Review of Literature
Chapter 2

REVIEW OF LITERATURE

2.1 ALUMINIUM

Aluminium (Al) is the third most abundant element (Kochian; 1995), making it about 8% of the earth’s surface (Bassam and Shakhashiri; 2007). It is a white, crystalline, ductile metal with an atomic weight of 27, atomic number 13, density 2.7 and small ionic radius of only 0.51 Å. Aluminium occurs only in oxidized form, mainly as alumina (Al₂O₃), and forms an important constituent of almost 300 different minerals such as feldspar and micas. It is insoluble in the form of hydroxides and complex of aluminosilicates, but acidification increases the level of free aluminium in the soil and lakes. The high charge and small size gives aluminium a strong polarizing effect on adjacent atoms (Greenwood et al, 1997). It has a strong affinity for phosphate ions and various organic phosphate compounds. A property that is responsible for its DNA binding affinity and formation of Al⁺³-ATP complexes (Anitha and Rao; 2002).

2.1.1 Aluminium Exposure

Aluminium is released to the environment both by natural processes and from anthropogenic sources, such as soil-derived dusts from erosion and particulate from coal combustion (Grant et al, 1990), mining and agriculture (Eisenreich, 1980). Humans are constantly exposed to aluminium as a result of an increase in industrialization and improving technology practice (Turgut et al, 2007). The estimated average human exposure of aluminium through food and water is between 2-30 mg/day. The major sources of aluminium exposure are listed in table 2.1.1

2.1.2 Metabolism of Aluminium

Absorption of aluminium

Alfrey (1983) reported that the respiratory system and gastrointestinal tract are thought to be relatively less permeable to aluminium as only 10% of the ingested aluminium is absorbed (Gorsky, 1981) and about 3% of aluminium is also
absorbed into the blood from the lungs (Jones and Bennett, 1986). The absorption of aluminium from gut depends on the chemical form of aluminium compound and also on the pH of the environment. Cam et al (1976) suggested that gastrointestinal absorption of aluminium occurs in the acidic environment of the stomach and duodenum. Generally aluminium is poorly absorbed due to its tendency to form insoluble salts especially with phosphates. Additionally the presence of other dietary agents, such as citrate, which forms complex with the metal decrease the aluminium absorption (Powell et al, 1999). Although, the exact mechanism of aluminium uptake from the gut has not been elucidated it is postulated that the iron carrier protein transferrin plays an important role in the uptake of aluminium along with iron (Purves et al, 1988). Specialised iron-absorption pathways in the gut may absorb ionic aluminium actively.

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Distribution of Aluminium

After gastrointestinal absorption (or intraperitoneal injection), aluminium travels via the portal circulation into the liver, where it is thought to undergo a first-pass clearance (Voto and Yokel; 1994).

1. In blood aluminium binds avidly to iron-transfer protein transferrin presumably to the same site as Fe$^{3+}$ (Harris, 1998) and shares this iron transporters protein for its transport. This fact strongly affects the distribution of aluminium tissues since there are receptors for transferrin in most tissues. The interaction with iron has many implications to aluminium toxicity. Trapp (1983) reported that aluminium also binds to low molecular weight compounds, predominantly citrate. Some non-specific aluminium binding may also take place with albumin, and at least two other aluminium-binding proteins, 8-kDa component (38,77) and 18-kDa component, albindin (Zatta et al, 2003).

2. Bone tissue, liver and the spleen are the primary depots for aluminium accumulation in the body which range from about 0.3 to 0.8mg/kg wet weight (Thurston et al, 1972; Marsden et al, 1979; Spencer et al, 1995; Lote et al, 1993) i.e. 100 to 300 times higher than the probable concentrations in blood plasma.

3. In the brain the normal concentration of aluminium is approximately 0.25 to 0.75 mg/kg wet weight (Alfrey et al, 1980).

Aluminium in Brain:

Aluminium gains entry into the brain by crossing the blood brain barrier with the help of carrier system presumably with transferrin (Tf)-mediated endocytosis. Studies from various laboratories have shown that brain cells possess high-affinity receptors for transferrin (TfR) that is independent of metal being transported (Trapp, 1983; Martin et al 1986). The Tf-TfR system is postulated to be the route whereby the brain can access iron from the general circulation to meet its high metabolic requirements. However, it is generally accepted that only 30% of the ion binding sites in plasma Tf has available in the circulation are saturated with iron at any given time (Cochran et al, 1987), leaving the remaining 70% available to the other ions/metals. Roskams and Connor (1990) examined the binding of aluminium – transferrin and iron – transferrin to homogenized rat brain and found evidence of a receptor that binds to both iron and aluminium. Studies have shown that 30% of binding sites are
actually occupied with aluminium (Trapp, 1983). Therefore aluminium is capable not only of binding transferrin but also of utilizing this pathway in gaining entry into the brain. However uptake mechanisms are not completely understood and may involve both para and transcellular routes. In order to avoid aluminium deposition in the brain, the blood-brain barrier has an active efflux of this cation through a monocarboxylate transporter (Yokel and McNamara, 2001). However this system can be overcome by an increase in blood aluminium concentration. Aluminium brain concentrations should be lower than 2µg/gm (Andrasi et al, 2005). A ten-fold increase in aluminium concentrations was reported in patients intoxicated with aluminium through the use of hemodialysis solutions containing high levels of aluminium (Alfrey et al, 1976). The accumulation of aluminium in brain has been connected to the onset of various neurodegenerative diseases such as Alzheimer’s disease (Paul et al, 2004), Parkinsonism dementia (Berthon, 1996; Corain et al, 1996; Becaria et al, 2002; Yokel, 2002), dialysis encephalopathy (Wisniewski et al, 1990) and amyotrophic lateral sclerosis (Flaten, 2001). The contribution of aluminium to these diseases remains controversial. There are epidemiological studies failing to reveal a connection with the intake of aluminium (Savroy et al, 1996) but even if aluminium is not the causative element it may initiate events leading to disease or accentuate the pathogenesis.

Aluminium in Neurons and Glial Cells

Several studies have suggested the distribution of aluminium in both neurons and glial cells (Café et al, 1995). The primary function of glial cells in the brain is to provide the appropriate microenvironment around neurons for their proper functioning and also the maintenance of the short-term constancy of BIF (Brain interstitial fluid). Glial cells respond to brain toxins by increased expression of glial fibrillary acidic protein. Disruption of glial cell function by aluminium could lead to accumulation of unwanted, possibly cytotoxic debris as well as modulation of synaptic transmission and neuron-glial signaling. Excitotoxicity by aluminium is one of the proposed mechanisms by several authors. Glutamate is the major excitatory neurotransmitter of the brain and the glutamate receptor mediated excitotoxicity contributes damage in many pathological entities. However the role of glutamate
handling is one of the important functions of glial cells and therefore it is important to study the glial cell functions in aluminium exposure.

Aluminium is preferentially found in glial cells e.g. astrocytes, oligodendrocytes, microglia and shows cellular changes indicative of oxidative stress (Stasio et al, 1994). However studies of Levesque et al (2000), who compared the toxicity and accumulation of aluminium in neuronal and astrocytic cells suggested greater aluminium uptake by neuronal cells as compared to astrocytic cells. Similarly Shaw and Petrik (2009) reported that aluminium treated mice showed significantly increased apoptosis of motor neurons and also increased reactive astrocytes and microglial proliferation within the spinal cord and cortex. Campbell et al (1999) have shown that exposure of cells to aluminium sulphate, increased the ROS formation which in turn is accompanied with elevated mitochondrial activity and glutathione depletion, in glial but not neuronal cell lines.

Excretion

Aluminium is excreted primarily in the urine and to a small degree in the faeces (largely as insoluble aluminium phosphate) by way of the portal circulation, liver and bile. Xu et al, (1992) found that biliary excretion accounted for < 1% of the aluminium administered as the sulphate to rats, whereas urinary elimination accounted for 9-17. Biliary excretion of aluminium and resultant impaired hepatic functions may contribute to aluminium toxicity. Williams, (1986) observed that patients ingesting aluminium-containing antacids have greater concentration of aluminium in bile.

2.1.3 Neurotoxicity of Aluminium

Mounting evidence in the recent years has suggested aluminium to have severe toxic manifestations in the central nervous system. High environmental levels of this element have been associated with pathological changes in central nervous system in the form of neurofibrillary tangles (Perl and Brody, 1980) and β-amyloid plaques (Abramov et al, 2004). Alfrey (1980) implicated aluminium as the possible cause of encephalopathy in uraemic patients on chronic haemodialysis who routinely received aluminium containing phosphate binders. Similarly infants subjected to prolonged feeding with aluminium containing intravenous solutions exhibited...
impaired neurological development (Becaria et al, 2002). Their study demonstrated that increasing aluminium exposure was associated with a reduction in the mental development index, an effect seen only in aluminium and not with other trivalent metals like boron, chromium, gallium etc.

Some of the neurotoxic symptoms of aluminium exposure include speech disturbances, dysparaxia, tremors, psychosis, personality changes, partial paralysis and finally death. Buchta et al (2003) reported the neurotoxicity of chronic occupational exposure to aluminium containing welding fumes in terms of delay in overall reaction time of the exposed workers. An autopsy case of aluminium encephalopathy as reported by Shirabe et al (2002), depicted excess amount of aluminium in brain following a 15-years of exposure with a dosage regime of 3.0 gms/day. Further, aluminium accumulation in the brain is proposed to be associated with neurodegenerative diseases that including Alzheimer’s dementia, Parkinson’s disease, amyotrophic lateral sclerosis and dialysis encephalopathy.

2.1.4 Role of aluminium in Alzheimer’s Disease

Considerable evidence suggests that Al may play a role in the etiology or pathogenesis of Alzheimer’s disease (AD), but whether the link is causal, is still open to debate. Early studies found elevated levels of aluminium in the brain of AD patients (Crapper et al, 1975) and later studies using more sensitive detection methods confirmed small, but significant elevations of aluminium in AD hippocampus, inferior parietal lobule and superior and middle temporal gyri, compared to their corresponding control tissues (Butterfield et al, 2001). However, other groups have failed to find any difference between the levels of aluminium in AD brains when compared with aged matched controls (Bjertness et al, 1996). In brain aluminium can potentiate the formation of ROS, and activate glial cells. Both these mechanism stimulate the inflammatory response that ultimately may lead to neurodegeneration. A direct link between aluminium and AD is yet to be established definitely. Nonetheless, it is clear that aluminium is capable of inducing both inflammation and the generation of ROS and thus contributes to the progression of the disease process (Campbell et al, 1999).
Dialysis Encephalopathy Syndrome (DES)

In the early 1970s, a new type of neurologic disease in patients on long-term hemodialysis was reported from several centers (Nadel and Wilson, 1976; Chokroverty et al, 1976). The disease most often began with speech disturbances and later, ataxia, dyspraxia, and increased dementia-like symptoms were observed. The first hypothesis that DES could be an aluminum poisoning came from the same group that had first described the actual clinical condition of DES (McDermott et al, 1979). Later several others have confirmed that blood and tissue concentrations of aluminium were significantly increased in DES (Exley and Korchaazhkina, 2001; Flaten, 2001) in comparison to normal individuals.

Amyotrophic Lateral Sclerosis and Parkinson’s Dementia

Aluminium has also been linked to amyotrophic lateral sclerosis and Parkinson’s disease (Flaten, 2001) both of which are characterized by the loss of motor neuron functions and the presence of neurofibrillary tangles in the brain (Perl et al, 1982). Investigations have shown that chronic nutritional deficiencies of calcium and magnesium and relative excesses of trace metals such as aluminium and manganese led to dementia-like conditions and also initiated the formation of neurofibrillary tangles (Garruto et al, 1988). Aluminium is known to accumulate in the tangle-bearing neurons in these patients (Perl et al, 1982). Epidemiological evidence from Guan and other foci of Parkinson-dementia syndrome in the western pacific have observed a state of secondary hyperparathyroidism following increased absorption of aluminium, and deposition of the metal within the central nervous system as a cause of disease occurrence (Yanagihara et al, 1984).

2.1.5 Aluminium and Oxidative Stress

Aluminium has no redox capacity in biological systems. However, extensive experimental evidence demonstrates both, in vitro and in vivo, that high aluminium concentrations cause oxidative stress. Sies and Jones defined this condition as “an imbalance between oxidants and antioxidants, in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage” (Sies and Jones, 2007). The nervous system is particularly sensitive to oxidant-mediated damage because of-
1. its high oxygen consumption
2. presence of highly oxidizable polyunsaturated fatty acids in brain membranes
3. deficient antioxidant enzymes. The activity of catalase, superoxide dismutase and glutathione peroxidase is comparatively lower in brain as compared to other tissues
4. and also high iron content in brain

Evidence of an oxidative stress status has been found in association with most neurodegenerative disorders in which aluminium is present in relative high amounts. These findings led to an extensive investigation on the possible link between aluminium and the promotion of oxidative stress. In vitro, aluminium stimulates iron-initiated lipid oxidation (Gutteridge et al, 1985). This ability of aluminium is enhanced by the presence of polar head groups with either negative charge (Oteiza, 1994) or polyhydroxylation (Verstaeten et al, 1998), which allows the binding of aluminium through electrostatic interactions. Aluminium binding to negatively charged lipids results in their lateral rearrangement with creation of discrete domains with lower fluidity than the original membrane (Verstraeten and Otezia, 1995; Verstraeten et al, 1997, 1998). In myelin fractions isolated from aluminium-intoxicated mice, the aluminium content was elevated and also the membranes were more rigid and contained higher amounts of lipid peroxidation products than controls (Verstraeten et al, 1997). Aluminium is also found to be associated in regions of substantia niagra and locus coeruleus, which contain neuromelanin and thus depicts a strong correlation between aluminium and PD. Fahn, (2003) have found increased deposits of melanin, aluminium and iron in patients suffering from PD. In vitro studies have suggested that aluminium facilitates both iron and copper supported oxidation of dopamine to melanin and also mediates per se the conversion of melanochrome to melanin, which then can bind to metals like iron, aluminium, zinc, selenium and Cobalt (Di and Bi, 2003 & Di and Bi, 2004). This further leads to lipid peroxidation.

2.1.6 Lipid Peroxidation

Free radical mediated peroxidation of polyunsaturated fatty acids with the loss of membrane integrity is currently recognized as a mechanism of tissue injury.
Role of Antioxidants in Al Induced Neurotoxicity

(Siddiqui, 1996). Aluminium as a trivalent cation, can bind to membrane components and modify membrane physical properties, ultimately affecting membrane-associated processes. Aluminium enhances iron-mediated lipid oxidation; the observed effects could be a resultant of aluminium interaction with the bilayer, but also to iron-induced membrane oxidation with the subsequent loss of poly-unsaturated fatty acids, which would make membranes more fluid. Lipid peroxidation causes cellular damage in several ways:

1. Reactive free radicals formed during the process, react and cause damage to cellular macromolecules
2. The decomposition of hydroperoxides formed from polyunsaturated fatty acids

Increased lipid peroxidation has been reported after long-term low-level aluminium exposure (Kaizer et al, 2005). Very recently, the products of lipid oxidation; malondialdehyde (MDA) have been quantified in rat brain exposed to aluminium and phenolic antioxidants (Candan and Tuzmen, 2008). Studies have also shown that aluminium compounds can enhance the lipid peroxidation in liposomes and alter membrane fluidity (Kaneko et al, 2007). However, Golub et al (1992) observed no change in lipid peroxidation in both brain and liver in adult mice fed on high aluminium diet.

2.1.7 Aluminium and Neurotransmission

Aluminium negatively impacts neurotransmission, either by directly inhibiting the enzymes responsible for the synthesis and/or utilization of neurotransmitters, or by affecting the physical properties of synaptic membranes, that could affect the release and/or uptake of these molecules. Goncalves and Silva (2007) has reviewed the impact of aluminium on neurotransmission. In animal models of aluminium intoxication, a 40% decrease in striatum dopamine content (Ravi et al, 2000), and an imbalance of dopamine metabolites were observed (Tsunoda and Sharma, 1999), which suggested an altered metabolism of this neurotransmitter. Aluminium does this via two methods –

1. By inhibiting the enzyme dopamine-β-hydroxylase, responsible for dopamine conversion into norepinephrine
2. By decreasing dopamine D1 and D2 receptors in brain cortex and striatum.
Cholinergic and GABAergic metabolisms are also affected by aluminium. In a model of four weeks administration of aluminium to rats, a severe impairment of cognitive functions was associated with a decrease in acetylcholine synthesis, degradation, and decreased muscarinic acetylcholine receptors (Julka et al., 1995). Further, aluminium exposure in rats caused a significant increase in the excitatory amino acids glutamine and glutamate, accompanied by a decrease in GABA content, suggesting a role for this metal in glutamate-mediated excitotoxic neuronal injury (El-Rahman, 2003; Nayak and Chatterjee, 2003).

Further, low serotonin levels have been associated with cholinergic hypofunction. Aluminium-intoxication causes a decrease in serotonin levels and its deaminated derivative is 5-hydroxyindole acetic acid in brain cortex and hippocampus (Kumar, 2002). Limited information is available on the effects of aluminium on neurotransmitter release.

2.1.8 Neurobehavioral Alterations

The importance of neurobehavioral studies in risk assessment lies in the fact that behavior can be regarded as the net output of the sensory, motor and cognitive functions occurring in the nervous system and can serve as potentially sensitive end points of chemical induced neurotoxicity. It has also been suggested that in some cases, behavioral changes may be more sensitive than neurochemical alterations, as indicators of neurotoxicity and may be observed early during exposure (Tilson 1993), especially with regard to aluminium exposure (Baydar et al. 2003). Aluminium neurotoxicity in children tends to be manifested by regression of verbal and motor skills observed at plasma levels above 100 µg/L which is 20–50 times higher than normal plasma levels (Sedman et al. 1984). Many behavioral disorders; in particular memory and motor dysfunctions are thought to be associated with impairments of specific neurotransmitters. Earlier work from our laboratory has shown cholinergic dysfunction following chronic aluminium exposure (Nehru and Bhalla, 2006). The involvement of brain cholinergic system in the mechanisms of learning and memory (Bartus et al. 1982) and the damage caused to them in neurodegenerative diseases (Perry 1986) are well known. Elevated levels of aluminium have been observed in autopsied brain samples of patients with certain neurological disorders such as Parkinsonism (Garruto et al. 1984) and Amyotrophic lateral sclerosis (Perl et al. 18.
A correlation was drawn between aluminium levels and neurocognitive functioning in patients on hemodialysis by Bolla et al. (1992). They found higher aluminium levels associated with a decline in visual memory, lower vocabulary scores and decline in concentration, suggesting that aluminium does have neurotoxic properties. Gonda and Lehotzky (1996) found that rats prenatally exposed to an intraperitoneal (i.p.) injection of aluminium lactate showed impairment on a passive avoidance task. Moreover, impairment in maze tasks and in a radial arm maze performance after high aluminium injection was also reported in prenatally exposed adult mice (Santucci et al. 1994; Alleva et al. 1998). Similar to these, learning impairments at high doses of aluminium have also been observed in prenatally exposed rabbits (Yokel 1987). Aluminium induced behavioral alterations as well as cognitive deficit have been widely reported in literature but exact mechanism is not yet reported. Sethi et al (2008) suggested that alterations in synaptic transmission could be possibly responsible for the decreased synaptic plasticity observed in their study in rats. No deleterious effect of aluminium ingestion on cognitive behavior was found when 22 days old rats were given aluminium hydroxide for 60 days (Thorne et al. 1986). Similarly, no significant differences between control and aluminium-treated rats were found in behavioural tests of short and long-term memory (Clauberg and Joshi 1992).

A number of studies published so far have shown the cognitive impairments following aluminium exposure but the results are not consistent. While many studies reported neurobehavioral changes, the uniformity of results was questioned by studies not showing exposure related deterioration of performance (Iregren et al. 2001; Kiesswetter et al. 2007). Taken together, these conflicting findings on learning impairments found in the literature may be attributed to the differences in duration of dose and route of exposure. More studies are required to be carried out with a broader range of concentrations (doses), different routes and durations of exposure to aluminium so as to corroborate these findings.

2.1.9 Aluminium and Mitochondrial Dysfunction

Mitochondria are important organelles involved in maintaining cell functions and represent a primary site of cellular energy generation and oxygen consumption. Mitochondria make two diverse/contradictory contributions to cell survival. The main function is the synthesis of ATP, necessary for endergonic reactions; the other is
generation of reactive oxygen species, which may compromise the long-term survival of cell. The changes in mitochondrial functions that are responsible for ROS formation may result in oxidative damage to mtDNA. It could be suggestive that the increased ROS load following aluminium exposures could be a result of mitochondrial dysfunction. Aluminium has been shown to perturb the oxidative phosphorylation in human hepatocytes (Mailloux et al. 2007). Numerous sources of free radicals are present in the brain but the most common is from oxidative phosphorylation of ADP to ATP via the electron transport chain in the inner membranes of mitochondria. ATP is generated through the reduction of molecular oxygen to water by the sequential addition of 4 electrons and 4 H+. Leakage of electrons along the electron transport chain causes O$_2^-$ to form with the potential of forming OH$^-$ via the Fenton reaction. Neurons are highly dependent on oxidative phosphorylation to generate ATP and because brain consumes larger amount of oxygen than any other organ it is more vulnerable to oxidative stress. More active neurons or more specific neuron compartments that contain mitochondria such as synapse may be particularly vulnerable to oxidative stress. It is therefore imperative to study mitochondria and oxidative stress.

Aluminium can also bind to phosphates and other oxygen-donating ligands to form stable complexes, disrupting enzyme activity in mitochondria and affecting the electron transport chain. De Marchi et al. (2004) have also reported enhanced ROS production, decreased mitochondrial membrane potential and decreased activity of enzymes in mitochondria after aluminium toxicity. Another study by Swegert et al. (1999) showed impaired ADP/O ratio, as well as, a reduction in the state three and four respiration in the brain mitochondria following 90–100 days aluminium chloride exposure in rats. Niu et al. (2005) have shown that aluminium induces ROS production and is involved in impairments of mitochondrial functions in vitro in rat neuronal cells. Mailloux et al. (2006) has also shown that aluminium toxicity is associated with the decrease in the activity of various TCA cycle enzymes in aluminium-exposed hepatocytes and may be in decreased ATP production. Moreover, the aluminium induced mitochondrial dysfunction promotes enhanced lipogenesis and decreases the oxidative-ATP production in human hepatocytes as shown by Mailloux et al. (2007) in HepG2 cells. Murakami and Yoshino (2004) believed that aluminium might be causing the oxidative damage to mitochondria by limiting the
NADPH supply in mitochondria and glutathione regeneration. Thus, aluminium might be implicated in the functional decline of mitochondria that may lead to ROS generation. This entire phenomenon may be associated with increased oxidative stress and decreased antioxidant defense system.

2.1.10 Carbohydrate Metabolism

Abnormalities in carbohydrate metabolic pathways are among the most outstanding lesions arising by the action of toxic compounds. The natures of these deviations as well as the possible mechanism through which they may occur are diverse (Ignacio et al, 1990). Impairments in carbohydrate metabolism may constitute a major cause of xenobiotic induced neuronal dysfunction (Spencer et al, 1998). According to some authors, impaired oxidative and energy metabolism are relevant features in Alzheimer’s disease due to compromised mitochondrial oxidative metabolism in brain cells. Therefore, defects in energy metabolism may play a role in the pathogenesis of neurodegenerative diseases in general and in AD in particular. The demonstration of a link between alterations in mitochondrial enzymes and neurodegeneration is of paramount importance since some of these enzymes are related to certain neurotransmitter systems.

Recent data raise the possibility of a genetically determined alteration in some enzymes of the Krebs cycle in a subgroup of patients affected by Alzheimer’s disease (Zatta et al, 2003). Abnormalities in oxidative metabolism have consistently been detected in biochemical assays of AD brain autopsies, with a functional defect at the level of the Krebs cycle. Impaired oxidative phosphorylation and activation of glycolysis is also been indicated during acute cyanide poisoning and under ischemic conditions.

Glucose enters the glycolytic pathways as glucose-6-phosphate (G-6-P) and the reaction is catalysed by hexokinase, which requires ATP and Mg (II) as driving factors. Aluminium is known to bind ATP ten times more tightly than does Mg (II) and thus is responsible for causing inhibition of hexokinase enzyme activity as has been observed in the studies of Nehru et al (2006). The inhibition may arise because of competitive binding between aluminium and Mg for the same binding site. Thus, one would anticipate that aluminium inhibit all ATP-dependent reactions and thus hamper the energy dependent processes. Further, aconitase is a protein that is an important enzyme for the proper functioning of TCA cycle in the mitochondria. It has
been shown that the enzyme has lower activity in Al-stressed cells due to the perturbation of the Fe-S cluster (Middaugh et al, 2005). Fumarate and succinate dehydrogenase, the two other Fe-S containing enzymes, were also down regulated in the Al-stressed cells. The reduction in the biosynthesis of these two enzymes would severely impede the ability of the TCA cycle to generate NADH, a crucial metabolite for the generation of ATP via oxidative phosphorylation. Thus, aluminium has profound effect on the energy metabolism in brain cells, which can lead to the decreased production of ATP and hence affects the ATP dependent processes.

2.1.11 Apoptosis

Apoptosis, which plays a critical role in the normal development and maintenance of tissue homeostasis, plays an relevant role in neurodegenerative diseases and aging (Ghribi et al, 2001). Alzheimer’s disease, a common neurodegenerative disorder, is characterized typically by intraneuronal neurofibrillary tangles, neuritic plaques and selective neuronal death, with evidence of apoptosis being observed as an early event preceding the formation of these classical neuropathological features.

Mitochondrial changes following cytotoxic stimuli represent a primary event in apoptotic cell death, since the apoptotic factor, cytochrome c, is released into the cytoplasm. Once this translocation occurs, cytochrome c binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspases, of which caspase-3 is a prominent member (Srinivasula et al, 1998). Release of cytochrome c from the mitochondria has been shown to involve two distinct pathways. One implicates the opening of the mitochondria permeability transition pore (MTP), and the second, triggered by the pro-apoptotic Bax, is independent of the MTP opening (Eskes et al, 1998). While Bax has been shown to trigger cell death (Gross et al, 1998; Wolter et al, 1997), the anti-apoptotic Bcl-2 can block cytochrome c release and caspase activation (Adams and Cory, 1998; Reed, 1998). Bcl-2 resides in the mitochondria and prevents activation of the effector caspases by mechanisms such as blockade of the MTP opening (Marzo et al, 1998), or by functioning as a docking protein (Reed, 1998).

Ghribi et al (2001) have shown that neurotoxic injury, induced in rabbits by the intra-cisternal administration of Al-maltolate, results in cytoplasmic cytochrome c translocation, endoplasmic reticulum Bcl-2 down regulation and Bax up-regulation, as
well as caspase activation. In his previous report Ghribi et al (2001) have pre
immunohistochemical evidence for a similar decrease in the Bcl-2: Bax ratio, to
with evidence of apoptosis, aged rabbits treated with aluminium maltolate. Res
Pedro et al. (2004) showed that aluminium induces cell death throu
mechanism, which could determine whether necrosis or apoptosis would be trig

Thus it is evident that aluminium from various diverse sources gains en
the brain, results in various metabolic derangements, which could be suggestive
neurotoxicological manifestations. Based on the above observations, we have ot
a few possible pathways leading to Al accumulation in brain and its po
consequences in figure 2.1.1.

2.2 CURCUMIN

Curcumin, is a potent anti-oxidant and anti-inflammatory agent (Meno
Sudheer, 2007) has a long history of use, as a food preservative both in trad
Indian and Asian medicine. It is often used as an oral or topical extracts for cor
where western medicine might employ non-steroidal anti-inflammatory
(NSAIDs) or vitamin E (Cole et al, 2003). It is derived from turmeric (Curc Longa L.) that is botanically related to ginger (Zingiberaceae family). It is a peren plant having a short stem with large oblong leaves and bears ovate, pyriform oblong rhizomes, which are often branched and brownish-yellow in colour. Its use has been well documented in Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Ammon and Wahl, 1991).

Based on all studies performed on curcumin, the FDA has approved curc as “generally regarded as safe”, and it is being used in the United States in mustard sauce, cheese, butter, chips, and other as both a preservative and a colouring agent. The safety of curcumin, combined with its efficacy and lower cost, makes it an effective chemo-preventive or chemotherapeutic agent. Various countries are already selling curcumin-based products; and it is also extensively being sold as food supplement in cream and other forms (Goel et al, 2008).

The wild turmeric is called C. aromatica and the domestic species is called longa. Its taxonomic position is as follows:

Class : Liliopsida
Subclass : Commelinales
Order : Zingiberales
Family : Zingiberaceae
Genus : Curcuma
Species : Curcuma longa

2.2.1 Physical Properties

IUPAC name: \((1E,6E\text{-}1,7\text{-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadi} \text{3,5-dione})\)
Other names: Curcumin, Diferuloylmethane, Natural Yellow 3
Molecular formula: \(C_{21}H_{20}O_{6}\)
Molar mass: 368.38g/mol
Appearance: Bright yellow to orange powder
Melting point: 183°C (361 K)
2.2.3 Chemical Composition

The yellow colour of turmeric is due to Curcumin (diferuloylmethane) (Fig 2.2.1) which is 3-4% and comprises of three subunits i.e. curcumin I (94%), curcumin (II) (6%) and curcumin III (0.3%) (Ruby et al, 1995). Demethoxy and bisdemethoxy derivatives of curcumin have also been isolated. It has a melting point at 176-177°C; forms a reddish-brown salt with alkali and is soluble in ethanol, alkali, ketone, acetic acid and chloroform.

2.2.4 Pharmacokinetics and Metabolism

Curcumin undergoes a rapid and efficient metabolism that severely curtails the availability of parent compound in the bio-phase. When curcumin bioavailability was investigated using a 3H-radiolabeled agent the vast majority of the oral dose was excreted in the faeces and one-third was excreted unchanged (Ravindranath and Chandrasekhara, 1981). Intravenous and intra-peritoneal administration of curcumin in rats resulted in large quantities of curcumin and metabolites in bile (Ravindranath and Chandrasekhara, 1981a). Measurements of blood plasma levels and biliary excretion showed that curcumin was poorly absorbed from the gut. The major amount of the drug was metabolised, which suggests its poor absorption and rapid metabolism (Goel et al, 2008). The metabolites were characterized mainly as glucuronides of tetrahydrocurcumin and hexahydrocurcumin. Curcumin undergoes biotransformation during absorption in the intestinal tract and enterohepatic re-circulation (Ravindranath and Chandrasekhara, 1981). Pan et al (1999) investigated that curcumin, administered intra-peritoneally to the mouse, was found to undergo metabolic reduction to dihydrocurcumin and tetrahydrocurcumin, which, in turn, were converted to monoglucuronide conjugates.

Subsequent studies were attempted with higher amounts of curcumin, hoping to get over its poor absorption, as curcumin was found to be safe even at higher doses. But the percentage of absorbed curcumin remained constant (60-66% of the given dose), regardless of the dose administered (Ravindranath and Chandrasekhara, 1980; 1981b) suggesting that administration higher curcumin dose does not result in better absorption. The liver was found to be the major organ responsible for metabolism of curcumin (Wahlstrom and Blennow, 1978; Garcea et al, 2004; Hoehle et al, 2006). A daily dose of 1.6gm of curcumin is usually recommended in humans (Sharma et al, 2001).
Many of these studies indicated that curcumin has a poor bioavailability once ingested. However, fasting increases the plasma curcumin levels, consistent with a previous report (Chan et al., 1998). Fasting slows stomach emptying and gastric motility, so it could result in an increase in absorption from the stomach, as opposed to the small intestine where glucuronidation is thought to occur. In an attempt to enhance bioavailability Shoba et al (1998) combined curcumin with piperine, a known inhibitor of hepatic and intestinal glucuronidation, and examined the serum levels of curcumin in rats and healthy human volunteers. When curcumin was given alone to rats, at a dose of 2gm, moderate serum concentrations were achieved over a period of 4hrs. Simultaneous administration of piperine resulted in a significant increase in the serum curcumin concentrations, while elimination half-life and the bioavailability of curcumin was increased by 154%. On the other hand, serum levels in humans were either undetectable or very low after a dose of 2mg/kg curcumin alone. Concomitant administration of piperine 20mg/kg produced much higher concentrations and the increase in bioavailability was 2000%. Thus piperine enhances the serum concentration and the extent of absorption and bioavailability of curcumin in both rats and humans.

2.2.5 Metal Chelation Property

Metals play an important role in neural toxicity. The significance of metal physiology and toxicity in the brain lies in the high metal sensitivity of various neurophysiological processes. The brain is a known concentrator of metals, where potentially toxic levels of copper, iron, aluminium, zinc and manganese could accumulate. Research currently points towards the involvement of metallochemical reactions in Alzheimer’s disease, prion disease, mitochondrial disorders and Parkinson’s disease (Bush, 2000). Daniel et al (2004) have reported the metal-ligand binding between iron and curcumin. Similar electrochemical studies for lead and cadmium indicate strong metal-ligand interactions, so extending the metal-binding scope of curcumin to these toxic metals is suggested in reducing their toxicity. Dairam et al (2007) have shown that curcumin reduces lead and cadmium-induced neurotoxicity in rat hippocampal neurons through its anti-oxidant and metal binding properties.
Further, the biological activity of curcumin has been attributed to the hydroxyl group substituted on the benzene rings and also to the diketonic structure. The β-diketo moiety of curcumin undergoes a keto-enol tautomerism. Crystal studies have shown that the symmetric structure of curcumin leads to a statistically even distribution of the enol proton between the two oxygen atoms. The strong chelating ability of diketones has been widely investigated towards a large number of metal ions and therefore, curcumin could be of great importance in the chelating treatment of metal intoxication and overload.

2.2.6 Biphasic Responses to Curcumin

The structure-function activity of curcumin as a free radical scavenger, metal chelator, and anti-oxidant has received considerable attention. It clearly reduces mRNA production for pro-inflammatory mediators including cytokines (Nanji et al., 2003; Al-Omar et al., 2006). However, the direct molecular targets at low doses are not entirely clear. Curcumin’s inhibition of AP-1 and NF-κB mediated transcription occurs at relatively low (<100nM) doses (Rahman, 2004). At high doses (>3μM) that are relevant to colon cancer and are to be unlikely achieved with oral delivery in plasma and tissues outside of the gut, curcumin can act as an alkylating agent (Fang et al., 2005). Some of the effects of curcumin at high in vitro doses are clearly toxic and undesirable beyond its use in cancer therapy. For example, inhibition of proteosomal function and potentiation of Huntington toxicity can be achieved with dosing >3 μM in vitro (Dikshit, 2006). The dose dependence of curcumin’s effects on proteasome is biphasic, with doses upto 1 μM causing 46% increased proteosomal activity and at higher doses, there is proteasome inhibition (Ali and Rattan, 2006).

Many protective effects, including anti-amyloid, anti-oxidant and anti-inflammatory activities, can be obtained with doses at or below 1 μM. For example, low-nanomolar doses can inhibit histone acetyltransferase (Kang et al., 2006). Low dose curcumin can limit the aggregation of multiple forms of amyloid-forming peptides that lead to intra-neuronal or extracellular aggregates in a variety of neurodegenerative diseases.
2.2.7 Biological Activities

Initially applied as a folk remedy, curcumin was extensively used for healing effect on both aseptic and septic wounds. However, an understanding of its complex structure, as well as, its ability to influence multiple signalling pathways and its extended use in diverse biological functions, suggests that curcumin has multiple targets. It has been applied in several biological functions both in clinical experimental studies that range from wound healing (Panchatcharam et al., 2007), chemoprevention (Johnson and Mukhtar, 2007), anti-arthritis (Joe et al., 2015), anti-inflammatory, anti-bacterial, anti-HIV and anti-amoebic (Li et al., 2009). Studies have suggested that curcumin affects different cellular processes such as activation of apoptosis, inhibition of platelet aggregation, inhibition of inflammatory cytokine production, inhibition of cyclooxygenase and lipoxygenase isoenzyme (Bishnoi et al., 2008a). It may also affect the activity of different key enzymes such as protein kinase C, protein tyrosine kinase calcium dependent endonucleases (Balasubramanyam et al., 2003). Some of the molecular targets of curcumin are listed in Fig. 2.2.2

Curcumin is a lipophilic molecule and rapidly permeates cell membranes affecting their structures and functions and also mimics typical events occurring during apoptosis. However, the cellular response to curcumin contrasted with typical apoptotic cell death because loss of membrane integrity was immediate, p53 reversible, and cells could recover in a relatively short time. The authors suggest that membranous changes evoked by curcumin might underlie some of its effects example, by changing access to phosphatidylserine, curcumin might modulate activity of enzymes such as protein kinase C (Hatcher et al., 2008).

Several human trial investigations on chemo-preventive effects of curcumin have been completed especially with regards to colon cancer, apparent crypt foci,
hepatocellular cancer and psoriasis. Curcumin exerts both pro- and antim effects (Šmerak et al., 2005). It shows anticoagulant activity by inhibiting and adrenaline-induced platelet aggregation in vitro as well as in vivo in rat aorta (Chattopadhyay et al., 2004). Its ability to inhibit human sperm suggests its potential for the development of a novel intravaginal conti (Rithaporn et al., 2003). It also has anti-fibrotic activity (Punithavathi Studies with regards to its anti-rheumatic activity of are in the subclinic: where significant improvement of symptoms is observed after administ curcumin (Balasubramanyam et al., 2003).

2.2.8 Therapeutic Potentials of Curcumin
A number of studies have addressed the effect of oral curcumin inflammatory diseases in humans as shown in Fig 2.2.3. Curcumin at 400 mg times daily for 5 days caused significant anti-inflammatory effect objectively and subjectively in post-operative patients (Satoskar et al., 1998) from pre-clinical models (Sharma et al., 2005) would suggest that suppt inflammatory response by curcumin might involve inhibition of the indu COX-2 and iNOS and the production of cytokines such as interferon-γ. Si Aggarwal (1995) have reported that curcumin offers its anti-inflammatory effect through its inhibition of NF-κB activation (Menon and Sudheer, 2007). Curcumin has also been shown to reduce the TNF-α-induced expression of the tissue factor gene in bovine aortic-endothelial cells by repressing activation of both AP-1 and NF-κB (Bierhaus et al., 1997, belongs to the TNF superfamily of pro-inflammatory cytokines and is an mediator for inflammatory tissue damage and has other immune-regulatory f
Curcumin treatment has profound effects on the modulation of TNF-induced signalling and has been consistently shown to inhibit the expression of TNF-α (Nan et al, 2005). It has been shown to inhibit LPS or phorbol-12-myristate-13 acetate (PMA)-induced TNF-α in dendritic cells, macrophages, monocytes, alveolar macrophages, and endothelial bone marrow cells. Curcumin also inhibits the production of pro-inflammatory monocyte/macrophage-derived cytokines [IL-8, monocyte inflammatory protein-1 (MIP-1), monocyte chemotactic protein-1 (MCP-1), interleukin-1β (IL-1β), and TNF-α] in PMA- or LPS-stimulated peripheral blood monocytes and alveolar macrophages (Sidhu et al, 2002). Studies of Sidhu et al (2002) suggested wound healing properties of curcumin in diabetic rats and mice.

2.2.9 Neuroprotection

Curcumin has multiple desirable characteristics for a neuroprotective drug that include its anti-inflammatory, anti-oxidant and anti-aggregate activities (Park et al, 2008). Additionally, it is orally safe and inexpensive and therefore has been tried in most of the neurodegenerative diseases. Curcumin has great potential for the prevention of multiple neurological conditions for which current therapeutics are less than optimal (Cole et al, 2003). Numerous studies have demonstrated that curcumin, amongst only a few other things such as high impact exercise, learning, bright light and anti-depressant usage, has a positive affect on neurogenesis in the hippocampus and concentrations of Brain-derived neurotrophic factor (BDNF), whose reductions are associated with stress, depression, and anxiety (Wu et al, 2006; Xu et al, 2007). Most neurodegenerative diseases involve multi-step cascade that induce slow degeneration and could best be dealt with long term prevention strategy, which is less expensive and safe. Curcumin therefore, has been applied in several neurodegenerative diseases both in experimental and clinical settings.

Alzheimer’s Disease

AD is characterised by the presence of a peptide called amyloid beta which forms aggregates (oligomers) and gets accumulated in the brain resulting in the formation of deposits known as amyloid plaques (Gandy, 2005). Inflammation and oxidative damage are also associated with the progression of Alzheimer’s disease.
(Cole et al, 2003). Frautschy et al (2001) have demonstrated that curcumin inhibits oxidative damage, inflammation and cognitive deficits in rats receiving infusions of toxic Aβ. Combined anti-oxidant and NSAID approach of curcumin AD prevention or therapeutics is shown in Fig 2.2.4.

A more extensive report showed that curcumin not only stained plaques and inhibited Aβ aggregation and fibril formation in vitro; but also inhibited the plaque formation in vivo, as curcumin readily entered the brain and labeled the plaques (Yang et al, 2005). It blocks Aβ aggregation at submicromolar concentrations in a dose dependent manner and this probably could be due to its structure, which is similar to Congo red, an amyloid binding dye (Cole et al, 2003). Further, curcumin also reduced Aβ accumulation and plaques in vivo in late stages of disease development.
AD (Das et al, 2001). This property of curcumin is of great significance since several other antioxidants and other treatments that fail to reduce amyloid in late stages in same mice model (Sung et al, 2004). Five different pathways have been suggested by which curcumin could limit amyloid production these include--

1. Metal chelation (Huang et al, 2004)
2. Limiting oxidative damage which is better than vitamin E (Sung et al, 2004)
3. Lowering cholesterol and reducing expression of β-secretase enzyme (Fassbender et al, 2001; Refolo et al, 2001)
4. Reducing pro-inflammatory cytokines (Sastre et al, 2003)
5. Lipid peroxidation product 4 hydroxynonenal acting on JNK-mediated transcription and protein oxidation (Tabaton, 2004)

Additionally several other authors have reported two other mechanisms that might contribute to amyloid reduction:

1. Curcumin at low dosing of (100–500-nM) can stimulate microglial phagocytosis, and clears amyloids in brain.
2. Induction of heat shock proteins (HSPs) that function as molecular chaperones to block protein aggregate formation. (Scapagnini, 2006)

Parkinson’s Disease

Another prevalent, age-related neurodegenerative condition, the movement disorder Parkinson’s disease, involves relatively selective vulnerability to the dopaminergic neurons. PD had the strongest association with elevated oxidative damage, including that associated with auto-oxidative dopamine breakdown. Low doses of curcumin can inhibit dopamine toxicity in vivo (Luo et al, 1999). Oxidative damage to vulnerable dopaminergic neurons and a PD syndrome can be produced in human and animal models by the MPTP toxin (Mehlhorn and Cole, 1985). MPTP toxicity is mediated by MPP+ and Chan et al (1998) have reported that curcumin can directly inhibit MPP+ toxicity to the PC12 neuronal cell lines. The α-synuclein protein is another aggregating, fibril-forming protein that is a major component of the Lewy body lesions characteristic of PD as well as certain cases of AD and several other neurodegenerative conditions (Hong, 2005). Recent studies have shown that curcumin can reduce the aggregation of α-synuclein (Takahashi et
al, 2002), and administration to cultured cells with α-synuclein aggregate formation results in fewer aggregates.

**Huntington’s Disease**

Huntington’s disease have extended C-terminal CAG repeats coding for polyglutamine, which causes protein aggregates to form at a rate determined by the repeat length. Caughey et al (2003) have reported that curcumin resembles Congo red and its chrysamine G homologue Congo red, and so its anti-amyloid-binding protein properties are generic and should extent to other protein-misfolding diseases with a β-sheet, including the polyglutamine diseases like Huntington’s disease. Bence and co-workers (2001) have reported a protective effect of curcumin in an HD transgenic model.

**Alcohol Induced Neurotoxicity**

Ethanol-induced toxicity involves lipid peroxidation, inflammation, and other well-established curcumin targets. Curcumin can effectively protect against ethanol-induced oxidative damage, inflammation and resulting liver damage and ethanol-induced CNS neurodegeneration in vivo (Rajakrishnan, 1999).

**Aging Brain**

Evidence for an impact of curcumin on aging brain has been recently suggested in rats. Chronic curcumin treatment was shown to result in reduced lipid peroxidation and accumulation of the age-pigment lipofuscin and to increase the antioxidant defense enzymes glutathione peroxidase and superoxide dismutase as well as sodium potassium ATPase, which normally declines during aging (Bala et al, 2006). Kitani et al (2004) have reported that one of its metabolite, tetrahydrocurcumin, increases the life span in middle-aged mice. Curcumin resembles resveratrol that is believed to have anti-aging activity via induction of sirtuins and HDAC activation. Therefore, curcumin’s ability to limit HAT and promote neurogenesis (Kang et al, 2006) might also impact longevity, promoting a sirtuin-like effect on HAT-regulated transcription.
Cerebrovascular Disease and Stroke

Curcumin has an ability to lower total cholesterol and raise high-density lipoprotein (HDL) cholesterol in humans that is relevant to dementia prevention (Bhattacharya et al., 2001). Curcumin effectively protects against homocysteine induced endothelial damage (Ramaswami et al., 2004) since homocysteinuria appears to be an important risk factor for both AD and cardiovascular disease (Dwyer et al., 2004). Prior and even delayed curcumin treatment, reduces the free-radical damage and inflammation, that contribute to ischemic damage after a stroke (Chen et al., 2006). Further, curcumin has been shown to be protective in a standard middle cerebral artery occlusion rat model for stroke (Wang et al., 2005).

2.3 LAZAROIDS

Glucocorticoids (GC) an important steroid of all vertebrate cells have an important role in the metabolism of glucose. They also contribute in turning on the immune system and have been therefore, exploited to treat diseases that are caused by an over-reactive immune system, such as allergies, asthma, autoimmune diseases and sepsis. GCs have many diverse (pleiotropic) effects, that may also include certain potentially harmful side effects (Rhen and Cidlowski; 2005). Methylprednisolone (MP), a potent glucocorticoid possesses a number of receptor-mediated anti-inflammatory actions and has been exploited in the spinal cord injury. It appears that neuroprotective mechanisms are not mediated via glucocorticoid receptor-mediated activity but by the inhibition of post-traumatic lipid peroxidation (Braughler et al., 1988a; Hall, 1997; Hall et al, 1994b). This led to speculation that modifying the steroid molecule so as to eliminate the glucocorticoid effects, (Jacobsen et al, 1990) would result in more targeted antioxidant therapy, and would also be devoid of the side effects of steroid therapy. This became the rational for the development of potent lipid peroxidation inhibitors i.e. 21-aminosteroids i.e. lazaroids (Hall, 1997; Hall et al, 1994b). These compounds had two benefits

a) They were devoid of the side effects of steroids
b) Clinically the high doses of MP could be substituted by lazaroids

There are three major types of lazaroids that are being tested in different neurological disorders i.e. U74500A, U74006F and U74389G. Fig 2.3.1 compares the structure of these three lazaroids with MP.
Among these compounds U-74500A has been used in the current study to investigate aluminium neurotoxicity. U-74500A (pregne-1,4,9(11)-triene-3,20-dione, 2 bis (diethylamino)-2-pyridinyl) - 1-piperazinyl) - 16-methyl-Cl (16 al) is reported to inhibit the cytotoxicity (Tanaka et al, 1997) and lipid peroxidation in iron-loaded cultured endothelial cells that had been submitted to an exogenous challenge.
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endogenous oxidant attack... They are effective in inhibiting hyperoxic lung injury, ischemic-reperfusion injury, bleomycin-induced lung injury, and lung inflammation. It also has a potential to ameliorate alcohol-induced liver injury (Hossein and Nanji, 1998). Studies have shown that lazaroids protect against oxidant-mediated injury in the isolated lung, in iron-loaded endothelial cells, in the reperfused canine myocardium after cardiac arrest, and also in models of intestinal ischemia and shock. These compounds are known to reduce H$_2$O$_2$ generation by stimulated human polymorphonuclear leukocytes and decreases both chemiluminescence and H$_2$O$_2$ produced by monocytes, which are harvested from the blood of patients affected by multiple sclerosis (Fischer et al, 1999). It has also been shown to diminish TNF-α generation and protects against endotoxic shock in neonatal calves (Semrad et al, 1993). However, when 21-aminosteroid was administered to healthy volunteers no major effect on global or regional cerebral blood flow, CMRO$_2$, or the reactivity of the cerebral vessels to changes in Pa$_{CO_2}$ was observed (Olesen et al, 1993). Lazaroid hold a great potential as therapeutic agents for pulmonary disease and are showing good experimental promise (Huang et al, 2004). Lazaroids are also known to improve the survival of cultured rat embryonic mesencephalic neurons (Frodl et al, 1996) as well as grafted dopamine neurons in patients of Parkinson’s disease (Brundin et al, 2000). The study of Anderson et al (1988) demonstrates the remarkable effectiveness of a non-glucocorticoid 21-aminosteroid, U-74006F, administered through the venous cannula, in enhancing neurological recovery in female adult mongrel cats traumatized by compression of the spinal cord with 180-gm weight for 5mins. Chen and co-workers (1994), Horton and Walker (1993), Stone and colleagues (1992) and Katz and associates (1995) reported amelioration of mucosal injury with lazaroids though Park and co-workers (1994) and Van Ye and associates (1993) found no protection.

However, apart from the pharmacological characteristics of specific compounds, these lazaroids represent very interesting molecules with important pharmacological features not yet explored. They have been effectively employed in animal models of head and spinal cord trauma, sub-arachnoid haemorrhage, neuronal hypoxia (Vignes and Hugon, 2006) and global ischemia in the CNS. Further, they also partially protect from an ischemic lesion or from an inflammatory reaction caused by endotoxins.
Lazaroids also increase the survival of transplanted dopaminergic neurons, improves behavioural recovery in rats and increases survival of dopamine neurons in cultures treated with the nitric oxide radical, which is toxic to cultured cells (Nakao et al., 1994).

2.3.1 Mechanism of Action

Braughler et al. reported earlier that analogs of 21-aminosteroids (U-774006E and U-74500A) are more potent at inhibiting lipid peroxidation in the membrane phospholipid than in a homogeneous solution of fatty acids. Lipid peroxidation refers to the oxidative degradation of lipids (Fig 2.3.2). It is the process whereby free radicals “steal” electrons from the lipids in cell membranes, resulting in cell damage. Peroxidation involves three phases i.e. initiation, propagation and termination. Initiation occurs when an hydrogen is abstracted from fatty acyl chain leaving a carbon based radical. Hydrogen can be abstracted from carbon, nitrogen, oxygen or sulphur based radicals. Among the oxygen based radicals OH is the most reactive. Polyunsaturated chains are more vulnerable to lipid peroxidation since hydrogen abstraction in their carbon hydrogen bond is more acidic by the carbon-carbon double bond. During propagation the carbon based radical of the fatty acyl chain forms hydoperoxyl radical which is very reactive and it abstracts a second hydrogen atom from the nearby fatty acyl chain to produce lipid hydroperoxide and a new carbon based radical thus, propagating peroxidation. Termination of lipid peroxidation occurs when two radical species react with each to form non-radical product. Cellular antioxidant systems for example SOD, catalase and iron chelator prevent the initiation of lipid peroxidation whereas ascorbate, alpha-tocopherol and reduced glutathione limit its propagation.

There are two major outcomes of lipid peroxidation i.e structural damage to membrane and generation of bioactive secondary products. Lipid hydroperoxyl radicals can undergo endocyclization to produce novel fatty acid esters that disrupt membranes. They can bring about alterations in biophysical properties that could have a profound effect on the activity of membrane bound proteins. Further the reactive aldehydes from lipid peroxidation also react with number of cellular nuceophiles including proteins, nucleic acids and lipids. They could also deplete glutathione induced dysfunction of structural proteins, reduce enzyme activity and cell death. This process proceeds by a free radical chain reaction mechanism. It most often
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affects polyunsaturated fatty acids, because they contain multiple double bonds between which lie methylene -CH2- groups that possess especially reactive hydrogens.

![Diagram of lipid peroxidation]

Fig 2.3.2: Basic reaction sequence of lipid peroxidation

It appears that the compound exerts its anti-lipid peroxidation action through co-operative mechanisms:

1. A radical scavenging action
2. A physicochemical interaction with the cell membrane that serves to decrease membrane fluidity.

Early animal studies of traumatic and ischaemic injury (Hall et al, 1991; et al, 1992; Sato and Hall, 1992) suggest, that the lazaroids inhibit membrane peroxidation by scavenging peroxyl radicals, a mechanism similar to vitamin E (et al, 1994). In experimental head injury models, lazaroids decreased hydroxyl radical production in the brain of mice produced by concussive head injury, and in ge with bilateral carotid occlusion indicating that it may also scavenge hydroxyl radical (Clark et al, 1993; Hall et al, 1992; Fisher et al, 1990). Huang et al (2001) shown that lazaroid is capable of scavenging 'OH radical with a reaction rate approx 1.0x10^{10} M^{-1}s^{-1}; that is capable with other well-established 'OH radical scavengers, such as ascorbate (K=1.2x10^{11} M^{-1}s^{-1}), GSH (1.5x10^{10} M^{-1}s^{-1})
cysteine (1.5x10^{10} \text{ M}^{-1}\text{s}^{-1}). Using competition reaction it was demonstrated that lazarooids does not block \(^\cdot\text{OH}\) generation from the Fenton reaction

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH}
\]

It appears that lazarooid inhibits \(^\cdot\text{OH}\) radical-induced cellular predominantly via scavenging \(^\cdot\text{OH}\) radical and not by attenuating its generation. Majority of scavengers have been found to be unsuitable and could not be del the site of cellular reactions. In this regard lazarooids have the advantageous of being lipophilic, easily penetrable at the cell membrane level, relatively st non toxic with a long biological half life. In addition to these properties they effective at inhibiting lipid peroxidation by scavenging free radicals.

The lazarooids also exert cell membrane-stabilizing effects by interact the hydrophobic steroid moiety located in the lipid phase of lipid bilayer interacting with the hydrophilic heterocyclic ring system near the phospholi groups (Wang et al 1996). They have a high affinity for the lipid bilayer incorporated into it (Braughler et al, 1989; Jacobsen et al, 1990; Hinzma 1992). The positively charged piperazine nitrogen in lazarooid interacts negatively charged phosphate containing head groups of the membrane lipids al, 1994a) (Fig 2.3.3). The membrane stabilizing effect of 21-aminosteroids is a property that, on one hand, contributes to the protection against lipid peroxidation. It reduces the cell damage by restricting the propagation of this process within the membranes where it may rise. On the other hand, this property could also be associated with reduced leakage of intracellular components, such as enzymes and other proteins.

Braughler et al (1988b) have demonstrated that the localization of 21-amino moiety towards the surface compresses the membrane phospholipid group. This membrane stabilizing action restricts the movement of lipid

![Fig 2.3.3: Membrane stabilizing effects of lazarooids](image-url)
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radicals within the membrane so that their interaction with neighbouring fatty acids is reduced.

Jacobsen et al (1990) have also reported that lazaro is inhibit propagation of lipid peroxidation by restricting the movement of peroxyl and alcohol radicals, which are formed during this process. Recently, lazaro has been shown to interact with the cross model membrane in an in vitro membranous system, which may show its protection to not only cell membranes but also intracellular components against peroxidative attack (Durmaz et al, 2003). Braughler et al (1989) have reported that lazaro is believed to intercalate into cell membranes by embedding their hydrophobic portion into the core of the lipid membrane and there potently inhibit lipid peroxidation, protecting the cellular membrane. It is believed that lazaro also can protect membranes via a direct interaction with the lipid bilayer and by shielding membrane proteins from proteolytic attack by their antioxidant action. Klein (1970) have reported that U-74500A inhibits lipid peroxidation at a step before diene conjugation (Fig 2.3.2), and diene formation is considered to be evidence of an early or moderate alteration in the structure of polyunsaturated lipids as a result of free radical attack (Klein, 1970).

2.3.2 Iron Chelating Property

Lazaroids containing an NC=CN fragment, such as U-74500A exert their protective effect largely from the inhibition of toxic hydroxyl radical production by chelating ferrous ions (Fe^{2+}) in Harber-Weiss reaction or in Fenton reduction of the free radical cascade (Tanaka et al, 1997; Braughler and Pregenzer, 1989). Iron plays a key role in the process of lipid peroxidation. The Fe^{3+}/2+ can react directly with the hydroperoxide radical of lipids

\[
R^- + LH \rightarrow RH + L^- \quad R^- = \text{OH}^-, \quad \text{O}_2
\]

\[
L^- + O_2 \rightarrow \text{LOO}^- \quad R^- = \text{LOO}^-; \quad L^-\n\]

\[
\text{LOOH} + (\text{Fe}^{3+}\text{-complex}) \rightarrow \text{LO}^- + (\text{Fe}^{3+}\text{-complex}) + H^+
\]

\[
\text{LOOH} + (\text{Fe}^{3+}\text{-complex}) \rightarrow \text{LO}^- + (\text{Fe}^{3+}\text{-complex}) + OH^-
\]

It was suggested that the absolute ratio of Fe^{3+} to Fe^{2+} was the primary determining factor for the initiation of lipid peroxidation reactions on the order of 1:1 to 7:1 (Braughler et al., 1986). However, lazaro does not alter the total iron content.
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of the brains. This lipophylic drugs with chelating activity displays spectral changes in the ultraviolet range in the presence of Fe^{2+} (Braughler et al, 1988a) and inhibits in vitro iron dependent lipid peroxidation of intact phospholipid membranes (Braughler et al, 1987). Ishizaki et al (1997) suggested that the early inhibition of lipid peroxidation by iron-chelation is more beneficial than scavenging lipid peroxyl radicals (Braughler et al, 1987; Hall et al, 1994a). A preliminary report suggests that U-74500A interacts with iron in a complex manner (Braughler et al, 1987). U-74500A has the ability to interact with ferrous ion and to lessen its oxidation, in contrast with tirilazad, which does not have this property. U-74500A also prevents adenosine diphosphate (ADP): Fe(II) autooxidation.

2.3.3 Lazaroids Versus a-tocopherol

Lipid peroxidation normally proceeds as a radical driven chain reaction involving oxygen, where the lipid peroxyl radical (LOO\(^-\)), formed through initiation, attacks a second unsaturated fatty acid (LH). An important endogenous inhibitor of lipid peroxidation in membranes is alpha-tocopherol (alpha-TC), which inhibits lipid peroxidation by scavenging (LOO\(^-\)):

\[
\text{LOO}^- + \text{alpha-TC} \rightarrow \text{LOOH} + \text{alpha-TC}^\cdot
\]

Thus preventing lipid radical chain reaction from occurring (Braughler and Pregenzer, 1989). The alpha-TC radical decomposes to tocopherol-quinone and effectively terminates the chain reaction (Braughler and Pregenzer, 1989). In addition, the alpha-TC radical is much less reactive in attacking adjacent fatty acid side chains and can be converted back to alpha-TC by vitamin C. Studies with intact membranes indicate that the 21-aminosteroids are as potent as alpha-TC as inhibitors of iron-dependent lipid peroxidation though their uptake in brain parenchyma is slow. U83836E is a steroid with the antioxidative chromanol ring of alpha-tocopherol. It shows 330-fold higher radical scavenger activity than the tocopherol. Similar differences in antioxidative capacity are found with U78517F, an enantiomer of U83836E, which inhibits iron-catalysed lipid peroxidation in rat brain homogenates 10 times more effective than U74006F and 100 times more effectively than alpha-tocopherol (Mertsch et al, 1998).
2.3.4 Pharmacodynamics

Although lazaroid is lipophilic, it penetrates the blood brain barrier poorly. It is highly concentrated in the vascular endothelial cell membrane (Raub et al, 1993) and this indicates an endothelial site of action. Lazaroid prevents increase in blood brain barrier permeability in acute trauma (Hall et al, 1994b) and subarachnoid haemorrhage (Zuccarello and Anderson, 1989) in animal models. This is mediated by preservation of endothelial function via reduction of oxygen radical damage, and maintenance of production and function of endothelial nitric oxide (Fernandez et al, 1997). In cerebral injury, a loss of endothelial function contributes to the loss of auto-regulation, microvascular hypoperfusion and vasospasm. However, direct neuronal protection by lazaroids cannot be ruled out because enhanced penetration into brain parenchyma, caused by increased blood brain barrier permeability (Hall et al, 1992), has been observed after trauma.

2.3.5 Pharmacokinetics

The pharmacokinetics of lazaroids has been studied after single and multiple doses. Lazaroid is extensively distributed in body tissues. It is 99% protein bound with a volume of distribution of ~ 3.33kg/l (Fleishaker et al, 1993). With multiple doses at 2 mg/kg/day and above its terminal half-life is ~35hrs, so that a steady state is achieved after 5 days of dosing. The hepatic clearance of lazaroid is dependent on hepatic blood flow suggesting that it has a medium to high hepatic extraction ratio (Fleishaker et al, 1993b). Lazaroid is metabolised in the liver to several inactive oxidative products and a reduced metabolite, U-89678, which is active (Weinkers et al, 1998). The clearance of lazaroid and its active metabolite is slightly greater in women (Hulst et al, 1994), due to gender differences in either hepatic blood flow, or metabolism of the steroid moiety of the molecule (Lew et al, 1993; Weinkers et al, 1998). The elimination of lazaroid is increased by enzyme-inducing anticonvulsants such as phenytoin (Fleishaker et al, 1998). Only 12% of the dose may be recovered in the urine and most of it is in the faeces (Stryd et al, 1992).

2.3.6 Side Effects

Extensive animal and human studies of single and multiple doses of lazaroids (upto 6mg/kg/day for 5days) demonstrate minimal changes in heart rate, blood pressure or cardiac rhythm. It does not significantly affect plasma glucose, cortisol or
adrenocorticotrophin concentrations and temperature regulation (Fleishaker et al, 1998, Fleishaker et al, 1993). A transient rise in serum alanine transaminase concentrations in human subjects receiving 6mg/kg/day for 5 days has been reported (Fleishaker et al, 1993). The most common side effect is pain at the site of injection caused by the vehicle (0.02M citric acid monohydrate, 0.0032M sodium citrate dihydrate, 0.077M NaCl, pH 3).