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Oxidative Stress and Toxicity Induced by the Nucleoside Reverse Transcriptase Inhibitor (NRTI) - 2', 3'-dideoxycytidine (ddC): Relevance to HIV Dementia

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Abstract

Human immunodeficiency virus dementia (HIVD) is the most common form of dementia occurring among young adults. In HIVD, neuronal cell loss occurs in the absence of neuronal infection. With the advent of highly active anti-retroviral therapy (HAART), the incidence of HIVD has drastically reduced, though prevalence of milder forms of HIVD continues to rise. Though these agents have been used successfully in suppressing viral production, they have also been associated with a number of side effects. Here we examine the possible role of NRTIs, in particular 2', 3'-dideoxycytidine (ddC), in the neuropathology of HIVD. Synaptosomes and isolated mitochondria treated and incubated for 6 h with CSF-achievable concentrations of ddC, i.e., 6–11 ng/ml, were found to show a significant increase in oxidative stress with 40 nM ddC as measured by protein carbonyls and 3-nitrotyrosine (3NT), effects that were not observed in the more tolerable NRTI; 3TC. Protection against protein oxidation induced by ddC was observed when brain mitochondria were isolated from gerbils 1 h after injection i.p with the brain accessible antioxidant and glutathione mimetic, tricyclodecan-9-yl-xanthogenate (D609). In addition, there is a significant reduction in the levels of anti-apoptotic protein Bcl-2 and a significant increase in cytochrome-c release and also a significant increase in the expression of pro-apoptotic protein caspase-3 after mitochondria were treated with 40 nM ddC. The results reported here show that ddC at 40 nM can induce oxidative stress, cause the release of cytochrome c, and in addition, reduce the levels of anti-apoptotic proteins, increase the levels of pro-apoptotic proteins, thereby increasing the possibility for induction of apoptosis. These findings are consistent with the notion of a possible role of the NRTIs, and in particular, ddC, in the mechanisms involved in HIVD.

Keywords

ddC; Oxidative stress; HIV-Dementia; Mitochondria and Highly Active Antiretroviral Therapy (HAART)

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Introduction

Infection of the brain with HIV often results in a dementing disorder known as HIV-dementia (HIVD), the most common form of dementia occurring in human populations of less than 60 years of age. This dementia is marked by loss in memory and impaired cognitive function, among other indices of dementia (Navia and Price 1987). Pathologically, reactive astrocytosis, myelin pallor, infiltration by macrophages and multinucleated giant cells, among others, characterize HIVD (Nath et al., 1998). Since the neuronal loss and dysfunction observed in HIVD occurs, though the virus rarely infects neurons (Mattson et al. 2005), the mechanism of this neurodegeneration are still unknown. Several studies have implicated the involvement of HIV viral proteins such as Tat, gp120 and gp 41, as well as neurotoxins that are produced from activated astrocytes and microglia, in the pathways leading to neurodegeneration (Adamson et al. 1996; Cheng et al. 1998; Nath et al. 2000; Pocernich et al. 2005).

In recent years, the use of highly active antiretroviral therapies (HAART) has revolutionized the treatment of AIDS, with a suppression of viral load and consequent reduction in complications observed in the late-stages of the disease (Gray et al. 2003). HAART can suppress the replication of the virus in the long term, but this is often accompanied by significant toxicities that can compromise treatment (Egger et al. 1997; Brinkman and Kakuda 2000). Anti-retrovirals such as the nucleoside reverse transcriptase inhibitors (NRTIs), which include, 2', 3'-dideoxycytidine (ddC), are of great significance and are an important component of HAART (White 2001). Among the toxicities reported with the NRTI's include: hepatic steatosis, lactic acidosis, encephalopathy, lipodystrophy, peripheral neuropathy, and myopathy (Scalfaro et al. 1998; Blanche et al. 1999). The mechanisms of these toxicities has been found to involve inhibition of neurite regeneration and inhibition of DNA polymerase gamma, leading to inhibition of mitochondria DNA synthesis, resulting in oxidative stress and eventual mitochondrial dysfunction (Cui et al. 1997). But recently, Mallon et al. (2005) have also shown that NRTI's could cause mitochondrial dysfunction through other mechanisms other than inhibition of DNA polymerase gamma (Mallon et al. 2005).

With the advent of HAART, the incidence of HIVD has fallen, while the cumulative prevalence of HIVD has risen (Neuenburg et al. 2002). Despite the decline of HIVD incidence, neurological complications still remain an important cause of disability and death associated with AIDS (Kandaneeratchi et al. 2003; McArthur et al. 2003). The rise in the prevalence of HIVD despite the use of HAART is consistent with the poor penetration of some of these anti-retrovirals into the CNS, hence increasing the longevity of the virus in the CNS (Deutsch et al. 2001). It has recently been reported that these drugs can effectively cross into the CNS, therefore, raising the question of their efficacy in the CNS and their possible role in HIVD (Enting et al. 1998; Sawchuk and Yang 1999; Gibbs et al. 2003a; Gibbs et al. 2003b). For example, one treatment study showed variable results on the efficacy of NRTIs in the brain: one group of patients on HAART and having HIVD remained stable from cognitive impairment, while the other group showed signs of progressive neurological impairment even with treatment (Kandaneeratchi et al. 2003). This disparity could possibly be due to the increased survival of the virus in the brain or could possibly be due to the NRTIs on their own being neurotoxic as has recently been shown with the increase in damage to brain mitochondria in HIV positive patients using NRTI's (Schweinsburg et al. 2005). The latter may be of particular concern, since several experimental strategies are currently being considered to increase the transport of NRTIs across the blood brain barrier.

Therefore, in the current study, we have investigated these NRTIs, and specifically ddC, with the hypothesis that NRTIs could in themselves be neurotoxic and thus play a role in the progression of HIVD through an oxidative stress mechanism, possibly involving induction of apoptosis through the mitochondria. We report here that ddC, at concentrations achievable in

the CSF, induces oxidative stress when added to synaptosomes and isolated mitochondria. The levels of oxidative stress as measured by protein carbonyls on synaptosomes were significantly reduced upon the use of a known brain accessible antioxidant and glutathione mimetic [D609] (Zhou et al. 2001; Lauderback et al. 2003; Sultana et al. 2004). In addition, we observed a significant reduction in the levels of the anti-apoptotic protein Bcl-2, a significant release of cytochrome c and an equally significant increase in caspase-3 protein levels upon treatment of mitochondria with ddC, suggesting the induction of apoptotic process following treatment with ddC. These results provide a possible mechanistic pathway for cell death through oxidative stress-induced apoptosis and are consistent with the notion of the possible contribution of ddC to HIVD.

Material and Methods

Unless stated otherwise, all chemicals and antibodies were purchased from Sigma-Aldrich (St. Louis, MO). The oxidized protein detection kit was purchased from InterGen (Purchase, NY). The nucleoside reverse transcriptase inhibitor (NRTI), 2', 3'-dideoxycytidine (ddC), was obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (NSC 606170). Assays for protein oxidation, lipid peroxidation, cytochrome c release and Bcl-2 levels were performed 6 h after NRTI treatment with some modifications as previously described (Butterfield et al. 1999; Yatin et al. 1999; Butterfield and Lauderback 2002).

Animals

The animal protocols were approved by the University of Kentucky Animal Care and Use Committee. For all studies in this paper, male Mongolian gerbils 3 months of age and approximately 100 g in weight were used. The rodents were housed in the University of Kentucky Central Animal Facility under 12-h light/dark conditions and fed standard Purina rodent laboratory chow.

Isolation of synaptosomes

Synaptosomes were isolated from three-month old male Mongolian gerbils as previously described (Whittaker 1993). Briefly, the gerbils (n=5) were sacrificed by decapitation and the brain immediately isolated and dissected. The cortex was placed in 0.32 M sucrose isolation buffer containing 4 µg/ml leupeptin, 4 µg/ml pepstatin, 5 µg/ml aprotinin, 2 mM ethylene diamine tetra-acetic acid (EDTA), 2 mM ethylene glycol-bis-tetraacetic acid (EGTA), 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20 µg/ml trypsin inhibitor, and 0.2 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4. The tissue was homogenized by 20 passes with a Wheaton tissue homogenizer. The homogenate was centrifuged at 1500 g for 10 minutes. The supernatant was retained and centrifuged at 20,000 g for 10 minutes. The resulting pellet was resuspended in ~1 ml of 0.32 M sucrose isolation buffer and layered over discontinuous sucrose gradients (0.85 M pH 8.0, 1.0 M pH 8.0, 1.18 M pH 8.5 sucrose solutions each containing 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES) and spun at 82,500 g for 1 hr at 4°C. Synaptosomes were collected from the 1.0/1.18M sucrose interfaces, and washed in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM glucose, 5 mM HEPES) twice for 10 minutes at 32,000 g. The resulting synaptosomal membranes were assayed for protein concentration by the Pierce BCA method. The synaptosomal preparation was relatively free of non-synaptic moieties (Whittaker 1993).

Isolation of Mitochondria

Mitochondria were isolated from gerbil brain following i.p. injection ip with saline (control) or with D609 (50 mg/kg body wt), 1 h after injection as previously described (Sultana et al. 2004). The brain mitochondria were isolated as previously described with minor modifications

(Sims 1990). Briefly gerbils, (n=6) were decapitated and the whole brain was isolated on ice. Whole brain was homogenized in ice-cold isolation buffer (250 mM sucrose, 10 mM HEPES, and 1 mM potassium EDTA, pH 7.2, 4 µg/ml leupeptin, 4 µg/ml pepstatin, 5 µg/ml aprotinin 20 µg/ml trypsin inhibitor) with 6 passes of a Wheaton tissue homogenizer. The homogenate was centrifuged for 3 min at 1,330× g at 4°C, and the resulting pellet was resuspended in isolation buffer and centrifuged at 1,330× g for 3 min. The supernatants from both spins were combined and spun at 21,200× g for 10 min at 4°C. The pellet was resuspended in 15% Percoll solution (v/v in isolation buffer) and layered onto discontinuous Percoll gradients of 23 and 40% Percoll (v/v in isolation buffer). Gradients were centrifuged at 30,700× g for 5 min at 4°C. At the 23–40% Percoll interface, mitochondria were isolated and resuspended in respiration buffer (250 mM sucrose, 2 mM magnesium chloride, 20 mM HEPES, and 2.5 mM phosphate buffer, pH 7.2) and centrifuged at 16,700× g for 10 min at 4°C. The pellet was resuspended in respiration buffer, centrifuged at 6,900× g for 10 min at 4°C, and the resulting pellet was washed in PBS at 6,900× g for 10 min at 4°C.

Incubation of synaptosomes and mitochondria with ddC

Stock solutions of ddC were diluted in phosphate buffered saline (PBS). Synaptosomal and mitochondrial concentrations of 4mg/ml were then treated with the ddC to a final concentration based on the CSF-achievable concentration range of 6–11 ng/ml (Sawchuk and Yang 1999). These samples were then incubated for 6 h at 37°C with frequent vortexing. Controls were incubated with PBS. These samples were then frozen for subsequent assays.

Oxidation of synaptosomes with Fe²⁺/H₂O₂

Protein oxidation was initiated by addition of hydroxyl radical generating mixture (30 µM FeSO₄/3 mM H₂O₂) as previously described (Pocernich et al. 2000). Briefly, synaptosomes (4 mg proteins/ml) were suspended in 0.987 ml of lysing buffer and treated with 30 µM Fe²⁺ (FeSO₄·7H₂O) and 3 mM H₂O₂ (diluted from 30% H₂O₂), bringing the total volume to 1 ml, for 30 min at room temperature. Fe²⁺ reacting with H₂O₂ produce hydroxyl free radicals (Butterfield and Stadtman, 1997). The protein suspension was then washed four times with lysing buffer at 14,000 rpm for 4 min. This sample was used as a positive control for protein oxidation.

Measurement of protein carbonyls

Protein carbonyls are an index of protein oxidation and were determined as described previously (Butterfield and Stadtman 1997). Briefly, 5µl of sample were derivatized with 10mM 2, 4-dinitrophenylhydrazine in the presence of 5µl of 12% SDS for 20 min at room temperature. The samples were neutralized with 7.5 µL of the neutralization solution (2 M Tris in 30% glycerol). Derivatized protein samples were blotted onto nitrocellulose membrane with a slot-blot apparatus (250 ng/lane). The membrane then was washed with wash buffer (10mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% Tween 20), blocked by incubation of 5% BSA, followed by incubation with rabbit polyclonal anti-DNPH antibody as primary antibody for 1 h. The membranes were washed with wash buffer and further incubated with alkaline phosphatase (ALP)-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Blots were developed using Sigma fast tablets (BCIP/NBT) and were quantified using Scion Image (PC version of Macintosh compatible NIH Image) software.

Measurement of lipid peroxidation

4-Hydroxynonenal (HNE) levels—HNE is a marker of lipid peroxidation, and this assay was performed as previously described (Lauderback et al. 2001). Briefly, 10 µl of sample both from cell culture or synaptosomes were incubated with 10 µl of Laemmli buffer containing 0.125 M Tris base pH 6.8, 4 % (v/v) SDS, and 20% (v/v) Glycerol. The resulting sample (250

ng) was loaded per well in the slot blot apparatus. Samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) BSA in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (PBST) for 1 hr and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in PBST for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in PBST. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in PBST and added to the membrane. The membrane was washed in PBST three times and developed using Sigma fast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image.

Measurement of cytochrome c release

Cytochrome c release was detected as previously described with slight modifications (Yang et al. 1997). Briefly, after incubation and centrifugation of mitochondrial samples, the supernatant was used for Western blot analysis for cytochrome c release. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS/Tween for 2 h and incubated with a 1:2000 dilution of anti-cytochrome c polyclonal antibody (C-5723; anti-sheep; Sigma) in PBS/Tween for 2 h. The membrane then was washed in PBS/Tween for 5 min three times after incubation. The membrane was incubated for 1 h, after washing, with an anti-sheep IgG alkaline phosphatase secondary antibody diluted in PBS/Tween in a 1:8000 ratio. The membrane then was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified using Scion Image software.

Measurement of Bcl-2 and Bax levels—The levels of anti-apoptotic protein Bcl-2 and pro-apoptotic Bax were detected as previously described (Yang et al. 1997) with slight modification as described above, except a 1:2000 dilution of anti-Bcl-2 monoclonal antibody (AAM-072; anti- mouse; stressgen) and anti-Bax polyclonal antibody (AB2930; chemicon) in PBS/Tween for 2 h was used.

Measurement of Caspase-3 protein levels—The levels of pro-apoptotic protein caspase-3 were detected through immunoblotting as previously described (Yang et al. 1997). A 1:1000 dilution of anti-caspase-3 monoclonal antibody (MAB4703 anti- mouse chemicon) in PBS/Tween for 2 h was used. Followed by an alkaline phosphate-conjugated secondary antibody. The membrane then was washed three times in PBS/Tween for 5 min and developed in Sigma Fast tablets. Blots were dried, scanned with Adobe PhotoShop, and quantified using Scion Image software.

Statistical analysis

Student's t-test was used to assess statistical significance; p values < 0.05 were considered significant.

Results

Effects of ddC on synaptosomes

Synaptosomes are a good model for studying neuronal synaptic function at nerve terminals and display markers of neurodegeneration upon treatment with various oxidants such as amyloid β -peptide (A β) (Butterfield and Boyd-Kimball 2005). Hence, synaptosomes were used for the present study to determine if ddC caused oxidative damage. Oxidative stress can lead to a variety of detrimental effects on neurons including protein cross linking, decreased protein turnover, loss of protein function, altered redox potential, disruption of Ca²⁺ homeostasis, and ultimately cell death (Butterfield and Stadtman, 1997). The production of protein carbonyls is

a marker of oxidative stress. As shown in Fig 1a, synaptosomes were exposed to a known hydroxyl radical generating mixture (30 μ M FeSO₄/1 mM H₂O₂) as a positive control and a range of ddC concentrations (29 nM- 52 nM). There was a significant increase in protein carbonyls levels on synaptosomes treated with 40 nM ddC $p < 0.05$. These levels were comparable to those of the synaptosomes treated with Fe²⁺/H₂O₂. Fig 1b shows levels 3-nitrotyrosine (3NT), another marker of protein oxidation, in ddC treated synaptosomes. A significant increase in the levels of 3NT with 40nM ddC was observed; however, there was no significant change in the levels of lipid peroxidation as indexed by HNE when synaptosomes were treated with ddC in all the concentration ranges.

Effects of ddC on mitochondria

Fig 2 shows the levels of protein carbonyls and HNE in mitochondria treated with ddC. Fig 2a shows a significant increase in the levels of protein oxidation as indexed by protein carbonyls. These results were significant at 40 nM ddC, seen previously with synaptosomes. Fig 2b shows the levels of lipid peroxidation as indexed by HNE was unaffected when mitochondria were treated with a varying range of concentrations of ddC, consistent with the results with synaptosomes.

Effects of ddC on mitochondria from D609-treated rodents

The use of antioxidants has been shown to provide protection against oxidative insult in a number of models of neurodegenerative disorders (DeAtley et al. 1999; Calabrese et al. 2003; Farr et al. 2003; Lauderback et al. 2003; Sultana et al. 2005; Perluigi et al., 2006). The levels of protein oxidation as indexed by protein carbonyls in mitochondria isolated from brain of gerbils injected 1 h before sacrifice with saline or D609 are shown in Fig. 3. These brain mitochondrial samples were subsequently treated with 40 nM ddC, the concentration that showed the most effect. The levels of protein carbonyls were significantly increased in mitochondria isolated from brain of saline-injected gerbils and treated with ddC compared to control. Mitochondria isolated from brain of D609-injected gerbils and subsequently treated with 40 nM ddC showed a significant decrease in the levels of protein carbonyl levels compared to mitochondria isolated from brain of saline-injected gerbils and subsequently treated with 40 nM ddC.

Effects of ddC on cytochrome c release

Mitochondria are the main source for the generation of reactive oxygen species (ROS), which has been known to contribute to the release of cytochrome c and subsequent induction of apoptosis (Sullivan et al. 1999). Fig. 4 shows the level of cytochrome c released from mitochondria isolated from gerbil brain and treated with various CSF concentrations of ddC. There was a significant increase in the amount of cytochrome c released by mitochondria treated with 40nM ddC compared to control.

Effects of ddC on levels of anti-apoptotic protein Bcl-2

Bcl-2 is a human proto-oncogene that modulates cell death or apoptotic pathways by regulating the release of pro-apoptotic molecules from the mitochondria. Fig 5 shows the level of Bcl-2 protein in mitochondria isolated from gerbil brain and treated with various CSF concentrations of ddC. Though there was a decrease in the levels of Bcl-2 with all concentrations of ddC used, there was a significant decrease in the levels of Bcl-2 with 52nM ddC treatment compared to control.

Effects of ddC on levels of pro-apoptotic protein caspase-3

As an additional evidence of apoptotic events taking place after the treatment of mitochondria with ddC, we measured the levels of caspase-3 protein levels as shown in figure 6. There was

an observable increase in the levels of caspase-3 in all treatment but there was a higher increase in the 40nM ddC treated mitochondria. It should be noted that since this was just additional proof of apoptotic events taking place, and $n = 4$ was used. The changes observed are about 40% of control with statistical significance $p < 0.05$.

Effects of ddC on levels of pro-apoptotic protein Bax

Bax is another pro-apoptotic protein that is involved in the release of cytochrome c from the inner mitochondrial membrane. There was an increase in the levels of Bax observed after treatment with the three concentrations of ddC as shown in fig 7; however this increase in the level of Bax was not significant. Nevertheless, we believe that this result may provide an indication of the initiation of the apoptotic cascade following treatment of mitochondria with NRTI's.

Effect of 3TC on protein carbonyls

As additional evidence for the oxidative stress-induced toxicities of NRTI's we have shown that 3TC, the most tolerable NRTI in HIV therapy, did not induce oxidative stress in synaptosomes treated using concentrations achievable in the CSF compared to DDC as shown in Figure 8. As a result the oxidative stress-related effects we are observing in the present study are specific to only ddC and not any other NRTI.

Discussion

The findings reported here show that ddC, at physiologically relevant concentrations achievable in the CSF and especially at 40nM, is able to induce oxidative stress in both synaptosomes and isolated mitochondria, as measured by the elevated levels of protein oxidation. DDC also reduced the levels of the anti-apoptotic protein Bcl-2, slightly increased the levels of Bax, induced the release of cytochrome c, and increased caspase-3 protein levels. Consistent with these findings, the glutathione mimetic and brain accessible antioxidant D609 (Lauderback et al., 2003; Perluigi et al., 2006) protects mitochondria against oxidative stress induced by ddC (Fig. 3). These results implicate oxidative stress and the mitochondria as possible pathways involved in NRTI-induced neuronal death.

Infection of the brain with HIV often results in a decline of neuropsychological performance, neurological impairments (McArthur et al. 1989; Karlsen et al. 1993, 1995; Baldewicz et al. 2004), and a dementing disorder known as HIV-dementia (HIVD). HIVD is the most common form of dementia in young adults, and it is estimated that 30% of adults infected with HIV usually develop HIVD (Janssen et al. 1992; Nath and Geiger 1998). The mechanism of this neurodegeneration remains unknown, since HIV rarely infects neurons (Nath and Geiger 1998). Recent studies have proposed various mechanisms for HIVD, for example, HIV over-activates immune cells within the brain to produce cytokines or chemokines, which can inhibit the growth and survival of brain cells or trigger cell death and low levels of transforming growth factor β 1 (TGF β 1) may enhance brain cell damage in people with HIV infection (Ensoli et al. 1999; Letendre et al. 1999). HIV proteins such as Tat, nef, gp120, and gp 41, have also been implicated in the pathways leading to this neurological dysfunction (Adamson et al. 1996; Cheng et al. 1998; Nath et al. 2000; Pocernich et al. 2004; Pocernich et al. 2005). Oxidative stress has been found to play a major role in most neurodegenerative disorders (Butterfield and Stadtman, 1997; Butterfield and Boyd-Kimball 2005; Keller et al. 2005; Pocernich et al. 2005; Butterfield et al., 2006a,b). This could be another possible mechanism involved in HIVD induced neurodegeneration, since recent studies have shown that there is increased oxidative stress in the brain and CSF of HIVD patients (Chauhan et al. 2003; Turchan et al. 2003).

The NRTIs, including ddC, form the basis of the HAART therapy (White 2001). These molecules are derivatives of nucleoside bases with varying modifications that give alternative substrates for the DNA polymerases. The most common chemical modification is the lack of 3'OH group of the sugar bases (Kakuda 2000). The mechanism for action of ddC is through inhibiting the attachment of nucleic acids and at the same time terminating DNA chain elongation once incorporated (Balzarini 1994). Just like endogenous nucleic acids, NRTIs have to undergo phosphorylation by a number of kinases to give the active triphosphorylated form (Kakuda 2000). It is known that the concentrations of NRTIs decrease progressively as they move from the plasma to the CSF (Sawchuk and Yang 1999; Kandaneeratchi et al. 2003). The penetration of NRTIs into the CNS has been an issue to date (Enting et al. 1998; Sawchuk and Yang 1999). An increase in the incidence of HIV dementia has been thought to be due to the prolonged survival of the HIV virus in the brain resulting from the poor penetration of the antiretroviral used in the therapy (Deutsch et al. 2001), but studies now suggest that some of these drugs do effectively penetrate the CNS (Enting et al. 1998; Arendt et al. 2001; Gibbs et al. 2003a). ddC is transported to the CNS through organic anion transporters (Hedaya and Sawchuk 1989). This could be a possible explanation for the lack of any significant difference observed in the levels of lipid peroxidation product HNE measured in both synaptosomes and isolated mitochondria treated with ddC in the current study. For HNE to be generated, the oxidant must be capable of inserting itself into the lipid bilayer to trigger the cascade for lipid peroxidation (Butterfield and Boyd-Kimball 2005). Since the transport of ddC into the CNS is through organic anion transporters ddC may not get into the bilayer and thus not induce lipid peroxidation, though this is speculative at present.

Though NRTIs are capable of controlling the replication of the HIV virus, they also have adverse effects during therapy (Scalfaro et al. 1998; Blanche et al. 1999). Mitochondrial dysfunction has been previously observed with NRTIs, and extensive studies on the mechanism of NRTI toxicity has been linked to mitochondrial dysfunction, mutations in mtDNA and oxidative stress through inhibition of DNA polymerase gamma, which encodes 13 oxidative phosphorylation (OXPHOS) genes (Lewis et al. 2001; Lewis et al. 2003). It has recently been suggested that NRTIs could cause mitochondrial dysfunction through mechanisms other than inhibition of DNA polymerase gamma (Mallon et al. 2005). Of the above mechanisms of toxicity, oxidative stress seems to play a major role. Oxidative stress is an imbalance between reactive oxygen species and cellular antioxidant defenses (Butterfield and Stadtman, 1997). The role of HIV infection and NRTI on induction of oxidative stress has been known for a while, but has not been well explored (Droge 2002). We have shown in the present study that, on treatment of synaptosomes and isolated mitochondria with CSF-achievable concentrations of ddC, there is a significant increase in the oxidative stress as indexed by the levels of protein oxidation biomarkers, i.e., protein carbonyls and 3NT. These results support the role NRTI induction of oxidative stress. DDC at lower concentrations had very little effect of the induction of protein oxidation; however at 40nM of ddC there was a significant increase in the levels of protein oxidation which on further increase in the concentration of ddC had no effect. This observed biphasic effect could be possibly due the saturation of ddC transporters at 40 nM ddC or could be due to may be through some mechanisms that ddC is acting as a quencher for ROS hence the reduced levels of oxidative stress observed.

The use of antioxidants has been found to be protective in various models neurodegenerative disorders (DeAtley et al. 1999; Calabrese et al. 2003; Farr et al. 2003; Lauderback et al. 2003). Tricyclodecan-9-yl-xanthogenate (D609) is an inhibitor of phosphatidylcholine-specific phospholipase C, an antioxidant, anti-tumor and a glutathione (GSH) mimetic (Zhou et al. 2001; Lauderback et al. 2003; Sultana et al. 2004). Mitochondria isolated from brain of D609-injected gerbils and treated with 52 nM ddC showed decreased oxidative stress as measured by the levels of protein carbonyls, demonstrating that though ddC can induce oxidative stress, these effects can be modulated when a brain accessible glutathione mimetic

antioxidant is used. We previously suggested that D609 possibly could have therapeutic relevance in treating HIVD (Pocernich et al. 2005).

Mitochondria are not only an essential component of the cell for energy generating capacity but are targets of oxidative stress because of their ability to generate ROS. In addition mitochondria also are prodigious regulators of cell death (Melov 2000). Several molecules contained within the mitochondria, such as cytochrome c, have a pro-apoptotic influence. In contrast, Bcl-2 members regulate the pores in the inner membrane of the mitochondria, which when opened lead to release of pro-apoptotic molecules and induction of apoptosis (Stefanis 2005). The inhibition of mtDNA replication can result in acquired defects that leads to most mitochondrial genetic diseases (Lewis et al. 2003). As a result, NRTIs could thus generate phenotypic OXPHOS defects that would eventually compromise the electron transport chain (ETC), resulting in energy depletion and eventually cellular dysfunction. It is from this consideration that the phrase “mitochondrial dysfunction hypothesis” was introduced (Lewis et al. 2003), linking NRTI toxicity to their effects on the mitochondria (Moyle 2000; Dalakas et al. 2001). In the present study, we have observed a significant decrease in the levels of anti-apoptotic protein Bcl-2 and consequently a significant increase in the release of cytochrome c, upon treatment of mitochondria with ddC specifically at 40 nM of ddC. We also observed an increase in the protein levels of caspase-3. These results present a possible mechanism through which NRTIs could induce cell death and neuronal loss in HIV-related dementia. The decrease of Bcl-2 protein, increase in cytochrome c and increase in caspase-3 provides a synergistic environment for the induction of apoptosis. The down regulation of Bcl-2 levels, leads to the release of cytochrome c, which binds to Apaf-1 and pro-caspase 9 forming the apoptosome. Also the slight observation of the increase in Bax levels would lead to an increase in the permeability of the mitochondrial membrane possibly leading to the release of cytochrome c (Susin et al. 2000). These mediators then activate caspase 9, which triggers the cascade of “effectors” caspases and eventual cell death (Stefanis 2005). Our findings are consistent with a recent report that showed that other than ddC, 2', 3'-didehydro-3'-deoxythymidine (d4T), also caused apoptosis in rat dorsal root ganglion neuronal cell cultures in HIV free environments (Bodner et al. 2004).

Despite the use of HAART, neurological complications still remain an important cause of disability and death associated with AIDS (Kandaneeratchi et al. 2003; McArthur et al. 2003). The efficacy of these drugs in the brain is still controversial and research into the effect of HAART on the signs and symptoms of HIVD has resulted into a number of conflicting findings. For example, there are reports showing that HAART is capable of reducing HIV encephalitis and leukoencephalopathy, improving neurocognitive function, mental concentration, speed of mental processing, memory, and visuo-spatial and constructional abilities (Gendelman et al. 1998; Dougherty et al. 2002; Vago et al. 2002). Another study reports a partial improvement of clinical abnormalities after patients were on HAART (Stankoff et al. 2001). A study by (Bouwman et al. 1998) has reported that after HAART treatment some patients remained free of cognitive impairment with prolonged life span, while others become neurologically impaired. Dougherty et al. (2002) treated 96 HIV- demented patients with HAART, and of the 30 patients that had adequate follow-up data, 60% showed neurological improvement (Dougherty et al. 2002). These studies among others continue to raise the question on the effect of the antiretroviral therapy on the incidence and prevalence of HIVD and the efficacy of the NRTIs in the brain, therefore supporting the possibility that these drugs could themselves contribute to the neurological impairments. In a recent study, it has been shown that there is brain mitochondrial injury in HIV positive patients who are on NRTI therapy (Schweinsburg et al. 2005), this provides additional support for the present study of the possible involvement of NRTI's in HIV-dementia.

As a result, the current study was an effort at studying a possible toxic effect of NRTI's and in particular ddC, with oxidative stress and mitochondrial dysfunction as the key mechanism of toxicity. Synaptosomes provide a good model of the synapse and display markers of neurodegeneration upon treatment with various oxidants. Equally, isolated mitochondria provide a good model to study mechanisms of neurodegeneration since they are a source of ROS and control cell death through regulation of apoptosis (Butterfield et al. 1999; Lauderback et al. 2001; Lauderback et al. 2003; Brito et al. 2004; Pocernich et al. 2004; Butterfield and Boyd-Kimball 2005). As noted above, despite the reduction of late stage HIV complications with the use of antiretroviral treatment, neurological complications are still being reported, questioning the efficacy of these drugs in the brain (Bouwman et al. 1998). It is therefore our view that in addition to the prolonged survival of the HIV virus in the brain (Deutsch et al. 2001) and increased penetration of the antiretrovirals to the CNS (Enting et al. 1998; Sawchuk and Yang 1999), these NRTIs could by themselves contribute to this neurological complication through increased oxidative stress as shown by the increase in oxidative stress biomarkers measured here. It is also possible that ddC could potentiate and act synergistically with other compounds such as Tat, gp120 and gp41 in inducing mechanisms implicated in HIVD (Pocernich et al. 2005). This is particularly possible since Bonder et al (2004) have recently reported that there exists a possible synergy in toxicity to DRG neuronal cell cultures by gp120 and another NRTI, d4T. In addition, a recent study has also provided evidence for the injury to brain mitochondria from patients taking NRTI's (Schweinsburg et al. 2005) and that NRTI-oxidative stress-induced DNA damage through inhibition of repair mechanism could lead to oxidative stress-induced apoptosis in neurons (Hashiguchi et al. 2004; Harrison et al. 2005). Therefore, evidence provided in the present study suggests that ddC could possibly contribute to the mechanisms involved in HIVD through induction of oxidative stress, reduction in anti-apoptotic protein Bcl-2, release of cytochrome c and increase in caspase 3 and bax proteins making this study the first to establish the possible involvement of NRTIs in HIV related dementia.

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Figure 1.

Protein oxidation and lipid peroxidation as indexed by protein carbonyls, 3NT and HNE in ddC treated synaptosomes. There is an increase in the levels of protein carbonyls (Fig 1a) and 3NT (Fig 1b) on synaptosomes treated with 40nM ddC. The levels of protein carbonyls were comparable to those of synaptosomes treated with the positive control of the hydroxyl radical generating mixture of Fe²⁺/H₂O₂. Fig 1c shows the levels of lipid peroxidation as indexed by HNE; there was no significant difference among the treatments. Results here are given as the mean $\pm$ S.E.M. expressed as percentage of control values. (*) $p < 0.05$ $n=5$.

Figure 2.

Protein oxidation and lipid peroxidation as indexed by protein carbonyls and HNE respectively on ddC treatment of isolated mitochondria. There is an increase in the levels of protein carbonyls (Fig 2a) on mitochondria treated with 40nM ddC. Fig 2b shows the levels of lipid peroxidation as indexed by HNE; there was no significant difference among the treatments. Results here are given as the mean $\pm$ S.E.M. expressed as percentage of control values. (*) $p < 0.05$ $n=6$.

Figure 3.

The levels of protein oxidation as indexed by protein carbonyl in mitochondria isolated from brain of gerbils injected with saline or D609. The levels of protein carbonyls were found to be significantly increased in mitochondria isolated from brain of saline- injected gerbils and treated with ddC compared to control. Mitochondria isolated from brain of D609-injected gerbils and treated with 52nM of ddC showed a significant decrease in the levels of protein carbonyl levels as compared to mitochondria isolated from brain of saline-injected gerbils brain and subsequently treated with 40 nM ddC. Results here are presented as the mean $\pm$ S.E.M. expressed as percentage of control values. (*) $p < 0.05$, $n=6$. D609 concentration used was 50 μ M, chosen based on prior studies of this agent (Perluigi et al., 2006)

Figure 4.

The level of cytochrome-c release from mitochondria isolated from gerbil brain and treated with different concentrations of ddC. There was a significant release in cytochrome-c when 40 nM ddC was added to isolated mitochondria. Data here are presented as the mean $\pm$ S.E.M. expressed as percentage of control values. (*) $p < 0.05$, $n=6$.

Figure 5.

The protein levels of anti-apoptotic protein Bcl-2 in brain mitochondria isolated from gerbil brain and treated with ddC. There was a reduction in the levels of Bcl-2 in with all ddC concentrations used, but a significant reduction was observed with 52 nM ddC. Data here are presented as the mean $\pm$ S.E.M. expressed as percentage of control values. (*) $p < 0.05$, $n=6$.

Figure 6.

The protein levels of pro-apoptotic protein Caspase-3 in brain mitochondria isolated from gerbil brain and treated with ddC. There was an increase in the levels of caspase 3 in with all ddC concentrations used, with 40 nM ddC showing the highest level. Data here are presented as the expressed as percentage of control values. $n = 4$, $p < 0.05$

Figure 7.

The protein levels of pro-apoptotic protein Bax in brain mitochondria isolated from gerbil brain and treated with ddC. There was an increase in the levels of caspase 3 in with all ddC concentrations used, though there were no significant differences observed. Data here are presented as the expressed as percentage of control values. $n=4$.

Figure 8.

Protein oxidation as indexed by protein carbonyls in ddC and 3TC treated synaptosomes. There is an increase in the levels of protein carbonyls (Fig 8a) on synaptosomes treated with 40nM ddC but no significant changes are observed in 3TC treated synaptosomes. Results here are given as the mean $\pm$ S.E.M. expressed as percentage of control values. $n = 4$ (*), $p < 0.05$.