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Aciclovir protects against quinolinic-acid-induced oxidative neurotoxicity

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Abstract

AIDS-related encephalopathy, including AIDS dementia complex (ADC) and the opportunistic disease, herpes simplex encephalitis (HSE), are postulated to arise due to the release of neurotoxic products, such as quinolinic acid (QUIN), by activated microglial cells in the brain. QUIN causes a cascade of events to ocurr, which leads to the production of reactive oxygen species (ROS), these being ultimately responsible for oxidative neurotoxicity. The antiherpes antiviral aciclovir has been reported to protect against neuron loss in HSE, but the mechanism for this neuroprotection is unknown. Therefore, this study was conducted to investigate whether aciclovir has the ability to inhibit QUIN-induced lipid peroxidation in rat brain homogenates, after in-vitro and in-vivo exposure to QUIN and aciclovir. The thiobarbituric acid (TBA) assay was the method used to analyse lipid peroxidation. Rat brains were also examined histologically after in-vivo exposure to visually assess whether neuron loss was suppressed. The results show that aciclovir inhibits the QUIN-induced lipid peroxidation, in a dose-dependent manner. Furthermore, aciclovir reduced necrosis of hippocampal neurons and retained the characteristic morphology, integrity and arrangement of these cells. Thus, it appears that aciclovir has neuroprotective properties, which could possibly be useful in the treatment of AIDS-related encephalopathy.

Introduction

Patients with AIDS-related encephalopathy, including AIDS dementia complex (ADC) and herpes simplex encephalitis (HSE), develop neurological signs (Gulliford et al 1987; Lopez et al 1996). There is hence a possibility that these conditions are neurodegenerative disorders.

HIV enters the brain via infected monocyte infiltration. These monocytes differentiate into macrophages, which shed viral proteins, such as HIV-1 transactivator protein (Tat) and HIV gp120. The viral proteins activate other macrophages and microglia, causing them to release many neurotoxic products (Nath 1999; Johnston et al 2001; Lawrence & Major 2002; Pu et al 2003), including cytokines, quinolinic acid (QUIN) and glutamate (Flavin et al 1997; Streit et al 1999). Previous studies have shown a correlation between increased cerebrospinal fluid QUIN levels and the presence of ADC (Heyes et al 1991; Št'astný et al 2003; Valle et al 2004), thus indicating that increased QUIN levels play a role in ADC.

Macrophage and microglial activation also has a role to play in the pathogenesis of HSE, a viral infection of the nervous system (Zielasek & Hartung 1999). Reinhard (1998) reported large increases in QUIN levels in the CNS of mice infected with the herpes virus. These levels were coincident with neurological signs.

QUIN is an endogenous tryptophan metabolite formed via the kynurenine pathway (Pérez-Severiano et al 2004). It has a role to play in the activation of glutamatergic NMDA receptors, increasing cystolic Ca^{2+} levels and, therefore, production of reactive oxygen species (ROS). ROS have the potential to lead to neuronal cell death (Lipton 1998; ChinLim et al 2003) because of their oxidizing action, which destroys membrane lipids, proteins and DNA (Alexia et al 2000). The brain is especially susceptible to the formation of ROS due to its high consumption of total body oxygen and relatively low concentrations of antioxidant enzymes (Coyle &

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Puttfarcken 1993). Brain tissue of patients with ADC showed evidence of lipid peroxidation after immunostaining (Turchan et al 2003). Neuronal lipid peroxidation following HSE has not been reported. However, it is likely to occur since QUIN, a well-known lipid peroxidant, is produced.

Since both herpes simplex virus type 1 (HSV-1) and HIV infection have the potential to induce neuronal loss in the CNS, by the actions of QUIN, neuroprotective strategies are required in the treatment of AIDS-related encephalopathy, to block the mentioned mechanisms of neurotoxicity (Clifford 2002).

Aciclovir is a nucleoside analogue antiviral drug that has proven efficacy in the treatment of HSE (Richards et al 1983). Aciclovir is known to achieve significant levels in the brain (O'Brien & Campoli-Richards 1989). By virtue of its antiviral mode of action, aciclovir currently has an important place in the drug regimen of AIDS patients with HSE as a secondary infection. When aciclovir was introduced after HSV-1 infection of hippocampal slice cultures of rat pups, not only was there the expected inhibition of HSV-1 replication, but protection from neuronal loss was also observed (Chen et al 2004). The mechanism for this protection was unclear. Thus the aim of this study was to investigate whether aciclovir has the ability to protect against QUIN-induced oxidative neurotoxicity and therefore has a possible role to play in the prevention or treatment of AIDS-related encephalopathy.

Materials and Methods

Chemical and reagents

The sodium salt of aciclovir (aciclovir for injection) was donated by Aspen Pharmacare (Port Elizabeth, South Africa). QUIN, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (98%) and butylated hydroxytoulene (BHT) were purchased from Sigma (St Louis, MO, USA). Trichloroacetic acid (TCA), butanol, glacial acetic acid and diethyl ether were purchased from Saarchem (PTY) (Krugersdorp, South Africa). Cresyl violet stain was purchased from BDH Laboratory supplies and distrene plasticizer in xylene (DPX) was purchased from Philip Harris.

Animals

Three-week-old adult male albino Wistar rats, 250–300 g, were used. The rats were housed in a controlled environment with a 12-h light–dark cycle, and were given free access to standard laboratory food and water. The experiments were approved by the Rhodes University Animal Ethics Committee.

Instrumentation

Samples were analysed on a Shimadzu UV-160A UV–visible recording spectrophotometer.

Sample preparation

For the in-vitro experiments, both QUIN and aciclovir were dissolved in Milli-Q water.

For the in-vivo experiments, QUIN was dissolved in phosphate-buffered saline (PBS) pH 7.4 and aciclovir was dissolved in saline solution.

In-vitro experiments

Five rats were sacrificed by cervical dislocation. The whole brain of each rat was removed and the hippocampi dissected out. The hippocampal homogenates were prepared for the lipid peroxidation assay.

In-vivo experiments

The rats were divided into three groups of five each (Table 1). On day one, all the rats were anaesthetized with diethyl ether (100%) by placing the rats in a desiccator jar containing diethyl ether until the rats were unconscious. Thereafter the rats were placed in a stereotaxic apparatus. Stereotaxic co-ordinates derived from lambda and according to the atlas of König & Klippel (1963) were 2.58 mm anterior, 3.5 mm lateral and 6.5 mm ventral of the dura.

Two microlitres of PBS was injected bilaterally into the hippocampi of the rats in group 1. The rats in groups 2 and 3 were injected with 240 nmol (2 μL of 120 nmol μL^{-1}) of QUIN instead of PBS.

The rats in group 3 were injected intraperitoneally with a daily dose of 10 mg kg^{-1} (0.25 mL) aciclovir, for four days following the injection of QUIN. For these four days, the rats in groups 1 and 2 received daily doses of 0.25 mL saline solution instead.

On the fifth day after the intra-hippocampal injections, all the rats were sacrificed by cervical dislocation. Only the hippocampi were removed and brain homogenates were prepared for the lipid peroxidation assay.

Homogenate preparation

The tissue was homogenized with 0.1 M PBS, pH 7.4, to give a final concentration of 10% w/v. This was necessary to prevent lysosomal damage of the tissues.

Table 1 Treatment regime for each group of rats

Treatment group	Intrahippocampal injection	Daily treatment (intraperitoneal injection) for 4 days after stereotaxic surgery
1 (control)	2 μL PBS	0.25 mL saline
2 (AC (−))	240 nmol QUIN	0.25 mL saline
3 (AC (+))	240 nmol QUIN	0.25 mL 10 mg kg^{-1} AC

AC, aciclovir; QUIN, quinolinic acid; PBS, phosphate-buffered saline.

Lipid peroxidation assay

Lipid peroxidation was determined using the TBA assay. This assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with TBA to yield a pink complex. A modified method of Ottino & Duncan (1997) was used in this assay.

To assess the effect of QUIN on lipid peroxidatoin in rat hippocampal homogenates in-vitro, homogenate (1 mL) containing varying concentrations of QUIN (0–1.5 mM) was incubated in an oscillating water bath for 1 h at 37°C. At the end of the incubation period, 0.5 mL BHT (0.5 g L^{-1} in absolute ethanol) and 1 mL TCA (25% in water) were added to the mixture. All samples were heated for 15 min at 95°C to release protein-bound MDA. The effect of aciclovir on QUIN-induced lipid peroxidation in rat brain homogenates in-vitro was then assessed by following the same procedure, but using homogenate (1 mL) containing 1 mM QUIN in the absence and presence of aciclovir (0, 0.25, 0.5, 0.75, 1 and 2 mM) instead. QUIN and aciclovir were absent in the control.

For the in-vivo experiments, homogenate (1 mL) in combination with 0.5 mL BHT and 1 mL TCA was incubated for 15 min at 95°C to release protein-bound MDA.

In all the experiments, both in-vitro and in-vivo, centrifugation at 2000 g for 20 min followed, to remove the insoluble protein. Then, 2 mL of protein-free supernatant was removed from each sample and 0.5 mL TBA (0.33% in water) was added to this fraction. The tubes were then incubated for 1 h at 95 °C. After rapid cooling on ice, the TBA–MDA complexes were extracted with 2 mL butanol. The absorbance was read at 532 nm and MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. The final results were expressed as nmol MDA (mg tissue)$^{-1}$.

Histological analysis

The same protocol as for the in-vivo experiments was followed. However, on the fifth day, when the rats were sacrificed, the hippocampi were removed and fixed in 10% formaldehyde for 48 h before embedding in paraffin wax.

The tissue was then processed and sectioned (10 μm thick) using a rotary microtome. The sections were Nissl stained using cresyl violet. The slides were then mounted with DPX and viewed under the light microscope and photographed using a digital camera.

Statistical analysis

The results of the lipid peroxidation assays were analysed using a one-way analysis of variance. If the P values were significant, the Student–Keuls test was used to compare the treated and control groups. The level of significance was accepted at $P < 0.05$ (Zar 1974).

Results

It is evident (Table 2) that the co-incubation of rat brain homogenate with increasing concentrations of QUIN (0.25–1.5 mM) caused a significant concentration-dependent increase in MDA level in the brain homogenate in comparison with the control value. There were no significant differences between the MDA level generated by 1 mM QUIN and that by 1.5 mM QUIN (Figure 1). Thus 1 mM QUIN was chosen for subsequent studies since it yielded the highest amount of MDA.

Table 2 The concentration-dependent effect of quinolinic acid on lipid peroxidation generation in rat hippocampal homogenates in-vitro

QUIN concn (mM)	MDA (nmol (mg tissue)$^{-1}$)
Control	0.0206 ± 0.00168
0.25	0.0086 ± 0.0064*
0.5	0.0115 ± 0.011**
1	0.1513 ± 0.0095***
1.5	0.1914 ± 0.0099***

QUIN, quinolinic acid; MDA, malondialdehyde. Data are means ± s.e.m., n = 5. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control, Student–Newman–Keuls Multiple Range Test.

Furthermore, aciclovir had a suppressive effect on the 1 mM QUIN-induced lipid peroxidation in rat brain homogenate (Table 3). The response proceeded in a dose-dependent manner and the suppression of oxidatively damaged lipid products was highly significant for each concentration chosen. The aciclovir concentrations of 0.25 mM and higher appeared to significantly reduce levels of MDA in comparison with QUIN treatment only. In addition, there appeared to be no significant difference between the protection offered by the 2 mM concentration of aciclovir in comparison with the control value.

The intrahippocampal injection of QUIN (240 nmol) caused a significant increase ($P < 0.001$) in MDA levels – 0.096 nmol (mg tissue)$^{-1}$ (s.e.m. ± 0.0053) in comparison with the control treated rats (0.028 nmol (mg tissue)$^{-1}$; s.e.m. ± 0.0037). However, the daily intraperitoneal injections of aciclovir for 4 days was able to significantly reduce the QUIN-induced MDA production (0.055 nmol (mg tissue)$^{-1}$; s.e.m. ± 0.0053) by ± 40% ($P < 0.001$).

From the photomicrographs shown in Figure 1, it is evident that the CA1 and CA3 regions of the hippocampi of the control rats (Figure 1A, B) show optimally sized, pyramid-shaped neuronal cells with a clearly observable cell nucleus and continuous cell membrane. The cells are grouped closely together to form a band-like appearance, which is characteristic of both the CA1 and CA3 regions. Thus the neurons in both the CA1 and CA3 region appear to be undamaged. The neurons and nuclei were stained an intense purple colour, while the background appeared light-purple to a pink colour. The same regions of the hippcampi of the rats treated with 240 nmol QUIN are shown in Figures 1C and 1D. The CA1 and CA3 neurons exhibit extensive degeneration by virtue of their roundness and swelling. The cells appear scattered with little integrity of cell membrane and appearance of dense nuclei. Necrosis of the neuronal cells in many areas is also evident. Figures 1E and 1F show that the neurons of the CA1

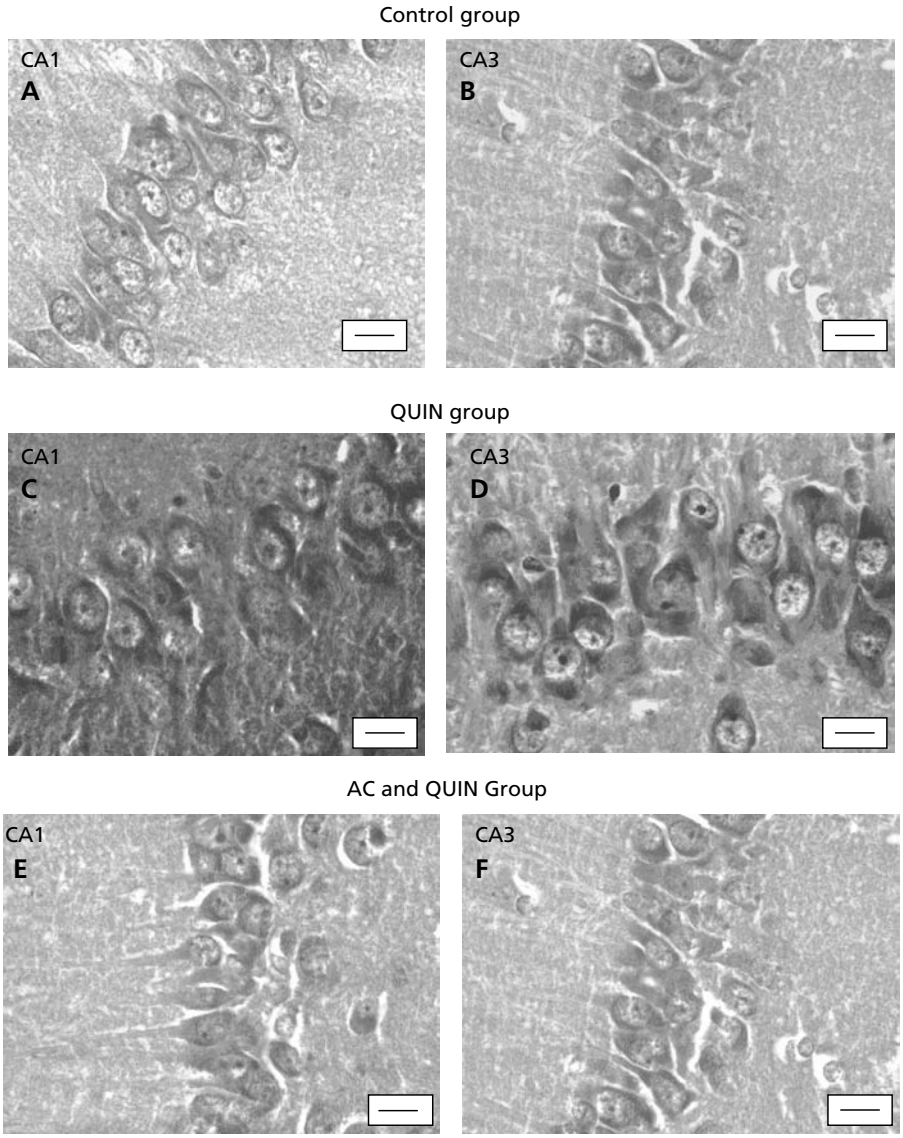

Figure 1 Quinolinic acid (QUIN) toxicity and the protective effects of aciclovir (AC) on rat hippocampal neurons. A, B. CA1 and CA3 regions of the control-treated rats, respectively. C, D. QUIN-treated CA1 and CA3 regions, respectively. E, F. Micrographs of the CA1 and CA3 regions of the QUIN plus AC-treated rats. Bar = 10 μm.

and CA3 regions of the rats co-treated with QUIN and aciclovir appear to have retained their pyramidal appearance and are not as inflamed as those present in the hippocampus of the rats treated with QUIN alone. The cells show better orientation, integrity of cell membrane and appear closer to each other as though in an attempt to reconstruct their typical band-like appearance.

Discussion

QUIN is an endogenous glutamate agonist with relative selectivity for NMDA receptors (Garthwaite & Garthwaite 1987). High concentrations of these receptors are found on the neurons of the CA1 region of the hippocampus (Monaghan & Cotman 1986). Since QUIN is not readily metabolized in the synaptic cleft, it stimulates the NMDA receptor for prolonged periods (Belle' et al 2004) and stimulation of these receptors allows Ca^{2+} and Na^{+} to enter the neurons (Choi 1987; Hopkins & Oster-Granite 1998). Large increases in intracellular Ca^{2+} may cause mitochondrial dysfunction, depressing oxidative phosphorylation (Dawson et al 1995), resulting in local free radical formation in the mitochondria of the neurons (Gunter et al 1993). Lipid peroxidation is a process whereby cell membranes are destroyed by oxidative attack from these free radicals (Mylonas & Kouretas 1999). Since the structure and integrity of the cell membrane is essential for the functioning of a cell, it is expected that lipid peroxidation in the brain would lead to neuronal cell death. This explains the increased MDA production, a

Table 3 The effect of different concentrations of aciclovir on 1 mM quinolinic-acid-induced lipid peroxidation in whole rat brain homogenates in-vitro

Concn of aciclovir (mM) + 1 mM QUIN	MDA (nmol (mg tissue)$^{-1}$
Control	0.0206 ± 0.00168
0.00	0.1513 ± 0.0095$^{@}$
0.25	0.0953 ± 0.0092**
0.50	0.07 ± 0.011***
0.75	0.0441 ± 0.013***
1.00	0.0545 ± 0.0123***
2.00	0.0185 ± 0.0136***

QUIN, quinolinic acid; MDA, malondialdeyde. Data are means ± s.e.m., n = 5. $^{@}P < 0.001$ QUIN vs control; $^{**}P < 0.01$ 0.25 mM aciclovir vs QUIN; $^{***}P < 0.001$ 0.5 mM, 0.75 mM, 1 mM and 2 mM aciclovir vs QUIN. Student–Newman–Keuls Multiple Range Test.

by-product of lipid peroxidation (Erba et al 2003) with use of QUIN alone in-vitro and in-vivo (Table 3).

Furthermore, the rapid influx of sodium ions into the neuron causes a passive entry of chloride ions and water into the cell through osmotic pressure, which results in the rapid swelling of the neurons due to the excessive depolarization, ion influx and water entry (Tilson & Mundy 1995). Together with lipid peroxidation, this can lead to loss of the integrity of cell membranes and ultimately cell necrosis. This is illustrated in Figures 1C and 1D. Thus it is postulated that aciclovir protects against lipid peroxidation and necrotic cell damage induced by QUIN by acting as a free radical scavenger.

Aciclovir demonstrated neuroprotection, shown by the lower levels of MDA produced in the presence of QUIN in both the in-vitro and in-vivo experiments and by its ability to reduce necrosis of hippocampal neurons and retain the characteristic morphology, integrity and arrangement of these cells.

Conclusion

The results of this study are the first to demonstrate that aciclovir reduces QUIN-induced oxidative neurotoxicity. Hence, apart from its antiviral activity, aciclovir also appears to exhibit neuroprotective properties. Since QUIN has a role to play in the neurodegeneration, which results in AIDS related encephalopathy, aciclovir is likely to provide effective neuroprotection to reduce the extent of neuronal damage in this disorder, improving the quality of life and extending the lifespan of such patients. Further studies are required to see if other antiviral analogues of aciclovir exhibit a similar neuroprotective effect.

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