nanoD609 ameliorates toxicity induced by Aβ in PC12 cells
Introduction

Alzheimer disease (AD) is an age-associated dementing disorder characterized by the loss of synapses and the presence of senile plaques and neurofibrillary tangles, affecting more than 18 million people worldwide (Katzman and Saitoh, 1991). Oxidative stress has been implicated in many neurodegenerative disorders, including AD (Hensley et al., 1995; Stadtman and Berlett, 1997; Markesbery and Lovell, 1998; Butterfield and Lauderback, 2002; Butterfield et al., 2001).

Reactive oxygen species (ROS) lead to lipid peroxidation (Markesbery and Lovell, 1998; Butterfield and Lauderback, 2002), DNA and RNA oxidation (Butterfield and Lauderback, 2002) and neuronal dysfunction or death. ROS generation hence becomes important in understanding oxidative stress and oxidative stress-related disorders. Many antioxidant therapies for neurological disorder are under investigation. Mitochondrial electron transport is a potential source of ROS production (Ide et al., 1999). It is now well known that ROS such as superoxide anion ('O2\textsuperscript{-}), hydroxyl radical (OH), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and peroxynitrite (ONOO\textsuperscript{-}) contribute to neurodegeneration (Butterfield et al., 2005; Rego and Oliveira, 2003; Zhang et al., 1994; Ansari et al., 2004; Tangpong et al., 2006). Mitochondrial membrane potential depolarization induces the release of cytochrome-c release into the cytoplasm and elevates the activity of caspase-3, suggesting a role for mtDNA-derived mitochondrial dysfunction in AD degeneration (Bosetti et al., 2002).

Glutathione (GSH) is an intracellular antioxidant that maintains redox balance in the cell. GSH is the major thiol participating in various cellular redox functions and biosynthesis (Schulz et al., 2000). Reduced levels of GSH in specific regions of the central nervous system of AD patients contribute to the oxidative stress-mediated neuronal cell dysfunction and/or loss (Benzi and Moretti, 1995). Studies have shown that an increase in endogenous GSH levels by dietary or pharmacological intake of GSH precursors or GSH mimetics or substrates for GSH synthesis protects the brain against oxidative stress (Butterfield et al., 2002; Halliwell, 2001; Anderson and Luo, 1998). Previously, it was shown that
endogenous elevation of GSH by intraperitoneal (ip) injection of N-acetyl cysteine (NAC) or γ-glutamylcysteine ethyl ester (GCEE) reduced oxidative stress markers in synaptosomes treated with various oxidants (Pocernich et al., 2001; Drake et al., 2003; Drake et al., 2002).

Tricyclodecan-9-yl-xanthogenate (D609) exhibits a variety of potent biological functions, including antiviral (Amtmann, 1996) and anti-inflammatory (Tschaiakowsky et al., 1998; Amtmann and Sauer, 1987; Sauer et al., 1990) activities. Most of these activities have been linked to the inhibitory effect of D609 on phosphatidylcholine-specific phospholipase C (PC-PLC) (Amtmann, 1996; Schutze et al., 1992). Such inhibition decreases production of the secondary messenger diacylglycerol (DAG) that activates protein kinase C (PKC) and acidic sphingomyelinase (aSMase) (Cifone et al., 1995). However, with a free thiol group, D609 may also possess strong antioxidant activity (Rao, 1971) with in vitro and in vivo radical scavenging properties and inhibition of free radical induced oxidative stress (Joshi et al., 2005; Zhou et al., 2001; Lauderback et al., 2003; Sultana et al., 2004; Perluigi et al., 2006).

The particular species of ROS that D609 can effectively scavenge is not clear, but this xanthate has ability to scavenge hydroxyl radicals (Joshi et al., 2005; Zhou et al., 2001; Lauderback et al., 2003; Sultana et al., 2004; Perluigi et al., 2006). The reaction with other ROS is also possible since xanthates generally have high reducing potential (Rao, 1971). D609 may protect intracellular GSH, which is important intracellular defense molecule against oxidative stress in neurons and has been shown to play an important role in radiation protection (Zhou et al., 2001, Halliwell, 2001; Anderson and Luo, 1998). Recently, it was shown that D609, a glutathione mimetic (Lauderback et al., 2003), protects primary neuronal culture against amyloid β-peptide (1-42) [Aβ (1-42)]-induced oxidative stress and neurotoxicity in vitro (Sims, 1990) and in synaptosomes in vivo (Perluigi et al., 2006).

Nanoformulation drastically increases surface area, hence making D609 more bioavailable, efficient, reduces side effects and makes the drug cost
effective. Furthermore, nanoformulated D609 (nD609) can also improve drug performance by speeding onset of action in the present study. We performed the current study to test the hypothesis that in vitro treatment with nD609 had antioxidant, antiinflammatory and antiapoptotic effects on differentiated PC12 cells exposed to amyloid beta 25-35 fragment (Aβ25-35).

Materials and Methods

Chemicals and reagents
As described in materials and methods, Chapter III

Formulation of nanoD609 drug
As described in materials and methods, Chapter III

Cell culture
As described in materials and methods, chapter III

Treatment
Cells were cultured in flasks or plates. After 8 days of differentiation, the medium was replaced and fresh medium containing nD609 was added for 24 h. Thereafter, the fresh medium containing Aβ25-35 was added for 24 h replacing medium containing nD609.

Cytotoxicity and cell viability
As described in materials and methods, chapter III

Biochemical estimation
The biochemical assays (LDH, ROS, MMP, NO, and protein) are described in materials and methods chapter III.

Immunocytochemistry
As described in materials and methods chapter III.

Statistical analysis
As described in materials and methods chapter III.

Results

Determination of size and shape of nanoparticle
The size and size distribution of the polymeric nanoparticles were measured by means of dynamic light scattering (DLS). In Figure 8.1, the

Figure 8.1. Dynamic light scattering of polymeric nanoparticles.
typical size distribution of the nanoparticles is illustrated, and the average size corresponds to less than 50 nm diameter at 25°C with a narrow size peak distribution. Transmission electron microscopy (TEM) of the polymeric nanoparticles is illustrated in Fig. 8.2, and demonstrates that the particles have spherical morphology and low polydispersity with an approximate size of around 45 nm diameter, which is comparable to the size, obtained from DLS measurements. The adsorption efficiency (E %) of nD609 was found to be ~90% by this method based on calculation described in material and methods.

**Determination of PC12 cells viability with different concentrations of Aβ25-35 and nD609**

The cytotoxicity of Aβ25-35 was evaluated based on its effect on cell growth (MTT Assay). Fig. 8.3 deals with PC12 cells exposed with Aβ25-35 at a series of concentrations (0, 0.01, 0.1, 1, 10, 20 and 40 µM) for 24 h.

Aβ25-35 induced marked decrease in cells viability in a dose dependent manner and 10 µM Aβ25-35 significantly reduced cells viability to 54.12 ± 3.81% (p < 0.05). The possible cytotoxicity of nD609 was evaluated which also based on the cells growth by MTT assay (Fig.8.4). Differentiated PC12 cells were exposed to varying concentrations of nD609 (0-100 nM) for 24 h. nD609 was found to be non...
toxic at all these doses. Therefore, three doses of nD609 were selected for further studies viz. 25 nM, 50 nM and 100 nM.

**nD609 pretreatment decreased the activity of lactate dehydrogenase in Aβ_{25-35} treated PC12 cells**

LDH release measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. This is a marker of cell damage. Aβ_{25-35} (10 µM) significantly increased the activity of LDH (p < 0.05) which was restored by 25 nM, 50nM and 100 nM nD609 (p < 0.01) (Fig. 8.5).

**nD609 protects the intracellular oxidative stress in Aβ_{25-35} treated PC12 cells**

The accumulation of ROS was evaluated to quantify the oxidative stress. As illustrated in Fig. 8.6, there was a significant increase in the intracellular ROS (p < 0.001) in cells treated with Aβ_{25-35} compared to control taken as 100%. nD609 (50nM)+Aβ (p < 0.05) and nD609 (100nM)+Aβ pretreatment (p < 0.01) has decreased the ROS level significantly when compared to Aβ_{25-35} treated cells.

**Protective effect of nD609 on mitochondrial membrane potential (ψ_m) in Aβ_{25-35} treated PC12 cells**

The data were expressed as the Mean ± SEM of three independent experiments carried out in triplicate. *p < 0.001 Aβ_{25-35} vs control, (#p < 0.05 nD609 (50 nM) vs. Aβ_{25-35}, ##p < 0.01 nD609 (100 nM) vs. Aβ_{25-35}.
JC-1 dye was used as the marker of membrane disruption. The green/red ratio was found to be increased significantly in Aβ25-35 treated cells (p < 0.001) as compared to control group suggesting decreased membrane potential. nD609 (50nM)+Aβ (p < 0.01) and nD609 (100nM)+Aβ (p < 0.001) pretreatment augmented the membrane potential significantly as compared to Aβ25-35 treated cells (Fig. 8.7).

Attenuation of NO level by nD609 in Aβ25-35 treated PC12 cells

Figure 8.8 shows significant increase (p < 0.01) of NO level in Aβ25-35 treated cells as compared to control group. nD609 (25 nM)+Aβ, nD609 (50 nM)+Aβ and nD609 (100 nM)+Aβ (p < 0.01) significantly protected the toxicity induced by Aβ25-35.

nD609 pretreatment attenuated the expressions of Apaf-1, Bak, Bax and Bcl2

The upregulation of Apaf-1, Bak and Bax is linked with apoptosis. The expression of Apaf-1, Bak and Bax was increased in PC12 cells treated with Aβ25-35 (Fig. 8.9 B, E and H). In nD609 (100 nM)+Aβ25-35 treated cells, the expression was decreased as compared to Aβ25-35 treated cells. Only nD609 (100 μM) did not show any marked effect on the cells as compared to control group (data not shown).

Bcl2 expression was decreased in Aβ25-35 treated PC12 cells (Fig. 8.9 K) as compared to control which was attenuated significantly in nD609 (100 nM)+Aβ25-35 treated cells as compared to Aβ25-35 treated cells (Fig. 8.9 L).

nD609 pretreatment attenuated the expressions of p53, Hsp-70 and ChAT

The upregulation of p53 and Hsp-70 was found in PC12 cells treated with Aβ25-35 (Fig. 8.10 B, E). In nD609 (100 nM)+Aβ25-35 treated cells, the expression was decreased as compared to Aβ25-35 treated cells (Fig. 8.10 C, F). nD609 (100 μM) did not show any
marked effect on the cells as compared to control group (data not shown). ChAT expression was decreased in Aβ$_{25-35}$ treated PC12 cells (Fig. 8.10 H) as compared to control which was attenuated significantly in nD609 (100 nM)+Aβ$_{25-35}$ treated cells as compared to Aβ$_{25-35}$ treated cells (Fig. 8.10 I).

nD609 pretreatment attenuated the expressions of COX-2, NOS-2 and NF-κB

The up regulation of COX-2, NOS-2 and NF-κB is linked with AD pathogenesis. The expression of COX-2, NOS-2 and NF-κB was increased in PC12 cells treated with Aβ$_{25-35}$ (Fig. 8.11B,E,H). In nD609 (100 nM)+Aβ$_{25-35}$ treated cells, the expression was decreased as compared to Aβ$_{25-35}$ treated cells (Fig. 8.11 C,F,I).

Discussion

Drug nanoparticles or nanoformulation
Chapter VIII

Figure 8.10. Effect of nD609 pretreatment on p53, Hsp-70 and ChAT expression. The profound expression of p53 and Hsp-70 was observed in Aβ$_{25\text{-}35}$ group while expression of ChAT was decreased in Aβ$_{25\text{-}35}$ groups (B, E, H) compared to control groups (A, D, G). Aβ$_{25\text{-}35}$ group pretreated with nD609 (C, F, I) has shown a moderate expression of p53, Hsp-70 and ChAT.

is an immersing field of research and has gained attention of scientist around the world. Nanoformulation drastically increases surface area, hence making substances more soluble, bioavailable, effective and efficient. This also enables the rescue of compounds that are otherwise discarded. Furthermore, nanoformulation can also improve drug performance by speeding onset of action enabling alternative delivery routes or even targeting specific tissues. The side effects of conventional drugs are reduced in nanoformulation as the quantity of drug used is very low in this method.

With this study, we have made an attempt to analyze the effect of nanoformulated D609 in the prevention of AD. The model selected in this study was in vitro PC12 cells differentiated with nerve growth factor (NGF) and subsequently treated with amyloid beta (Aβ$_{25\text{-}35}$) (Khan et al., 2012).

Oxidative stress reflects a marked imbalance between ROS and their removal by antioxidant systems. This imbalance may originate from an overproduction of ROS or from a
reduction in antioxidant defenses or both (Butterfield and Stadtman, 1997). It was earlier shown that D609 could effectively scavenge hydroxyl radicals (Zhou et al., 2001). It was also shown that the xanthate D609, protects primary neuronal culture against Aβ (1-42)-induced oxidative stress and neurotoxicity in vitro (Sultana et al., 2004) and against Aβ (1-42) in vivo (Perluigi et al., 2006). Our results have shown that nD609 has reduced the ROS generation significantly and was supported by the above literature. The identification of nD609 as a potent antioxidant implies that D609 may exert some of the reported activities that have been largely attributed to the inhibition of phosphatidyl choline-specific phospholipase C (PC-PLC) by its antioxidant properties. Among these activities are the inhibition of TNF-α induced NF-κB activation and inflammatory cytokine production (Amtmann, 1996; Schutze et al., 1992). NF-κB is prerequisite and ubiquitous transcription factor for the expression of many inflammation-related genes, including NOS-2 and COX-2. It has been reported that NOS-2 and COX-2 are induced in various types of CNS injuries and diseases (Hunot et al., 1996; Teismann et al., 2003). These two enzymes are often coexpressed in disease states associated with gliosis. In our study also, the expression of NF-κB, NOS-2 and COX-2 was upregulated as compared to control. Moreover the number of cells was significantly lower in Aβ25-35 group as compared to control. nD609 pretreatment has reversed these effects to normal levels. Nitric oxide (NO) level was also found to be increased by Aβ25-35 treatment in PC12 cells which was significantly restored by nD609 pretreatment. Aβ disrupts Ca²⁺ homeostasis in neurons (Mattson et al., 1993), and increased intracellular Ca²⁺ level can increase sphingomylinase activity to produce ceramide (Di Paola et al., 2004). Activation of the apoptotic sphingomyelin-dependent signaling pathway is mediated by ceramide (Di Paola et al., 2004) during oxidative stress to play role in the pathogenesis of neuronal disease (Michel et al., 1999). Apoptosis induced by the membrane-permeable second messenger ceramide, followed by the release of cytochrome-c and Ca²⁺ from the mitochondria with the loss of mitochondrial transmembrane potential has been observed in AD pathogenesis (Michel et al., 1999). Cytochrome-c
Figure 8.11. Effect of nD609 pretreatment on COX-2, NOS-2 and NF-κB expression. The profound expression of COX-2, NOS-2 and NF-κB was observed in Aβ25-35 group (B, E, H) compared to control groups (A, D, G), while Aβ25-35 group pretreated with nD609 (C, F, I) has shown a moderate expression of COX-2, NOS-2 and NF-κB.

binds with Apaf-1 to activate Caspase-9 ultimately leading to apoptosis. In our study, there was loss of mitochondrial membrane potential ($\psi_m$) along with the increase in lactate dehydrogenase activity suggesting disrupted membrane integrity, as a result of Aβ25-35 treatment. nD609 has significantly restored the $\psi_m$ and lactate dehydrogenase activity suggesting its protective potential. Apaf-1 expression was also increased by Aβ25-35 treatment. nD609 has prevented this increase expression.

It is known that caspase activation is also regulated by Bcl-2 family proteins. Antiapoptotic members (Bcl-2) of the Bcl-2 family prevent caspase activation by blocking factor release (Susin et al., 1996; Yang et al., 1997; Kluck et al., 1997), whereas proapoptotic members (Bak and Bax) of the family promote release (Shimizu et al., 1999; Shimizu et al., 2000). Cytosolic Bax translocates to mitochondria upon death stimulus (Guegan et al., 2001; Gross et al., 1998), promoting cytochrome c release (Gross et al., 1998). Bcl-2 binds to
Apaf-1, inhibiting the association of caspase-9 with Apaf-1 (Hu et al., 1998). Depletion of the endogenous neuroprotective Bcl-2 family signals directly contributes to neuronal loss in neurodegenerative diseases (Lukiw and Bazan, 2006). With this point in mind we have also seen the effect of Aβ25-35 on Bcl-2 family proteins. The expression of Bak and Bax was increased significantly and the expression of Bcl-2 was downregulated in Aβ25-35 treated cell as compared to control. nD609 has maintained the normal levels of these proteins suggesting anti-apoptotic activity of D609.

p53 expression was also analysed in this study as p53 is a tumor-suppressor protein that plays an important role in maintaining genomic integrity (Ko and Prives, 1996) during the exposure of cells to radiation, genotoxic chemicals, hypoxia or oxidative stress (Cenini et al., 2008a; Cenini et al., 2008b). Several studies have reported an increase in p53 immunoreactivity in sporadic AD (Kitamura et al., 1997; Ohyagi et al., 2004; Cenini et al., 2008a). There was increased expression of p53 in this model also suggesting the toxic effect of Aβ25-35. nD609 was able to downregulate the expression of this transcription factor. We have also analyzed HSP-70 and ChAT expression and it was found that HSP-70 was upregulated and ChAT was downregulated in Aβ25-35 treated cell which was significantly restored by nD609.

From all these results, taken together with the results of previous studies, it could be concluded that D609 is a potent antioxidant, antiinflammatory and antiapoptotic agent and its nanoformulation also exhibited the similar results as the pure chemical. So, further studies are needed to promote this pilot study for suggesting nD609 as a potent drug for the prevention of AD.

**Conclusion**

The present study summarizes the antioxidative, antiinflammatory and antiapoptotic role of nD609 to ameliorate the adverse condition of the cells treated with Aβ25-35. Our results indicated that nD609 has potential to mitigate the toxic effects induced by Aβ25-35 on differentiated PC12 cells. Further extensive studies are needed to promote the usage of nD609 for the prevention of AD and other neurological disorders.
Figure 8.12. Summarized diagram of the prevention of nD609 on amyloid beta induced toxicity in PC12 cells: This figure shows the induction of oxidative stress, inflammation, NO production and apoptosis by Aβ25-35 toxicity. Briefly, PC12 cells exposed to Aβ25-35 results in oxidative stress as evident from increase in ROS which activated NF-κB to induce production of NOS-2 and COX-2 leading to inflammation. p53 also accumulates as a result of oxidative stress and inflammation leading to apoptosis. Aβ25-35 insult also leads to mitochondrial damage and results in decrease mitochondrial membrane potential ($\psi_m$). HSP-70 expression is also increased as a result of Aβ25-35 insult. In the present study, we demonstrated that nD609 effectively decreased the oxidative stress and inflammation. Apoptosis, NO production and $\psi_m$ was also prevented by nD609 along with the down regulating of the expression of p53 and HSP-70 thereby suggesting its preventive role in AD pathogenesis.