblue diformazan standard curve

Adult, male Wistar rats were used for the experiment. Animal care was as described in section 4.3.1. Protocols for the experiment were approved by the Rhodes University Animal Ethics Committee.

4.4.1.1. *In vitro* assay

On the morning of the experiment the brains were removed as described in section 4.3.1 and weighed. Whole brain homogenates were used and homogenised in ice cold 0.1M PBS in a glass homogeniser to produce a 10% (w/v) homogenate.

Stock solutions were prepared so that on addition of 250 μl of test compound dissolved in Milli-Q water, the stock solution would be diluted down to the correct incubation concentration. The following compounds were tested at concentrations of 0.25, 0.5 and 1mM: diphenhydramine, cimetidine, roxatidine, thioperamide, clobenpropit, impentamine, aspirin and ibuprofen. All the

compounds were dissolved in Milli-Q water except aspirin and cimetidine (10% ethanol) and ibuprofen (60% ethanol).

KCN was tested at 0.25, 0.5 and 1mM. KCN was dissolved in Milli-Q water, 10% ethanol and 60% ethanol to be able to compare the toxin to each of the different dissolved test compounds.

The NBT assay was performed by using 0.5 ml samples of brain homogenate, 0.25 ml KCN (1mM), 0.25 ml of the relevant drug being tested (0.25, 0.5 and 1mM) and 0.4 ml of 0.1% NBT solution. The brain homogenates were allowed to incubate for 1 hour at 37°C in an oscillating water bath. Termination of the assay and extraction of reduced NBD was carried out by centrifugation of the samples at 2000 x g. The supernatant was poured off and the pellet was dissolved in 2 ml glacial acetic acid.

4.4.1.2. *In vivo* assay

Ether anaesthesia was employed for all surgical procedures. A rat was placed in a dessicator containing cotton wool that was soaked in ether. Once the animal was sedated it was removed and placed on the operating surface. A small flask containing cotton wool soaked in ether, was placed approximately 3 cm from the rat's nose. This flask remained in this position throughout surgery, except in cases where respiration became too weak. The depth of anaesthesia was monitored by the colour of the limbs and tail (pale pink). This was indicative of the optimum level of anaesthesia with a satisfactory rate and depth of respiration. A purple colour of the limbs was an indication of cyanosis. In instances where breathing ceased, the rat was held and gently squeezed in the area of the lungs. This method usually succeeded in reviving the animal.

Diethylether is a desirable anaesthetic to use because the mortality rate of the animals is lower than if halothane or phenobarbitone was used. Ether is also easy to administer and it is easy to monitor the depth of anaesthesia.

MPP^+ (32 nmol in 2 µl) was dissolved in PBS (pH 7.4) and infused bilaterally into the striatum employing a rat brain stereotaxic apparatus (Stoelting, IL, USA). The skull was orientated according to the stereotaxic atlas (Köning & Klippel, 1963).

After a saggital cut in the skin of the skull, the bregma and lambda suture were located and holes were drilled with a Bosch electrical drill fitted with a drill bit of 0.5 mm in diameter at the following coordinates of l = 0.24; AP = 0.30 and DV = 0.78, from lambda point (Paxinos & Watson, 1998). Care was taken not to lesion the meninges.

A Hamilton syringe, with a cannula of diameter in 0.3 mm, held rigidly in the stereotaxic micromanipulator, was used to inject 2 µl MPP^+. The injection was administered at a rate of 1 µl per minute and the cannula was left *in situ* for a further 3 minutes to allow for passive diffusion and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. The rats recovered from the anaesthesia after approximately 10-15 minutes. Throughout the process, the animals were monitored for any possible behavioural changes, but no visible effects were observed.

The rats used as controls were subjected to the same surgical procedures as described earlier. The stereotaxic injections were free of MPP^+, comprised solely of 1 µl saline and was injected into the left nigral region.

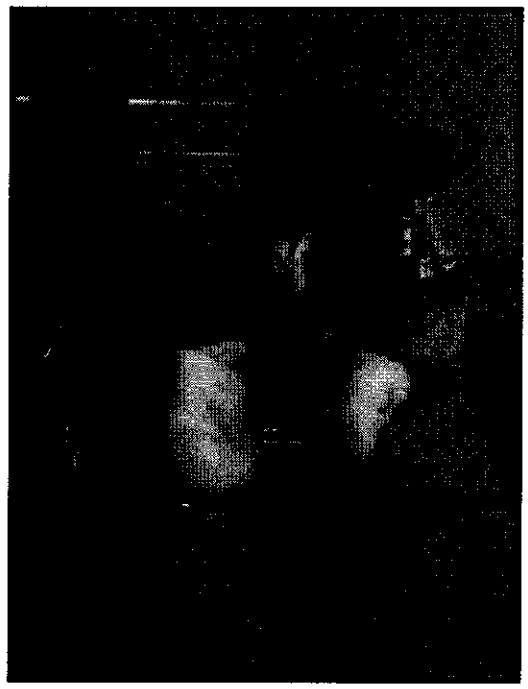

Figure 4.7: A view of the stereotaxic apparatus used for the bilateral intrastriatal injection of MPP^+.

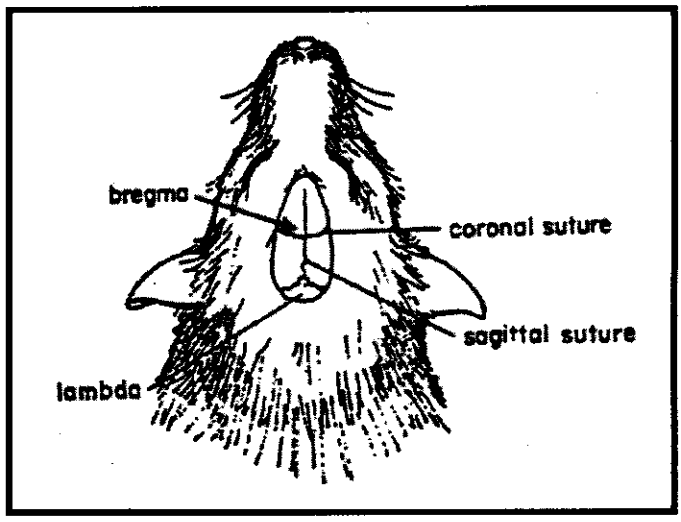

Figure 4.8 A view of the rat skull after the skin has been cut. The sutures shown are used as a reference point for the measurement of the coordinates for the intrastriatal injection.

The MPP^+ treated animals were separated into seven groups of four rats each and the following compounds were administered (i.p.) at a dose of 50 mg/kg: diphenhydramine, cimetidine, thioperamide, clobenpropit, aspirin and ibuprofen[1]. All the compounds were dissolved in 50% ethanol, except thioperamide and clobenpropit that were dissolved in 0.9% saline. Intraperitoneal administration of compounds followed immediately when the animals recovered from anaesthesia and again after four hours. 24 hours later another injection was given and it was repeated after four hours. On day four when MPP^+ reached maximum toxicity, animals were decapitated and the striatum rapidly dissected as described in section 4.3.1

Two control groups were used, one group received 50% ethanol (i.p.) and the other group 0.9% saline (i.p.).

One milliliter of rat striatum homogenate and 0.4 ml NBT was added. The homogenates were allowed to incubate for 1 hour at 37°C in an oscillating water bath. Termination of the assay and extraction of reduced NBD was carried out by centrifugation of the samples at 2000 x g. The supernatant was poured off and the pellet was dissolved in 2 ml glacial acetic acid.

[1] Roxatidine and impentamine were not tested *in vivo* due to insufficient amounts of the test compounds.

4.4.2. Results

The results obtained from the NBT assay are presented in table 4.4 and 4.5 and figure 4.9 and 4.10.

Table 4.4: The *in vitro* effect of selected compounds on KCN-induced superoxide anion formation in rat brain homogenate.

Compounds	**Concentrations used (mM)**	**Superoxide anion formation ± SEM (n = 5) (µmol diformazan / mg tissue)**
Control		0.75 ± 0.07
KCN	0.25mM	1.08 ± 0.06
	0.5mM	1.64 ± 0.10
	1mM	1.90 ± 0.10
Diphenhydramine + 1mM KCN	0.25mM	1.34 ± 0.02
	0.5mM	1.38 ± 0.03
	1mM	1.57 ± 0.09
Cimetidine + 1mM KCN	0.25mM	1.80 ± 0.12
	0.5mM	1.62 ± 0.03
	1mM	1.37 ± 0.04
Roxatidine + 1mM KCN	0.25mM	1.78 ± 0.09
	0.5mM	1.32 ± 0.03
	1mM	0.97 ± 0.02
Clobenpropit + 1mM KCN	0.25mM	1.35 ± 0.05
	0.5mM	1.11 ± 0.06
	1mM	0.82 ± 0.03
Impentamine + 1mM KCN	0.25mM	2.08 ± 0.02
	0.5mM	1.94 ± 0.04
	1mM	1.56 ± 0.02
Thioperamide + 1mM KCN	0.25mM	1.59 ± 0.12
	0.5mM	1.15 ± 0.03
	1mM	0.92 ± 0.05
Aspirin + 1mM KCN	0.25mM	1.48 ± 0.11
	0.5mM	1.26 ± 0.07
	1mM	1.00 ± 0.07
Ibuprofen + 1mM KCN	0.25mM	1.57 ± 0.07
	0.5mM	1.34 ± 0.03
	1mM	1.02 ± 0.02

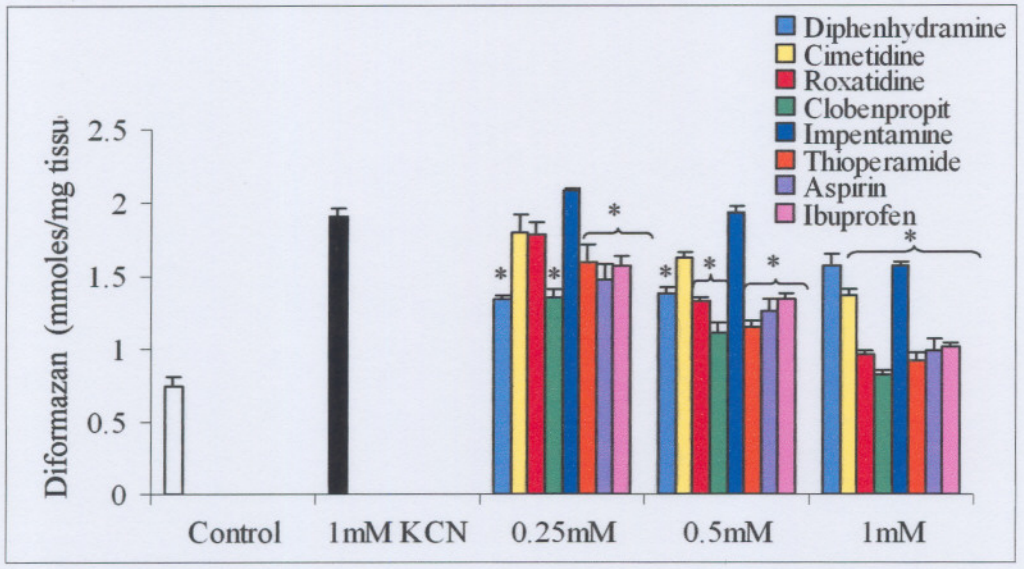

Figure 4.9: The effect of selected compounds on KCN-induced superoxide anion formation in rat brain homogenate. Each bar represents the mean ± S.E.M.; n = 5. * $p \leq 0.05$ compared to KCN.

The *in vitro* exposure of whole rat brain homogenate showed that KCN caused a significant concentration dependent rise in superoxide anion generation. As can be seen from the results, all the compounds were able to reduce superoxide anion generation compared to KCN. Diphenhydramine was more effective at lower concentrations and clobenpropit, thioperamide and ibuprofen reduced superoxide anion generation in a dose dependent manner (table 4.4; figure 4.9).

As can be seen from the *in vivo* results of the NBT assay, diphenhydramine, clobenpropit, thioperamide and ibuprofen significantly reduced the superoxide anion generation evoked by KCN (table 4.5; figure 4.10).

Table 4.5: The effect of *in vivo* administration of selected compounds on intrastriatal injected MPP^+-induced superoxide anion formation in rat striatal homogenate

Administered compounds	**Superoxide anion formation ± SEM (n = 4) (µmol diformazan / mg tissue)**
Control	1.90 ± 0.15
MPP^+	3.19 ± 0.14
Diphenhydramine + MPP^+	2.01 ± 0.12
Cimetidine + MPP^+	3.27 ± 0.05
Clobenpropit + MPP^+	2.00 ± 0.07
Thioperamide + MPP^+	1.58 ± 0.04
Aspirin + MPP^+	3.69 ± 0.06
Ibuprofen + MPP^+	1.89 ± 0.03

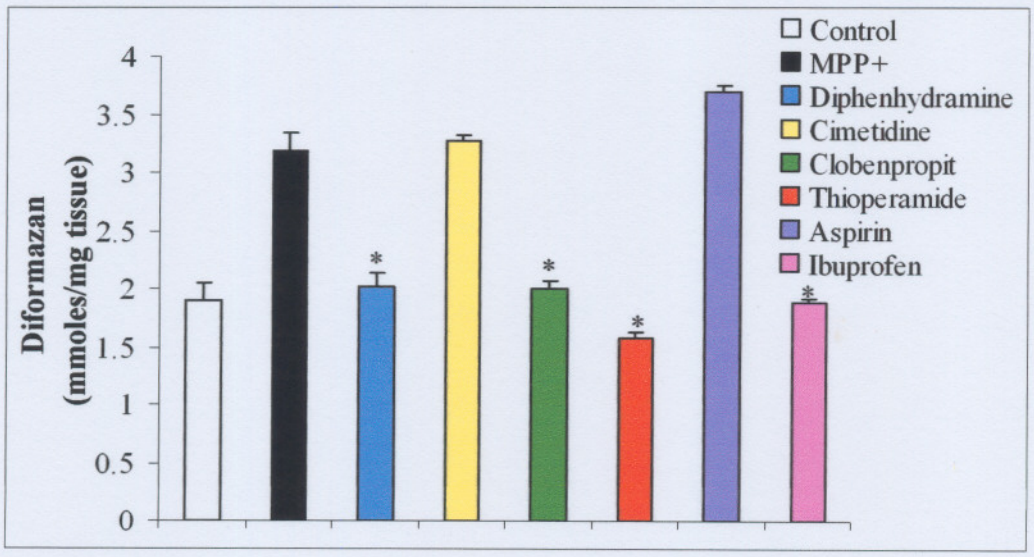

Figure 4.10: The effect of the *in vivo* administration of test compounds on intrastriatal injected MPP^+-induced superoxide anion formation in rat striatal homogenate. Each bar represents the mean ± SEM; n = 4. * $p \leq 0.05$(ibuprofen; diphenhydramine; clobenpropit and thioperamide compared to MPP^+).

4.5. Lipid peroxidation

Lipid peroxidation was determined using the TBARS assay. This assay involves the reaction of malondialdehyde (MDA) equivalents, a marker of lipid peroxidation, with thiobarbituric acid to yield a pink complex.

4.5.1. Experimental

Potassium cyanide, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, butylated hydroxytoluene and MPP^+ were purchased from Sigma Chemical Co., St Louis, MO. Trichloroacetic acid and butanol were purchased from SAARCHEM (PTY) Ltd., Krugersdorp, South Africa.

1,1,3,3-Tetramethoxypropane was used as a standard. A series of reaction tubes, each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1 ml. A calibration curve was generated by measuring the absorbance at 5 nmole concentration increments. The absorbance was read at 532 nm using an ultraviolet-visible spectrophotometer and plotted against the molar equivalent weight of malondialdehyde in the complex assayed (figure 4.12).

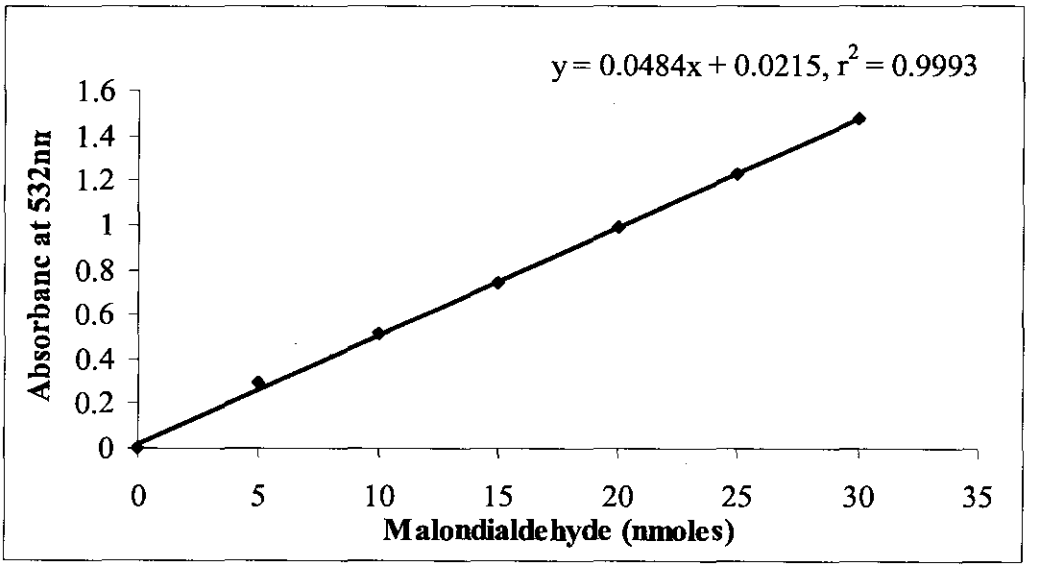

Figure 4.11: Lipid peroxidation standard curve

Adult, male Wistar rats were used for the experiment. They were cared for as explained in section 4.3.1. Protocols for the experiment were approved by the Rhodes University Animal Ethics Committee.

In vitro experiments were performed on whole brain homogenates and *in vivo* experiments on the striatum.

4.5.1.1. *In vitro* assay

On the morning of the experiment, the brains were removed and weighed, before homogenisation in ice cold 0.1M PBS in a glass homogeniser to produce a 10% (w/v) homogenate (section 4.4.1.1).

Stock solutions were prepared so that on addition of 100 µl of antagonist the stock solution would be diluted down to the correct incubation concentration. The compounds that were dissolved and tested were the same as in section 4.4.1.1.

The reaction mixture was incubated in an oscillating water bath for 1 hour at 37°C. At the end of the incubation period, 0.5 ml butylated hydroxytoluene (BHT) and 1 ml trichloroacetic acid (TCA) were added to the mixture. The samples were centrifuged at 2000 x g for 20 minutes to remove insoluble proteins. Following centrifugation, 2 ml of protein free supernatant was removed from each tube, and a 0.5 ml aliquot of 0.33% thiobarbituric acid was added to this fraction. All tubes were heated for an hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2 ml butanol. The absorbance of the complex was read at 532 nm and malondialdehyde levels were determined from the generated standard curve (figure 4.12).

4.5.1.2. *In vivo* assay

Adult male Wistar rats were used and the treatment of the animals, administration of the test compounds and controls were as described in section 4.3.1 and 4.4.1.2.

On the fourth day following intrastriatal injection of the toxin, the rats were sacrificed and the brains removed. The striatum was rapidly dissected after the cerebral cortex was opened (section 4.3.1).

The homogenate (1 ml) for each treated group was heated for 15 min with 0.5 ml BHT and 1 ml TCA in a boiling water bath. The samples were centrifuged at 2000 x g for 20 minutes to remove insoluble proteins. Following centrifugation, 2 ml of protein free supernatant was removed from each tube and a 0.5 ml aliquot of 0.33% thiobarbituric acid was added to this fraction. All tubes were heated for an hour at 95°C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2 ml butanol. The absorbance of the complex was read at 532 nm and malondialdehyde levels were determined from the generated standard curve.

4.5.2. Results

The *in vitro* and *in vivo* lipid peroxidation results obtained from the TBARS assay are presented in table 4.6 and 4.7 and figure 4.13 to figure 4.15.

Table 4.6: The effect of KCN-induced lipid peroxidation in rat brain homogenate.

Compounds	**Concentrations used (mM)**	**Lipid peroxidation ± SEM (n = 5) (nmol MDA / mg tissue)**
Control		0.19 ± 0.01
KCN	0.25mM	0.23 ± 0.01
	0.5mM	0.30 ± 0.01
	1mM	0.33 ± 0.01
Diphenhydramine + 1mM KCN	0.25mM	0.23 ±0.01
	0.5mM	0.28 ± 0.01
	1mM	0.31 ± 0.01
Cimetidine + 1mM KCN	0.25mM	0.19 ± 0.01
	0.5mM	0.16 ± 0.01
	1mM	0.14 ● 0.01
Roxatidine + 1mM KCN	0.25mM	0.20 ± 0.01
	0.5mM	0.17 ± 0.02
	1mM	0.15 ± 0.02
Impentamine + 1mM KCN	0.25mM	0.28 ± 0.01
	0.5mM	0.22 ± 0.01
	1mM	0.21 ± 0.01
Thioperamide + 1mM KCN	0.25mM	0.20 ± 0.02
	0.5mM	0.19 ± 0.01
	1mM	0.17 ± 0.01
Clobenpropit + 1mM KCN	0.25mM	0.21 ± 0.01
	0.5mM	0.19 ± 0.01
	1mM	0.15 ± 0.01
Aspirin + 1mM KCN	0.25mM	0.33 ± 0.02
	0.5mM	0.33 ± 0.02
	1mM	0.26 ± 0.01
Ibuprofen + 1mM KCN	0.25mM	0.34 ± 0.02
	0.5mM	0.31 ± 0.02
	1mM	0.28 ± 0.02

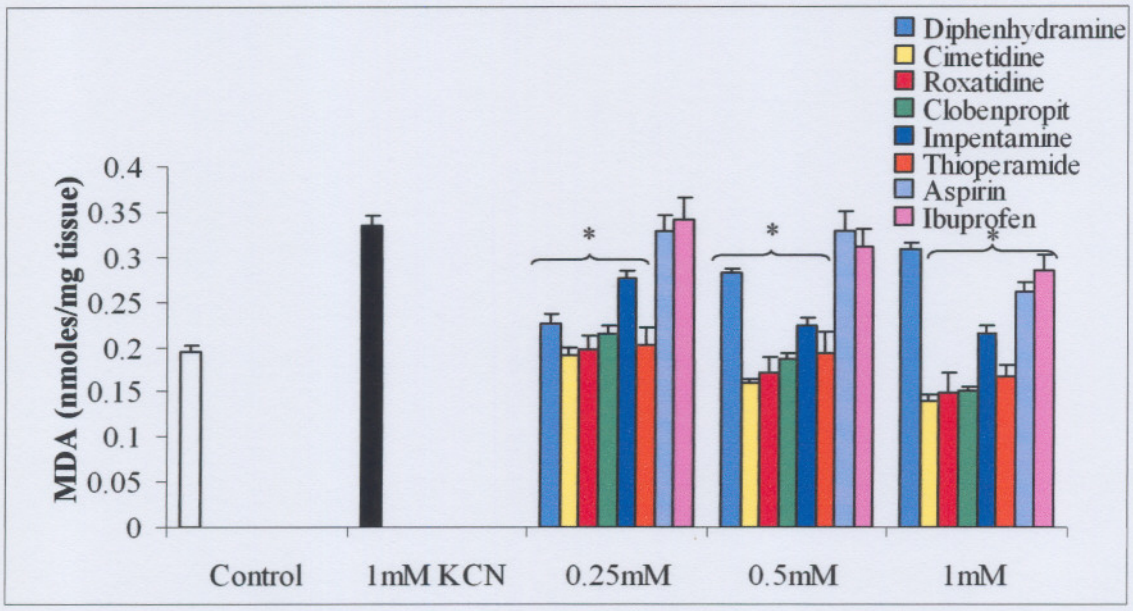

Figure 4.12: The effect of the test compounds on KCN-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean ± S.E.M.; n =5. $p \leq 0.05$ compared to KCN.

The *in vitro* exposure of whole rat brain homogenate showed that KCN caused a significant concentration dependent rise in malondialdehyde generation. As can be seen from the results, all the compounds were able to reduce malondialdehyde generation. Diphenhydramine was more effective at lower concentrations. Cimetidine, roxatidine, clobenpropit, impentamine and thioperamide reduced lipid peroxidation in a dose dependent manner (table 4.6; figure 4.12).

In the *in vivo* TBARS assay, diphenhydramine, clobenpropit, thioperamide and ibuprofen significantly reduced the malondialdehyde induced generation of lipid peroxidation (table 4.7; figure 4.13).

Table 4.7: The effect of the *in vivo* administration of test compounds on intrastriatal injected MPP^+-induced lipid peroxidation in rat striatal homogenate.

Administered compounds	**Lipid peroxidation ± SEM (n = 4) (nmol MDA / mg tissue)**
Control	0.020 ± 0.002
MPP^+	0.032 ± 0.003
Diphenhydramine + MPP^+	0.028 ± 0.000
Cimetidine + MPP^+	0.032 ± 0.003
Clobenpropit + MPP^+	0.014 ± 0.002
Thioperamide + MPP^+	0.016 ± 0.001
Aspirin + MPP^+	0.030 ± 0.003
Ibuprofen + MPP^+	0.017 ± 0.002

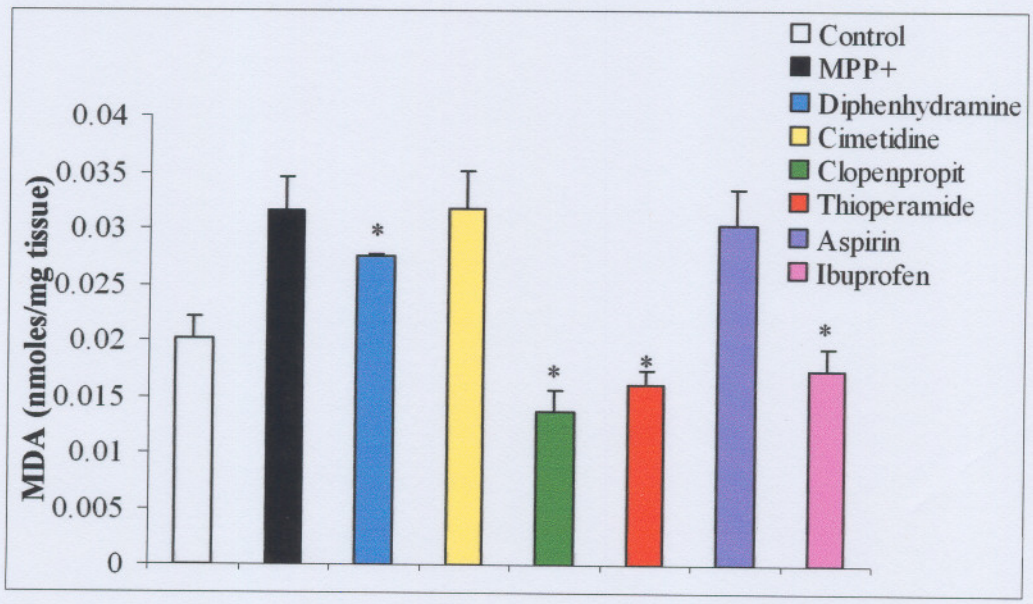

Figure 4.13: The effect of the *in vivo* administration of test compounds on intrastriatal injected MPP^+-induced lipid peroxidation in rat striatal homogenate. Each bar represents the mean ± SEM; n=4. * $p \leq 0.05$ compared to MPP^t.

The results were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

4.6. Summary

From these results it is evident that all the test compounds have free radical scavenging activities. The most effective compounds were the histamine H_3 antagonists, clobenpropit and thioperamide. These compounds significantly reduced superoxide anion generation and lipid peroxidation in a dose dependent manner *in vitro*. They also significantly reduced both variables *in vivo*. The test compounds did not have any meaningful activity in the FRAP and ORAC assays.

CHAPTER 5:

Discussion and Conclusion

Parkinson's disease is having an immense effect on the aging society and has a high incidence of occurrence. In Parkinson's disease it is the free radicals that ultimately bring about death of the dopaminergic neurons in the substantia nigra (Schapira *et al.*, 1993). It is thus important to identify compounds that are able to reduce free radical generation and that have effects on dopamine depletion.

Antioxidants act as free radical scavengers by converting them to less harmful molecules and thereby preventing the formation of new free radical species (Paździoch-Czochra & Widenska, 2001). They are believed to be important in health maintenance through the modulation of oxidative processes in the body (Ozben; 1998).

5.1. Test compounds

The histamine H_3 receptor acts as a heteroreceptor while its stimulation inhibits the release of dopamine. Histamine H_3 antagonists will reduce the mediated inhibition of dopamine release (Rodrigues, 1996). If histamine H_3 antagonists are able to scavenge free radicals in addition to increasing dopamine levels, a dual therapeutic effect will be obtained and these compounds might have tremendous effects in reducing the progression of Parkinson's disease. Clobenpropit, impentamine and thioperamide were used in this study to determine their free radical scavenging activities.

Diphenhydramine, a histamine H_1 antagonist, is used to treat mild cases of Parkinson's disease and cimetidine, a histamine H_2 antagonist, has been shown to be an effective hydroxyl scavenger *in vitro* (Kenneth & Serafin, 1996; Lapenna *et al.*, 1994). If these compounds are able to reduce free radical generation *in vivo* they might have therapeutic efficacy in neurological disorders associated with oxidative stress. Roxatidine was included as a structurally unrelated histamine H_2 antagonist.

Aspirin, a well-known free radical scavenger was used as a positive control to evaluate the effects of the histamine antagonists (Aubin, *et al.*, 1998). Ibuprofen was included to determine whether another NSAID is also able to scavenge free radicals.

5.2. Indicators of oxidative stress

The superoxide anion leads in the onset of the oxidative stress cascade. This anion is significant as the source of hydrogen peroxide and is able to act as a reductant of transition metals (Cheeseman & Slater, 1993). Superoxide anions are also key factors in the initiation step of the Fenton reaction which generates the highly reactive hydroxyl radical (figure 2.2; Buettner *et al.*, 1978). If the superoxide anion can be eliminated, the participation of transition metals in the generation of free radicals and lipid peroxidation will decrease (section 2.1.2.1). This will also lead to a reduction in the formation of hydroxyl radicals and hydrogen peroxide. During the initiation step of lipid peroxidation, polyunsaturated fatty acids are attacked by ROS including superoxide anions. This leads to the next step where the peroxyl radical is formed (figure 2.9). Scavenging of the superoxide anion will lead to a reduction in the incidence of peroxyl radicals and thereby, lipid peroxidation. It is thus evident that the superoxide anion plays an important role in the generation of free radicals and free radical processes. A compound able to significantly scavenge superoxide anions will remarkably affect the reduction of oxidative stress and diseases associated with free radical generation.

The FRAP assay is used to determine the reduction ability of test compounds (Benzie & Strain, 1996). It only involves a chemical reaction that includes electron donating antioxidants. However, antioxidants affect the oxidation processes by various mechanisms (section 2.3) and therefore this assay is not a reliable method to determine if compounds have free radical scavenging effects.

The ORAC assay is based on the ability of compounds to inhibit generated peroxyl radicals (Prior *et al.*, 2003). None of the test compounds showed meaningful activity in this assay (table 4.2 and figure 4.3). The damaging effect of the peroxyl radical lies in its ability to react with polyunsaturated fatty acids and thereby lead to lipid peroxidation (Cadenas, 1995). As mentioned earlier, scavenging of the superoxide anion will lead to a lower incidence of lipid

peroxidation. Therefore, this screening method does not necessarily indicate whether a compound is able to act as a scavenger of all free radicals.

In the *in vitro* nitroblue tetrazolium and lipid peroxidation assays, cyanide was used to induce neurotoxic effects. Cyanide neurotoxicity is attributed to a Parkinson-like condition and produces dopaminergic toxicity. Reactive oxygen species are generated as a result of increased intracellular calcium, thus leading to lipid peroxidation (section 2.2.3.2). MPP^+ was used in the *in vivo* Parkisonian model and leads to damage in dopaminergic neurons. This compound blocks the electron transport chain, thus leading to energy failure and an increase in the formation of free radicals. Severe energy impairment leads to intraneuronal calcium-overload, with the same consequences as seen in cyanide toxicity (Schmidt & Ferger, 2001). Although different toxins were used to study the *in vitro* and *in vivo* effects, it is evident that their neurotoxicity has the same result: dopaminergic toxicity and free radical formation.

5.3. Discussion

In the FRAP assay the compounds gave values in the same range as the control value and when compared to ascorbic acid, the compounds did not have any meaningful effects (table 4.1 and figure 4.1). Therefore it is evident that the mechanism of the compounds does not include the reduction of iron and are not acting directly as antioxidants.

As evident from the results shown, none of the selected compounds had meaningful effects in the ORAC assay (table 4.2 and figure 4.3). The compounds are thus not able to scavenge peroxyl radicals. As seen above, if the superoxide anion is scavenged, the formation of the peroxyl radical and lipid peroxidation will decrease. This assay does not measure the ability of compounds to scavenge other free radicals and is thus not adequate.

As can be seen from the results of the comet assay, MPTP did not cause any damage to the striatum after administration (table 4.3; figure 4.5). Therefore, the protective effects of the compounds could not be evaluated. Studies have shown that the age of the animals and the treatment regime plays an important role in MPTP toxicity. In young mice, repair and compensatory mechanisms seem to be more efficient and a higher dose is required to reach a comparable lesion than in aged mice where recovery is comparably lower (Schmidt & Ferger,

2001). It is thus suggested that one year old mice are used and that sub chronic rather than acute treatment is used to assure effective striatal dopamine depletion. Alternatively, the comet assay can be performed after the intrastriatal injection of MPP^+ to assure effective concentrations in the brain. Indeed, this was done in the NBT and lipid peroxidation studies and marked fosinity was evident, as discussed below.

5.3.1. Histamine antagonists

5.3.1.1. Histamine H_1 antagonists

In the nitroblue tetrazolium assay and lipid peroxidation assay diphenhydramine significantly reduced free radical generation at 0.25 and 0.5mM *in vitro*. At a concentration of 1mM, the drug did not reduce free radical levels, thereby suggesting that higher concentrations induce toxic effects (figure 4.9; figure 4.12). The compound also significantly reduced both variables *in vivo* (figure 4.10; figure 4.13). When compared to aspirin, diphenhydramine had values in the same range as this well known free radical scavenger. Although the compound did not have meaningful effects in the FRAP and ORAC assays, its ability to significantly reduce superoxide anions and lipid peroxidation makes it a promising tool in the treatment of diseases associated with oxidative stress.

5.3.1.2. Histamine H_2 antagonists

Roxatidine (0.5 and 1mM) and cimetidine (1mM) significantly reduced superoxide anion generation *in vitro*. Roxatidine (1mM) decreased superoxide anion formation by more than 50%. These compounds were able to significantly reduce malondialdehyde concentrations in a dose dependant manner in the *in vitro* experiments. Roxatidine had values in the same range as aspirin in both experiments (figure 4.9; figure 4.12). Cimetidine was not able to reduce *in vivo* free radical generation (figure 4.10; figure 4.13). Previous studies have shown that high concentrations of this drug is needed to cross the blood-brain barrier appreciably (Borrow *et al.*, 1981). The significant *in vitro* effect did not correlate with the *in vivo* effect, therefore it is evident that an efficient concentration did not cross the blood-brain barrier.

5.3.1.3. Histamine H_3 antagonists

Impentamine significantly reduced lipid peroxidation at all concentrations used and superoxide anion generation at 1mM. Thioperamide and clobenpropit significantly reduced superoxide anion generation as well as lipid peroxidation *in vitro* across the concentration range used. Clobenpropit and thioperamide at a concentration of 1mM was able to reduce lipid peroxidation to values lower than that of the control, thus suggesting that the drugs are able to reduce the free radical concentration from normal processes in the brain and not only the induced free radical generation (figure 4.9; figure 4.12). The two compounds also had significant *in vivo* effects and it was evident that they were able to reduce superoxide anion generation and lipid peroxidation to values lower than that of the control (figure 4.10; figure 4.13). This confirms the fact that these two compounds are able to attenuate normal free radical processes in the brain. The *in vitro* values of clobenpropit and thioperamide were in the same range as that of asprin and the *in vivo* effects were significantly better. Although the compounds did not give meaningful results in the FRAP and ORAC assays, their significant superoxide anion scavenging abilities will reduce the formation of peroxyl radicals and lipid peroxidation as well as the effect of transition metals on free radical generation. The reduction in superoxide anions will also prevent the formation of the highly reactive and damaging hydroxyl radical and the formation of hydrogen peroxide, making them promising compounds to act as effective free radical scavengers in the body. The ability of the compounds to induce dopamine release and to reduce superoxide anion generation and malondialdehyde generation, might have a dual therapeutic effect in treating and slowing down the progression of Parkinson's disease.

5.3.2. NSAIDs

Ibuprofen significantly decreased superoxide anions in a dose dependant manner and lipid peroxidation at a concentration of 1mM (figure 4.9; figure 4.12). The compound was able to significantly reduce both variables *in vivo* (figure 4.10; figure 4.13). It compared favourably to aspirin with *in vitro* values in the same range and significantly better *in vivo* effects. Ibuprofen is

a stronger anti-inflammatory agent than aspirin and therefore it is suggested that the anti-inflammatory activity of these compounds is a consequence of free radical scavenging abilities.

5.4. Conclusion

From the results it is evident that diphenhydramine at lower concentrations, ibuprofen and the histamine H_3 receptor antagonists, clobenpropit and thioperamide are more potent in scavenging free radicals than aspirin and that these drugs have the potential to be used in the treatment of neurodegenerative disorders. The activities of ibuprofen in the assays confirm that the NSAIDs used in this study are able to act as free radical scavengers.

The histamine H_3 receptor antagonists are also known to promote the release of dopamine and together with the ability of the compounds to scavenge free radicals, significant effects might be obtained in the treatment of Parkinson's disease.

Neurodegenerative diseases are having an immense effect on an aging society and it is important to identify beneficial agents that might be useful in reducing the risks of these diseases. From this research it is evident that the above mentioned compounds might have potential benefits as neuroprotectants and that further studies must be done to investigate their mechanism of action and to prove their efficacy and safety.

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