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Incorporation of sodium channel blocking and free radical scavenging activities into a single drug, AM-36, results in profound inhibition of neuronal apoptosis

*,[1]Jennifer K. Callaway, [1]Philip M. Beart, [1]Bevyn Jarrott & [1]Sarah F. Giardina

[1]Department of Pharmacology, Monash University, Victoria, 3800, Australia

1 AM-36 is a novel neuroprotective agent incorporating both antioxidant and Na^+ channel blocking actions. In cerebral ischaemia, loss of cellular ion homeostasis due to Na^+ channel activation, together with increased reactive oxygen species (ROS) production, are thought to contribute to neuronal death. Since neuronal death in the penumbra of the ischaemic lesion is suggested to occur by apoptosis, we investigated the ability of AM-36, antioxidants and Na^+ channel antagonists to inhibit toxicity induced by the neurotoxin, veratridine in cultured cerebellar granule cells (CGC's).

2 Veratridine (10–300 μM) concentration-dependently reduced cell viability of cultured CGC's. Under the experimental conditions employed, cell death induced by veratridine (100 μM) possessed the characteristics of apoptosis as assessed by morphology, TUNEL staining and DNA laddering on agarose gels.

3 Neurotoxicity and apoptosis induced by veratridine (100 μM) were inhibited to a maximum of 50% by the antioxidants, U74500A (0.1–10 μM) and U83836E (0.03–10 μM), and to a maximum of 30% by the Na^+ channel blocker, dibucaine (0.1–100 μM). In contrast, AM-36 (0.01–10 μM) completely inhibited veratridine-induced toxicity (IC_{50} 1.7 (1.5–1.9) μM, 95% confidence intervals (CI) in parentheses) and concentration-dependently inhibited apoptosis.

4 These findings suggest veratridine-induced toxicity and apoptosis are partially mediated by generation of ROS. AM-36, which combines both Na^+ channel blocking and antioxidant activity, provided superior neuroprotection compared with agents possessing only one of these actions. This bifunctional profile of activity may underlie the potent neuroprotective effects of AM-36 recently found in a stroke model in conscious rats.

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Abbreviations: AM-36, 1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3, 5-bis(1, 1-dimethyl)-4-hydroxyphenyl)methylpiperazine; CGCs, cerebellar granule cells; CI, confidence intervals; HBSS, Hank's balanced salt solution; MTT, 3-(4,5-diethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TTX, tetrodotoxin; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin (DIG) nick end labelling; U74500A, 21-[4-[5,6-bis (diethylamino)-2-pyridinyl]-1-piperazinyl]-16-methyl-pregna-1,4,9(11)-triene-3,20-dione, hydrochloride; U83836E, (−)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol, dihydrochloride

Introduction

Alterations in cellular ion homeostasis due to loss of ATP levels, and a state of oxidative stress resulting in the accumulation of reactive oxygen species (ROS), are thought to contribute to neuronal damage in cerebral ischaemia (Dirnagl *et al*., 1999; Kuroda *et al*., 1997). Loss of ATP results in a drop in membrane potential to the point where voltage-dependent Na^+ channels open, causing Na^+ influx, continual depolarization and initiation of a cascade of cellular events (Stys *et al*., 1992). Down-regulation of Na^+ channels has been proposed as a potential way of reducing energy expenditure in cerebral ischaemia since much of the energy required by excitable cells is used to maintain Na^+ and K^+ gradients across the cellular membrane (Carter, 1998; Urenjak *et al*., 1996). ROS, which are well known to induce lipid peroxidation, DNA damage and alterations in enzyme activation, contribute substantially to neuronal death in cerebral ischaemia (Dirnagl *et al*., 1999; Kuroda *et al*., 1997). Both Na^+ channel antagonists and free radical scavengers individually have been shown to reduce neuronal damage after cerebral ischaemia in experimental animals (Callaway *et al*., 1999; Rataud *et al*., 1994; Smith *et al*., 1996; Takamatsu *et al*., 1998; Umemura *et al*., 1994; Zhao *et al*., 1994). The importance of the role of free radicals is exemplified by the fact that even delayed treatment with antioxidants can be effective in reducing neuronal damage (Callaway *et al*., 1999; Huh *et al*., 2000; Takamatsu *et al*., 1998; Zhao *et al*., 1994).

Previous studies *in vivo* have localized apoptotic cells to the outer border of the ischaemic core indicating that progression

*Author for correspondence at: Department of Pharmacology, P.O. Box 13E, Monash University, Victoria 3800, Australia
E-mail: Jennifer.Callaway@med.monash.edu.au

of the ischaemic lesion and the ensuing neuronal death involve apoptotic mechanisms (Chopp *et al*., 1996; Du *et al*., 1996; Guegan *et al*., 1998; Linnik *et al*., 1995). ROS have been suggested to play a central role in the induction of apoptosis following exposure to a range of cytotoxic insults (Dirnagl *et al*., 1999; Jabs, 1999; Leist *et al*., 1998; Stoian *et al*., 1996). Apoptosis is induced by exposure to ROS in a variety of cell types (Bonfoco *et al*., 1995; Hanson *et al*., 1996; Hockenbery *et al*., 1993; Jacobson *et al*., 1995; McGowan *et al*., 1998; Stoian *et al*., 1996), is associated with oxidative stress induced by diverse cytotoxic insults (Devitt *et al*., 1999; Jabs, 1999; Leist *et al*., 1998) and is inhibited by antioxidants both *in vitro* and *in vivo* (Devitt *et al*., 1999; Hirose *et al*., 1993; Malorni *et al*., 1993; Melchiorri *et al*., 1998; Slater *et al*., 1995).

AM-36 has recently been shown to have potent neuroprotective activity in a model of focal forebrain ischaemia by an endothelin-1 induced occlusion of the middle cerebral artery in conscious rats (Callaway *et al*., 1999). AM-36 is a novel arylalkylpiperazine with combined antioxidant and Na^+ channel blocking activity within the one compound (Jarrott *et al*., 1997; 1999). The activity of AM-36 has been tested in *in vitro* assays where it was found to inhibit lipid peroxidation in the thiobarbituric acid reacting substances assay with activity (IC_{50} 48 (39–64) μM, 95% CI in parentheses) comparable to that of known antioxidants such as 3,5 tert-butyl-4-hydroxytoluene (BHT), U74500A and U83836E (Callaway *et al*., 1998; Jarrott *et al*., 1999). In binding assays, AM-36 inhibits ^{3}H-batrachatoxinin binding to site 2 of Na^+ channels in rat brain homogenates with a IC_{50} of 0.28 ± 0.14 μM ($\pm$s.e.mean, unpublished data).

Persistent activation of voltage-dependent Na^+ channels with veratridine causes Na^+ influx and ionic imbalance, and is known to induce neurotoxicity in cultured cerebellar granule cells (CGCs) (Dargent *et al*., 1996). We have found that veratridine also induces apoptosis and using this model system, we have evaluated the ability of AM-36 to inhibit veratridine-induced toxicity and apoptosis in murine cultured CGCs. The activity of AM-36 was compared with the antioxidants, U74500A and U83836E, and the Na^+ channel antagonists, tetrodotoxin (TTX) and dibucaine. We suggest the unique pharmacological profile of activity of AM-36, incorporating both Na^+ channel blocking and free radical scavenging, contributes to the current findings of complete inhibition of apoptosis. These properties could contribute to the potent neuroprotection recently observed in a model of transient focal cerebral ischaemia (Callaway *et al*., 1999) and suggest AM-36 would be beneficial for the management of acute stroke in humans.

Methods

Primary cell culture

All animal experimentation was performed according to the code of ethics approved by the National Health and Medical Research Council (Australia). Primary cultures of CGCs were prepared from 7-day-old Swiss-White mice as previously described (Cheung *et al*., 1998a). The cerebella were dissected and maintained in Hank's balanced salt solution (HBSS; pH 7.4) containing 3 mg ml^{-1} BSA and 1.2 mM $MgSO_4$ and digested at 37°C with trypsin (0.2 mg ml^{-1}), DNase (80 μg ml^{-1}) and $MgSO_4$ (1.2 mM) in HBSS for 30 min in a shaking water bath. Digestion was terminated by the addition of trypsin inhibitor (80 μg ml^{-1}) and DNase (80 μg ml^{-1}) in HBSS, and the cells were mechanically dissociated by trituration (10–15 strokes). The cell suspension was removed and resuspended in Neurobasal™ medium containing B27 components (Vanderbilt *et al*., 1982), 25.4 mM KCl, 100 u ml^{-1} penicillin-streptomycin and 500 μM L-glutamine. Cells were seeded in 24-well plates (Nunc) previously coated with poly-D-lysine (50 μg ml^{-1}) to give a final density of 0.4×10^6 cells cm^{-2}. Cultures were plated in medium containing 10% dialyzed foetal calf serum for 24 h on day 0 *in vitro* and were subsequently maintained in serum-free medium. Aphidicolin (2 μg ml^{-1}) was added to the medium 18–24 h after plating to inhibit non-neuronal cell proliferation (Miller *et al*., 1996). Previous studies have shown that these cultures contain greater than 95% neurons (Cheung *et al*., 1998a).

Veratridine exposure

Cultures were exposed to veratridine (1–300 μM) on day 8 *in vitro*. Stimulation was carried out for 1 h in N2-supplemented Neurobasal™ medium containing 100 u ml^{-1} penicillin-streptomycin, 0.25% bovine serum albumin, 83 μM D(+)-galactose, 16 μM ethanolamine, 6 μM L-carnitine, 0.4 μM biotin, 500 μM L-glutamine and 25.4 mM KCl (Bottenstein *et al*., 1979). Cultures were then washed and left for 16–18 h in N2-supplemented Neurobasal™ medium. A concentration of veratridine (100 μM), producing approximately 75–80% cell injury was chosen for all further exposure studies. Control sister cultures underwent similar treatment, but veratridine exposure was omitted. Cells were pre-incubated with AM-36 (0.01–10 μM), U74500A (0.1–10 μM), U83836E (0.03–10 μM), dibucaine (0.1–100 μM), or TTX (0.01–30 μM) for 30 min prior to a 60 min exposure to veratridine (100 μM).

Cell viability assays

Cell viability was examined 16–18 h after drug stimulation by phase-contrast microscopy and by the measurement of formazan production by the reduction of 3-(4,5-diethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells (Mosmann, 1983). The procedure was carried out as described previously (Cheung *et al*., 1998a). Briefly, MTT (5 mg ml^{-1}) was prepared in RPMI 1640 growth medium and was incubated with cells at 37°C for 30 min. This solution was then removed and replaced with 20% sodium dodecyl sulphate solution (SDS) in 40% dimethylformamide, and solubilized overnight at room temperature. The absorbance of samples was read using a spectrophotometer (Ceres UV900C) at a wavelength of 570 nm. Results were expressed as a percentage of vehicle treatment in control sister cultures. Cells were visualized under bright field microscopy and photographs were taken of random fields.

In situ *labelling of DNA fragmentation*

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin (DIG) nick end labelling (TUNEL) was carried out as previously described (Cheung *et al*., 1998a). Briefly, cells were fixed in 4% paraformaldehyde overnight at 4°C and

permeabilized in 2% Triton X-100 in Tris buffered saline. Cultures were then exposed to TdT reaction mixture (34 u ml^{-1} TdT, 280 pmol dATP, 90 pmol digoxigenin (DIG) labelled d-UTP was detected using 75 mu ml^{-1} anti-DIG-alkaline phosphatase (AP), followed by exposure to AP substrate solution (60 mM 4-nitro blue tetrazolium chloride and 170 nM 5-bromo-4-chloro-3-indolylphosphate-4-toluidine salt). Cells were visualized under bright field microscopy, photographed and cell counts were conducted from random fields.

DNA fragmentation on agarose gels

DNA fragmentation was carried out according to a previously published method (Cheung *et al*., 1998a). Cells were lysed in Tris-EDTA buffer (5 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, pH 8.0) and digested with proteinase K (100 μg ml^{-1}) overnight at 37°C. DNase free RN (5 μg ml^{-1}) was then used for 1 h at 37°C to break down any RNA present in the sample. DNA was extracted from approximately 2×10^6 cells with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) followed by one volume of chloroform, precipitated with a 1 : 10 dilution of 7.5 M ammonium acetate and two volumes of ethanol at −20°C overnight. This solution was then centrifuged at $12{,}000 \times g$ at 4°C for 30 min. The pellet was then washed in 100% ice-cold ethanol, air dried and resuspended in Milli Q water. DNA concentration was determined at 260–280 nm (Gene Quant II, Pharmacia Biotech, U.K.). DNA samples (1–2 μg) were loaded onto 1.5% agarose gels containing 1 μg ml^{-1} ethidium bromide and run at 80 V for 2 h before being visualized under u.v. light.

Materials

AM-36 [1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis(1,1-dimethyl)-4-hydroxyphenyl)methylpiperazine] was designed and synthesized as described (Jarrott *et al*., 1997; 1999). Neurobasal™ medium, TdT, B27 and N2 supplements were purchased from GibcoBRL Life technologies (Melbourne, Australia). U74500A and U83836E were kindly donated by (Pharmacia and Upjohn, Inc., Kalamazoo, U.S.A.). All other reagents were purchased from Sigma and were cell culture or molecular biology grade.

Data analysis

All data were expressed as mean ± s.e.mean. Statistical significance was determined by one- and two-way ANOVA and appropriate *post-hoc* tests performed on original data. Data was standardized relative to veratridine exposure and expressed as a percentage of vehicle control treated sister cultures. Concentration-response curves and IC_{50} values were generated using the computer-assisted curve fitting program GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.).

Results

AM-36 and blockade of veratridine neurotoxicity

Exposure of CGCs to veratridine for 60 min caused a concentration-dependent cell death as assessed by MTT assay 18 h after exposure (Figure 1A). As shown by phase contrast photomicroscopy (Figure 2A,B) 18 h after veratridine exposure, cells showed morphological characteristics of apoptosis, including cell shrinkage and neurite blebbing relative to vehicle treated control cultures (Sloviter *et al*., 1996; Walkinshaw *et al*., 1993). No signs of necrotic cell death (swelling and lysis) were observed.

As determined by the MTT assay, veratridine (100 μM) induced toxicity was inhibited by a maximum of 80.3 ± 5.6% by the specific sodium channel blocker tetrodotoxin (IC_{50} = 2.9 (0.7–11.5) μM, 95% CI in parentheses, Figure 1B). Treatment with the antioxidant compounds U74500A or U83836E resulted in only partial inhibition of veratridine-induced toxicity (Figure 1C,D, IC_{50} = 0.4 (0.07–2.6) and 1.0 (0.5–2.0) μM, respectively, 95% CI in parentheses). The Na^+ channel blocker dibucaine inhibited toxicity by a maximum of 60.1 ± 4.1% with an IC_{50} value of 7.0 (3.0–16) μM, 95% CI in parentheses (Figure 1E). AM-36 which possesses both Na^+ channel blocking and antioxidant activity completely inhibited cell death due to veratridine exposure with an IC_{50} of 1.7 (1.5–1.9) μM, 95% CI in parentheses (Figure 1F). AM-36 concentration-dependently attenuated morphological signs of veratridine-induced neurotoxicity. Cells treated with 5 μM AM-36 appeared morphologically similar to untreated control cultures (Figure 2C).

AM-36 attenuates veratridine-induced apoptosis

Exposure of CGC's to veratridine (100 μM) resulted in approximately 95% TUNEL-positive staining compared with control sister cultures (Table 1, Figure 3A,B). AM-36 concentration-dependently reduced the percentage of apoptotic (TUNEL-positive) cells (Table 1, Figure 3C,D). TUNEL-positive staining was not significantly different from untreated control cultures after treatment with 5 μM AM-36. TUNEL-positive cell counts are summarized in Table 1. Dibucaine reduced the percentage of apoptotic nuclei produced by veratridine by approximately 30%, but did not show a concentration-dependent effect for the three concentrations tested here. Treatment with the antioxidant compound U83836E, resulted in about 50% TUNEL positive staining at the three concentrations tested relative to veratridine alone (Table 1).

DNA laddering on agarose gels allowed the visualization of internucleosomal fragmentation of DNA which is considered a hallmark of apoptosis (Vanderbilt *et al*., 1982). All samples demonstrated a clear band at >20 Kbp indicative of genomic DNA from intact cells. DNA extracted from veratridine-treated cultures demonstrated a clear increase in lower molecular weight fragments ranging from <0.9 to 20 Kbp that was not evident in control sister cultures (Figure 4, lanes 2 and 1 respectively). Treatment with 5 μM AM-36 completely abolished DNA laddering, indicative of the attenuation of apoptosis (Figure 4, lane 3).

Discussion

The key finding of the present investigation was that AM-36, which has bifunctional pharmacological activities as a Na^+ channel blocker and free radical scavenger, provided superior neuroprotective activity against neurotoxicity compared with

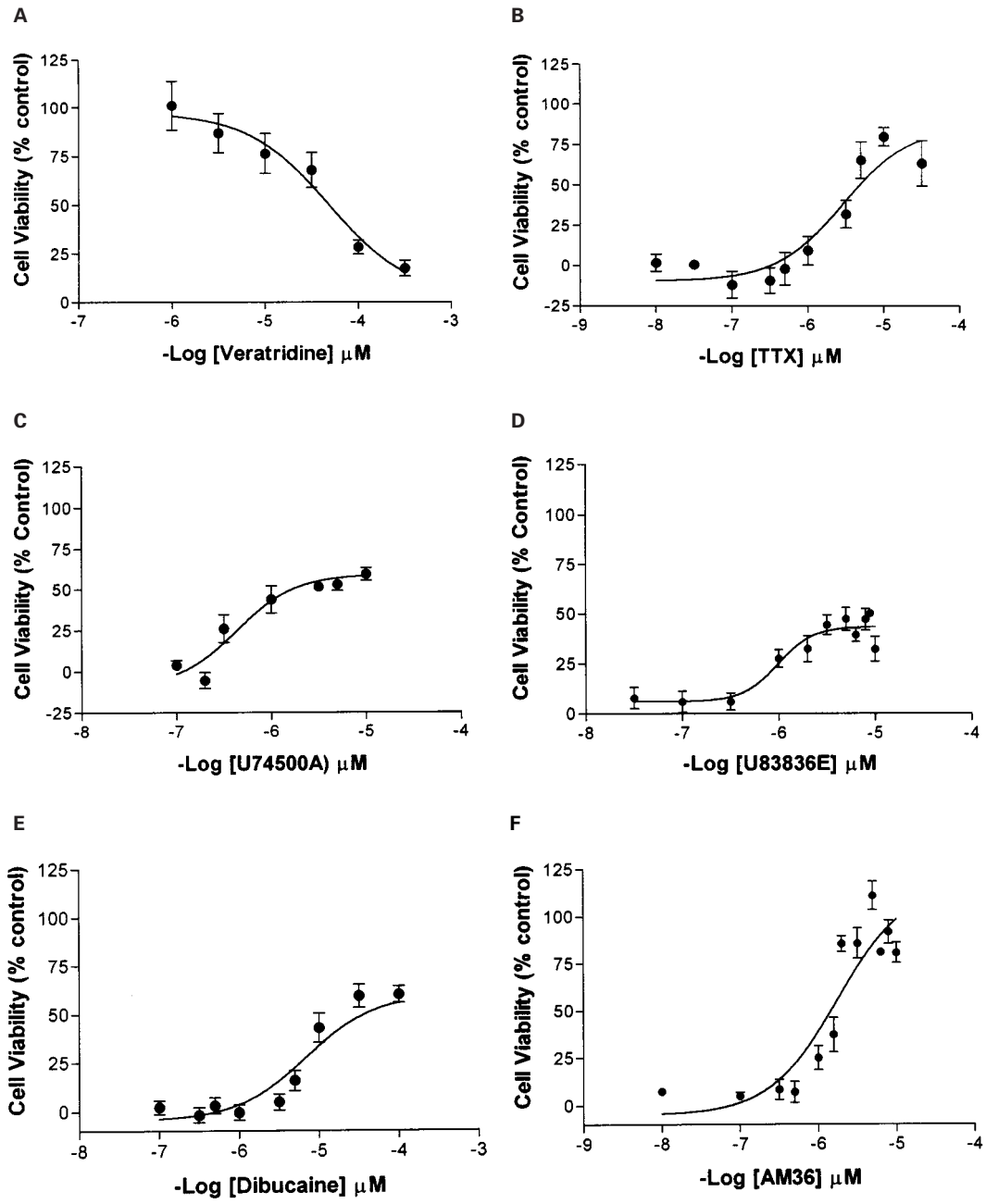

Figure 1 Concentration-dependent cytotoxicity induced by 1 h exposure to veratridine (A) in cultured CGCs as determined by MTT cell viability assay 18 h after exposure. The effects on 100 μM veratridine-induced toxicity of (B) TTX, (C) U83836E, (D) U74500A, (E) dibucaine and (F) AM-36, were determined. Each point represents 3–8 separate experiments conducted over 2–3 cultures $\pm$s.e.mean. Concentration response curves were generated using computer assisted, non-linear regression.

agents having only one of these actions. Using CGCs, we found that activation of Na^+ channels by the neurotoxin veratridine resulted in concentration-dependent cell death as previously reported (Dargent *et al.*, 1996). In our experimental paradigm, veratridine-induced cell death exhibited the characteristics of apoptosis as determined by morphology, TUNEL staining and DNA fragmentation on agarose gels. The veratridine-induced decrease in cell viability and apoptosis was partially inhibited by the antioxidant compounds U74500A and U83836E, and by the Na^+ channel antagonist, dibucaine. However, the novel compound, AM-36, which possesses both antioxidant and Na^+ channel blocking activity, completely inhibited the veratridine-induced reduction in cell viability and concentration-dependently inhibited apoptosis as measured by both TUNEL staining and DNA agarose gel electrophoresis.

Veratridine is thought to induce toxicity in cultured cells by causing persistent activation of voltage-dependent Na^+ channels, causing Na^+ influx and ionic imbalance (Catterall, 1980). However, the present findings that the Na^+ channel antagonist dibucaine only partially inhibited veratridine-induced cell viability as determined by MTT assay, and

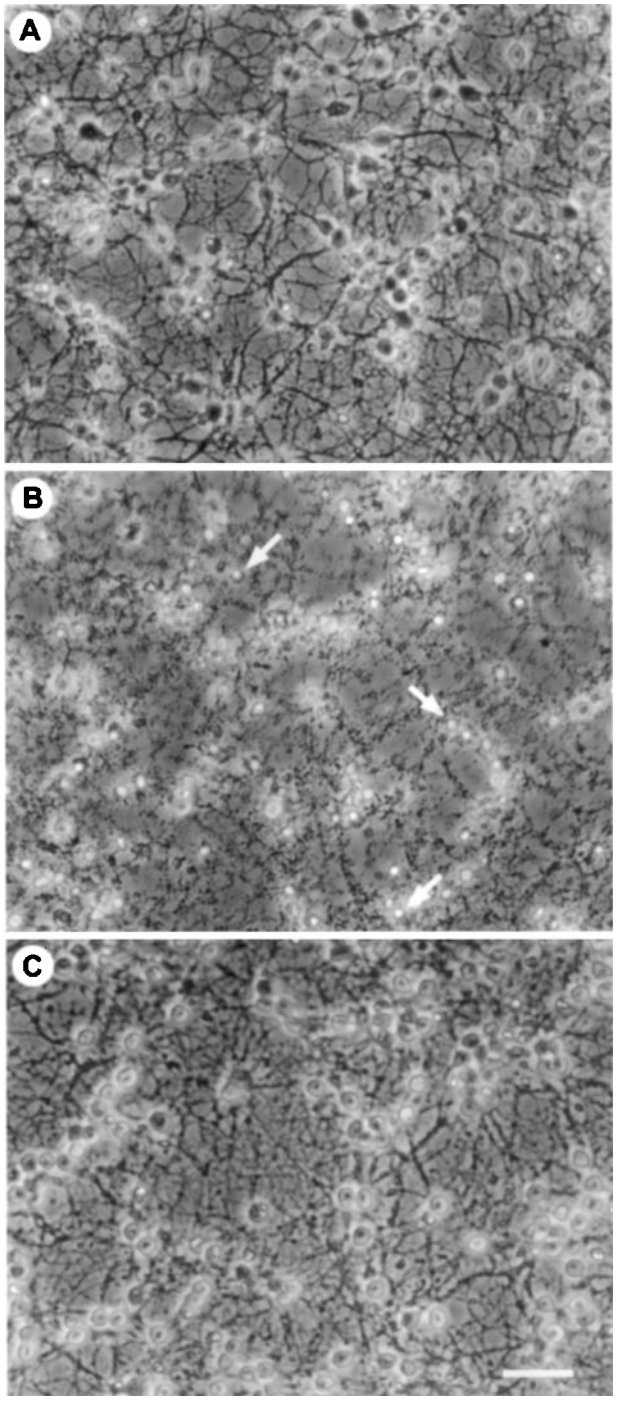

Figure 2 Phase contrast photomicrographs showing morphology of veratridine-induced neurotoxicity and its attenuation by AM-36 in cultured CGCs. Photomicrographs were taken 18 h after exposure to (A) vehicle control, (B) 100 μM veratridine, (C) 100 μM veratridine plus 5 μM AM-36. Cells exposed to veratridine showed morphological characteristics of apoptosis (arrows). Bar = 5 μm.

Table 1 Cell counts following immunocytochemical staining of neurons using the TUNEL technique

Treatment	*Concentration* (μM)	*TUNEL positive cells* (% total)
Control		26.0 ± .5
VER	100	95.6 ± 1.5
VER + AM-36	1	61.5 ± 4.9**
	3	45.4 ± 3.8**†
	5	28.2 ± 2.3**†‡
VER + dibucaine	10	75.1 ± 1.6*
	30	71.2 ± 3.8**
	100	77.8 ± 2.7*
VER + U74500A	3	59.9 ± 3.0**
	5	53.4 ± 3.8**
	8	56.0 ± 6.9**

Treatments were present 30 min prior to, and during exposure to 100 μM veratridine (VER). TUNEL staining, indicative of apoptosis, was determined 18 h after a 60 min exposure to veratridine. tunel-positive cells were counted in 2–3 representative fields from $n = 2-3$ separate experiments. The number of TUNEL-positive cells was expressed as a percentage of the total number of cells in each representative field. Data are mean ± s.e.mean. *$P < 0.05$, **$P < 0.01$ versus veratridine; †$P < 0.05$ versus 1 μM AM-36; ‡$P < 0.05$ versus 3 μM AM-36.

apoptosis as determined by TUNEL staining, suggests an additional mechanism unrelated to Na^+ channel blockade contributes to apoptosis. The ability of antioxidants to inhibit the effects of veratridine suggests the involvement of ROS in the mechanism of toxicity induced by this neurotoxin. The involvement of ROS in veratridine-induced toxicity has not previously been reported. However, ROS have been shown to mediate neuronal death caused by a range of other cytotoxic agents in cultured cells including glutamate (Dawson *et al*., 1991; Tan *et al*., 1998), kainic acid (Sun *et al*., 1998), dopamine (Offen *et al*., 1996), and serum deprivation (Satoh *et al*., 1996). In addition, a large body of experimental evidence implicates the involvement of ROS in apoptosis (Bonfoco *et al*., 1995; Jabs, 1999; McGowan *et al*., 1998; Sloviter *et al*., 1996; Sun *et al*., 1998). Antioxidants inhibit apoptotic cell death induced by a variety of different agents. Thiol antioxidants inhibit dopamine-induced apoptosis in PC12 cell cultures and depletion of cellular glutathione potentiates dopamine-induced toxicity (Offen *et al*., 1996). Phenidone, an inhibitor of lipoxygenase and cyclo-oxygenase, inhibits free radical injuries and staurosporine-induced apoptosis in mouse cortical cultures, by modulation of oxidative stress (Wie *et al*., 1999). A component of veratridine neurotoxicity is therefore likely to be mediated by production of ROS. This proposal is supported by the current finding of partial inhibition of the effects of veratridine on cell viability and apoptosis by antioxidants, and complete inhibition by AM-36 which has both Na^+ channel blocking and antioxidant activity (Callaway *et al*., 1999; Jarrott *et al*., 1999).

Veratridine-induced toxicity does not appear to be mediated by glutamate release as several studies have found no attenuation of toxicity by the glutamate antagonist MK-801 (Dargent *et al*., 1996; Diaz-Trelles *et al*., 1999; May *et al*., 1995). We too found no effect of MK-801 (0.3–100 μM) in our system (unpublished observations). Toxicity due to veratridine has been shown to be only partially related to increased intracellular Ca^{2+} (Dargent *et al*., 1996; May *et al*., 1995). The effects of AM-36 are unlikely to be related to inhibition of Ca^{2+} channels since this compound was not found to have any significant Ca^{2+} channel blocking activity (unpublished observations).

In comparison to a previous study which employed a long-term (18 h) exposure to veratridine (Urenjak *et al*., 1996), we

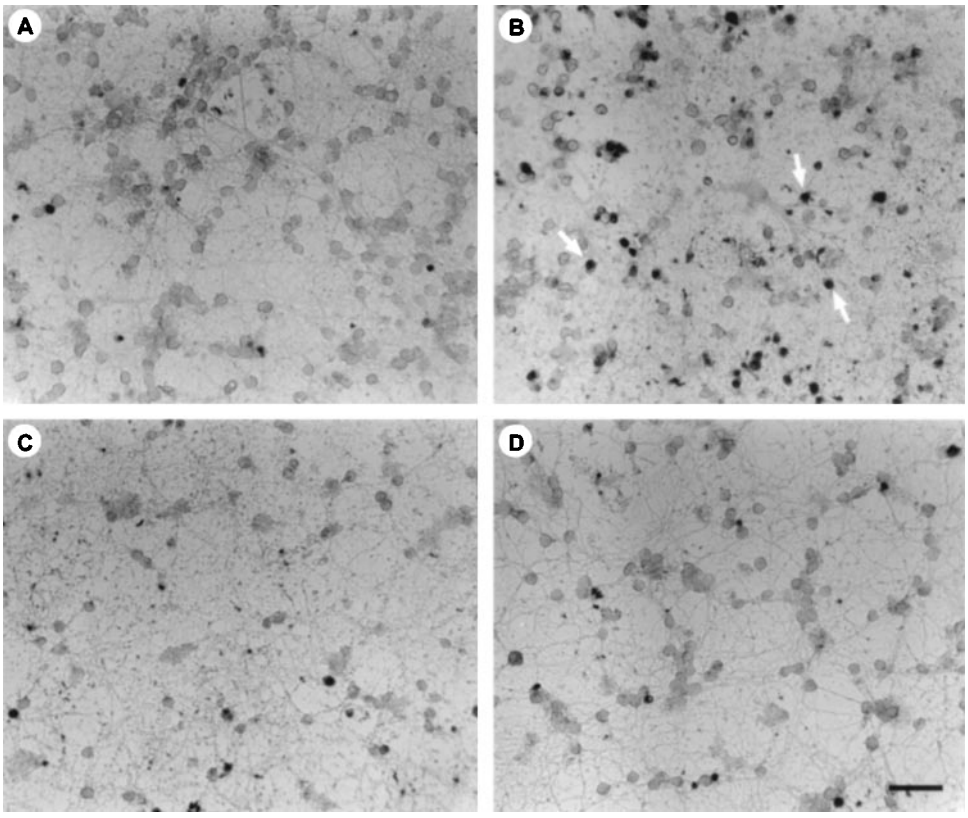

Figure 3 Immunocytochemical staining of neurons using the TUNEL technique, indicative of apoptosis. The percentage of TUNEL-positive cells was quantified by counting stained cells in 3–7 representative fields in ($n=3$ cultures). Bright field photomicrographs from representative fields indicate few TUNEL-positive neurons in untreated control cultures (A): a high degree of TUNEL positive staining after veratridine exposure; arrows (B); and marked attenuation of TUNEL positive neurons after treatment with AM-36 (3 or 5 μM, C and D, respectively). Bar = 5 μm.

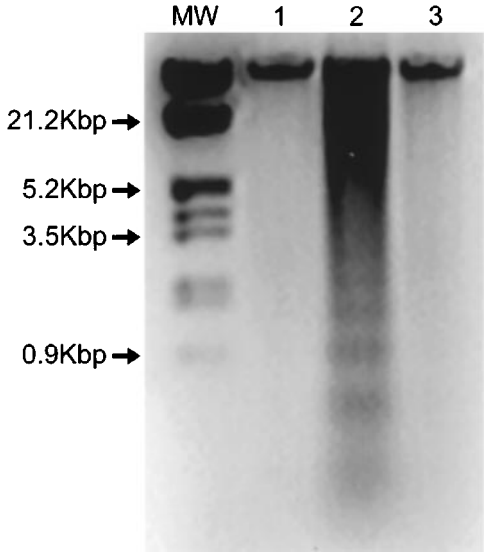

Figure 4 Representative agarose gel electrophoresis showing DNA laddering after exposure to veratridine (lane 2). DNA was extracted from cultures 18 h after 1 h veratridine (100 μM) exposure for gel electrophoresis. Control cultures (lane 1). Pre-incubation of cells with AM-36 (5 μM) for 30 min prior to and during veratridine exposure completely inhibited DNA laddering (lane 3) compared to treatment with veratridine alone (lane 2). DNA (*Hind*/*Eco*III) marker.

found that a 1 h exposure induced cell death characteristic of apoptosis. The presence of apoptotic cell death was determined by morphology, TUNEL staining and DNA laddering on agarose gel electrophoresis. Whether cell death occurs by necrosis or apoptosis, is now widely believed to depend on the intensity of the insult (Bonfoco *et al*., 1995; Cheung *et al*., 1998b; Chopp *et al*., 1996; Dirnagl *et al*., 1999; Leist *et al*., 1998). Mild insults induce apoptosis, whereas more severe insults induce necrotic cell death. In our system it is possible that a 1 h exposure to veratridine represents a 'mild' insult inducing apoptosis, whereas an 18 h exposure (Urenjak *et al*., 1996) is sufficiently severe to produce predominantly necrotic cell death. In cerebral ischaemia, neurons at the core of the occlusion, where blood flow is severely reduced, are thought to die rapidly and mainly by necrosis, whereas neurons in the surrounding penumbra, where blood flow is less severely reduced, die by apoptosis (Dirnagl *et al*., 1999). Many investigators have localized apoptotic neurons to the border of the ischaemic lesion where energy depletion and excitotoxic stimulation are less severe and prolonged (Chopp *et al*., 1996; Du *et al*., 1996; Guegan *et al*., 1998; Linnik *et al*., 1995). The ability of AM-36 to inhibit apoptosis due to persistent Na^+ channel opening may contribute to the potent neuroprotection found *in vivo* in a rat model of cerebral ischaemia (Callaway *et al*., 1999).

Pharmacological modulation of voltage-sensitive sodium channels is considered to be a rational and effective

therapeutic strategy against neuronal damage in cerebral ischaemia (Carter, 1998; Rataud *et al*., 1994). Sodium influx is an important initiating event leading to anoxic damage and a cascade of cellular events (Stys *et al*., 1992). Blockade of Na^+ channels during periods of reduced oxygen supply has therefore been proposed as an effective way of limiting energy expenditure since a large part of the energy consumed by excitable cells is used to maintain ionic gradients across the cellular membrane (Carter, 1998; Urenjak *et al*., 1996). Thus, there would be more energy available for maintaining vital cellular functions especially those pertinent to cellular survival when exposed to insult. Blockade of Na^+ channels has been proposed as an inherent survival mechanism in some neurons (Urenjak *et al*., 1996). The present finding of a secondary involvement of ROS following Na^+ channel activation in the induction of apoptosis suggests Na^+ channel blockade alone may not be sufficient to inhibit all of the consequences of Na^+ channel activation. Our findings support a multi-factorial approach to neuroprotection in cerebral ischaemia.

In conclusion, we report that cell death induced by Na^+ channel activation with veratridine in CGCs is characteristic of apoptosis and that this cell death is likely to be due to secondary mechanisms related to free radical production. Our novel compound, AM-36, which has combined Na^+ channel antagonist and antioxidant activity, completely inhibited veratridine-induced toxicity and apoptosis. Hence, AM-36 may act by both Na^+ channel blockade and antioxidant mechanisms to inhibit veratridine-induced apoptosis. The combined activity of AM-36 together with inhibition of apoptotic cell death may underlie the potent neuroprotective action shown by this compound in a middle cerebral artery occlusion model of transient focal ischaemia in the rat. Our findings indicate that the strategy of incorporating bifunctional activity into a molecule such that multiple cellular mechanisms are targeted, as here for processes contributing to neuronal injury, can provide a beneficial pharmacological profile superior to that given by the individual activities.

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