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Synergistic neuroprotective effects by combining an NMDA or AMPA receptor antagonist with nitric oxide synthase inhibitors in global cerebral ischaemia

Caroline A. Hicks, Mark A. Ward, Jennifer B. Swettenham, Michael J. O'Neill *

Eli Lilly & Company, Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey GU20 6PH, UK

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Abstract

We have investigated the neuroprotective effects of combining an NMDA or AMPA receptor antagonist with a nitric oxide synthase (NOS) inhibitor in the gerbil model of global cerebral ischaemia. Ischaemia was induced by occlusion of the common carotid arteries for 5 min. (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801, 2.5 mg/kg i.p.) or (3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1(2)*H*-tetrazole-5-yl)]decahydroisoquinoline-3-carboxylic acid (LY293558, 20 mg/kg i.p.) and 7-nitroindazole (25 mg/kg i.p.) or *N*-[4-(2-{[(3-chlorophenyl)methyl]amino}ethyl) phenyl]-2-thiophenecarboximidamide dihydrochloride (ARL17477, 25 mg/kg i.p.) were administered alone or in combination (i.e., MK-801 with 7-nitroindazole or ARL17477 or LY293558 with 7-nitroindazole or ARL17477). In the present studies, both MK-801 and LY293558 provided significant degree of neuroprotection, while 7-nitroindazole and ARL17477 also provided some neuroprotection, which failed to reach significance in every case. However, the combination of MK-801 with 7-nitroindazole or ARL17477 provided 21% or 44% greater protection than the total protection or either alone. Likewise, the combination of LY293558 with 7-nitroindazole or ARL17477 provided 14.5% and 35% greater protection than total protection of either compound alone. These results indicate that several pathways contribute to ischaemic cell death and combining excitatory amino antagonists and NOS inhibitors provides greater protection than either alone. Therefore, combination therapy should be considered as an approach for treating ischaemic conditions.

Keywords: Cerebral ischaemia; MK-801; LY293558; 7-Nitroindazole; ARL17477; Hippocampus; Combination

1. Introduction

Cerebral ischaemia leads to a selective pattern of neuronal damage in animals and in man. The exact mechanisms of damage remain to be fully elucidated, but several mechanisms (activation of voltage-gated calcium channels, excitotoxicity, free radicals, mitochondria and apoptosis) appear to be involved (Boxer and Bigge, 1997; Del Zoppo et al., 1997). Several earlier studies demonstrated that L-glutamate was neurotoxic and the ''excitotoxic'' hypothesis of cell death was proposed (Olney, 1978). Glutamate is the major excitatory neurotransmitter in the central nervous system (Collingridge and Lester, 1989). Glutamate activates ligand gated ion channels (ionotropic glutamate receptors or iGlu receptors) and receptors coupled to second messenger systems (metabotropic glutamate receptors or mGlu receptors). The ionotropic receptors are divided into two distinct subtypes, namely, the NMDA receptor and AMPA and kainate receptor (also known as non-NMDA receptors) subtypes (Watkins and Olverman, 1987; Collingridge and Lester, 1989).

The excessive increase of glutamate in the synaptic cleft following ischaemia is thought to play a critical role in the development of neuronal damage (Butcher et al., 1990). Several studies have indicated that many compounds acting at excitatory amino acid receptors have beneficial effects against cerebral ischaemia (Sheardown et al., 1990; Park et al., 1992; Bullock et al., 1994). Many of the early studies demonstrated that NMDA receptor antagonists are neuroprotective in animal models of global and focal cerebral ischaemia (Boast et al., 1988; Park et al., 1988, 1992; McCulloch, 1992). Other studies have focused on the neuroprotective actions of AMPA receptor antagonists

* Corresponding author. Tel.: +44-1276-853547; fax: +44-1276-853525; e-mail: oneillmichaelj@lilly.com

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in animal models of global (Sheardown et al., 1990; O'Neill et al., 1998) and focal (Bullock et al., 1994; Gill, 1994; Gill and Lodge, 1995; Graham et al., 1996; Yatsugi et al., 1996) cerebral ischaemia.

The role of nitric oxide (NO) in cerebral ischaemia has also been investigated. It has been demonstrated in mice deficient in neuronal nitric oxide synthase (NOS) compared with normal mice that there is a reduction in infarct volume after middle cerebral artery occlusion (Huang et al., 1994) and reduced hippocampal damage after global ischaemia (Panahian et al., 1996). In contrast, endothelial NOS knockout mice have larger infarcts after focal ischaemia (Huang et al., 1996). Therefore, NOS inhibitors have been examined as possible neuroprotective agents (Nowicki et al., 1991; Dawson et al., 1994; Yoshida et al., 1994; O'Neill et al., 1997). Earlier studies examined the effects of N^G-nitro-L-arginine methyl ester (L-NAME) in global and focal ischaemia (Caldwell et al., 1994; Dawson et al., 1994). However, L-NAME at higher doses is not selective for neuronal NOS over endothelial NOS. Recently, it has been reported that 7-nitroindazole is a specific inhibitor of neuronal NOS, which does not effect blood pressure and reduces infarct volume in focal ischaemia (Yoshida et al., 1994). It has also been demonstrated that 7-nitroindazole also protects against ischaemic brain damage in the gerbil (O'Neill et al., 1996). Zhang et al. (1996) have reported that a newer compound, *N*-[4-(2-{[(3-chlorophenyl)methyl]amino}ethyl) phenyl]-2-thiophenecarboximidamide dihydrochloride (ARL17477) is a potent and selective neuronal NOS inhibitor that decreases infarct volume after transient middle cerebral artery occlusion in rats.

There is also a large amount of evidence suggesting that apoptotic mechanisms also contribute to neuronal cell death in many disease states (Thompson, 1995; Thornberry and Lazebnik, 1998). However, to date none of these strategies for neuroprotection have had any success in the clinic and several recent reviews have suggested combination therapy may be an alternative approach to the treatment of neurodegenerative disorders (Koroshetz and Moskowitz, 1996; Boxer and Bigge, 1997; Del Zoppo et al., 1997; Sacchetti et al., 1997). Indeed, it has been reported that there is an added neuroprotective effect with dextrorphan and cycloheximide in a rat model of focal cerebral ischaemia (Du et al., 1996) and further experiments have demonstrated synergistic effects with caspase inhibitors and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) after transient focal cerebral ischaemia in mice (Ma et al., 1998). In another recent study, a synergistic protective effect was observed by combining basic fibroblast growth factor and citocholine after temporary focal ischaemia (Schäibtz et al., 1999). As stroke is a multifactorial disease we wanted to evaluate the effects of combining excitatory amino acid antagonists with NOS inhibitors. Preliminary studies in our laboratory indicated that combination of MK-801 or (3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1(2)*H*-tetrazole-5-yl)]decahydroisoquinoline-3-carboxylic acid (LY293558) with L-NAME provided greater neuroprotection than either alone (unpublished results). However, L-NAME is not selective for neuronal NOS and at higher doses can actually potentiate ischaemic damage (Dawson, 1995) so we decided to carry out the experiments with more selective neuronal NOS inhibitors, namely, 7-nitroindazole and ARL17477.

In the present studies, we have carried out two series of experiments. In the first we have examined the effects of administration of an NMDA receptor antagonist (MK-801, 2.5 mg/kg) or NOS inhibitors (7-nitroindazole or ARL17477, 25 mg/kg) alone or in combination on hippocampal damage produced by 5 min bilateral carotid artery occlusion (BCAO) in the gerbil. In the second, we have examined the effects of administration of an AMPA receptor antagonist (LY293558, 20 mg/kg) or NOS inhibitors (7-nitroindazole or ARL17477) alone or in combination on hippocampal damage produced by 5 min BCAO in the gerbil.

2. Methods

2.1. Surgery

Male Mongolian gerbils (Bantin and Kingman, Hull, UK) at least 3 months old and weighing in excess of 60 g were used. The animals were maintained in standard lighting conditions and food and water were available ad libitum. The animals were anaesthetised with a 5% halothane/oxygen mixture and maintained using 2% halothane delivered with oxygen at 1 l/min via a face mask throughout the procedure. Through a midline cervical incision, both common carotid arteries were exposed and freed from surrounding connective tissue. In animals to be rendered ischaemic both common carotid arteries were clamped for 5 min. At the end of the occlusion period, blood flow was re-established. In sham-operated animals, the arteries were exposed but not occluded. The wound was then sutured and the animals allowed to recover. Throughout surgery body temperature was maintained at 37°C using a ''K-TEMP'' temperature controller/heating pad (International Market Supply, Cheshire, UK) and brain temperatures were maintained using a heating lamp. After surgery, the animals were placed in a four compartmental thermacage (Beta Medical and Scientific, UK) which maintained the environmental temperature at 28°C and rectal temperatures were measured for a 12-h period after occlusion.

2.2. Histology

Five days after surgery, the animals were perfused transcardially with 30 ml of 0.9% saline followed by 100 ml of 10% buffered formalin solution. The brains were

removed and placed in 10% buffered formalin for 3 days, processed and embedded in paraffin wax. Five micrometers coronal sections were taken 1.5, 1.7 and 1.9 mm caudal to bregma using a microtome (Leitz 1400 sledge microtome). The sections were stained with haematoxylin and eosin and the neuronal density in the CA1 subfield of the hippocampus was measured using a microscope with grid lines (0.05 mm × 0.05 mm). The neuronal density is expressed as the number of viable cells per mm CA1 hippocampus. Statistical analysis of histological data was assessed using ANOVA followed by Students *t*-test with Bonferroni correction. *P*-values < 0.05 were considered statistically significant.

2.3. Experimental protocols

MK-801 (2.5 mg/kg i.p.) was administered 30 min prior to occlusion, LY293558 (20 mg/kg i.p.) was administered 30 min before occlusion reducing the dose to 10 mg/kg for subsequent injections at 2.5 and 5.5 h after occlusion. ARL17477 (25 mg/kg i.p.) and 7-nitroindazole (25 mg/kg i.p.) were administered immediately and again 3 h post-occlusion. All treatments were given by the intraperitoneal route.

3. Results

Five micrometers sections taken 1.5–1.9 mm caudal to the bregma in the anterior hippocampus were examined under a microscope with grid lines. The CA1 pyramidal neurones were found to be degenerated in the 5-min occluded animals. The neuronal death involved nearly all the pyramidal neurones and this neurodegeneration was not obviously evident in any other forebrain region. The

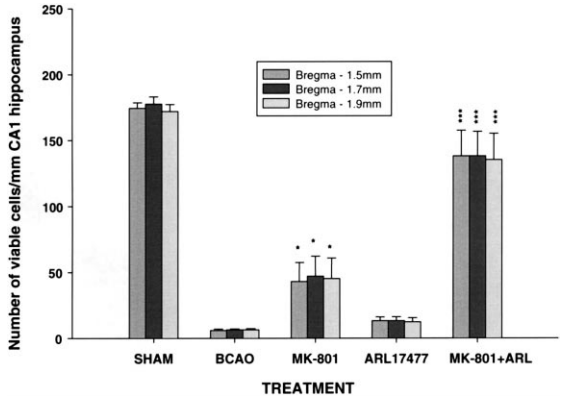

Fig. 2. The effects of MK-801 administered at 2.5 mg/kg i.p. 30 min before, ARL17477 (25 mg/kg i.p.) 0 and 3 h after 5 min BCAO or the combination of MK-801 and ARL17477 on the neuronal density in the CA1 region of the hippocampus 5 days after surgery. Histological results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampal region ($n = 8$ animals per group). Five minutes BCAO produced a severe loss in neurones in the CA1 region ($P < 0.0001$). MK-801 alone provided a significant ($P < 0.05$) degree of neuroprotection against the ischaemia-induced cell death in the hippocampus. ARL17477 (25 mg/kg i.p.) failed to provide any protection, but the combination of MK-801 and ARL17477 produced a much greater degree of protection than either compound alone ($P < 0.01$). Student's *t*-test.

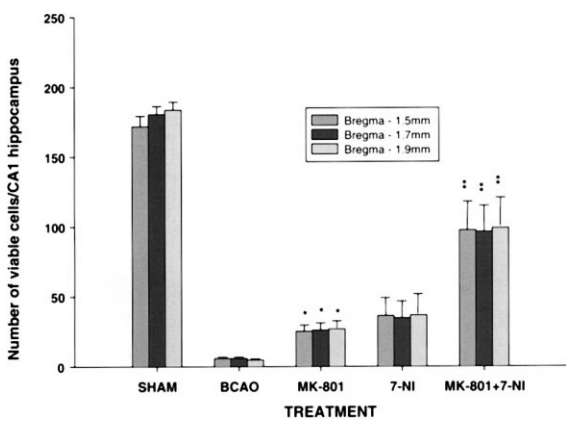

Fig. 1. The effects of MK-801 administered at 2.5 mg/kg i.p. 30 min before, 7-nitroindazole (25 mg/kg i.p.) 0 and 3 h after 5 min BCAO or the combination of MK-801 and 7-nitroindazole on the neuronal density in the CA1 region of the hippocampus 5 days after surgery. Histological results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampal region ($n = 8$ animals per group). Five minutes BCAO produced a severe loss in neurones in the CA1 region ($P < 0.0001$). MK-801 alone provided a significant ($P < 0.05$) degree of neuroprotection against the ischaemia-induced cell death in the hippocampus. 7-Nitroindazole (25 mg/kg i.p.) also provided some protection, but this failed to reach significance. The combination of MK-801 and 7-nitroindazole produced a much greater degree of protection than either compound alone ($P < 0.01$). Student's *t*-test.

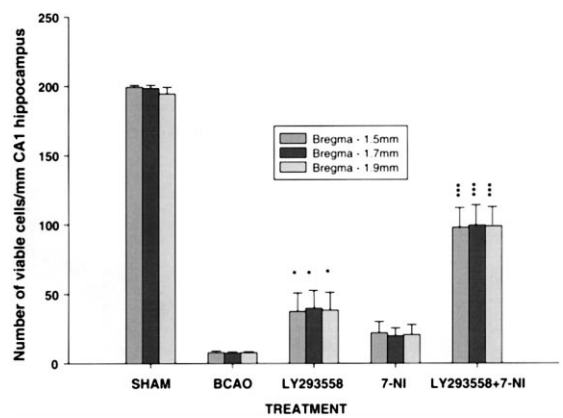

Fig. 3. The effects of LY293558 administered at 20 mg/kg i.p. 30 min before and at 10 mg/kg at 2.5 and 5.5 h after, 7-nitroindazole (25 mg/kg i.p.) 0 and 3 h after 5 min BCAO or the combination of LY293558 and 7-nitroindazole on the neuronal density in the CA1 region of the hippocampus 5 days after surgery. Histological results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampal region ($n = 8$ animals per group). Five minutes BCAO produced a severe loss in neurones in the CA1 region ($P < 0.0001$). LY293558 alone provided a significant ($P < 0.05$) degree of neuroprotection against the ischaemia-induced cell death in the hippocampus. 7-Nitroindazole (25 mg/kg i.p.) also provided some protection, but this failed to reach significance. The combination of LY293558 and 7-nitroindazole produced a much greater degree of protection than either compound alone ($P < 0.01$). Student's *t*-test.

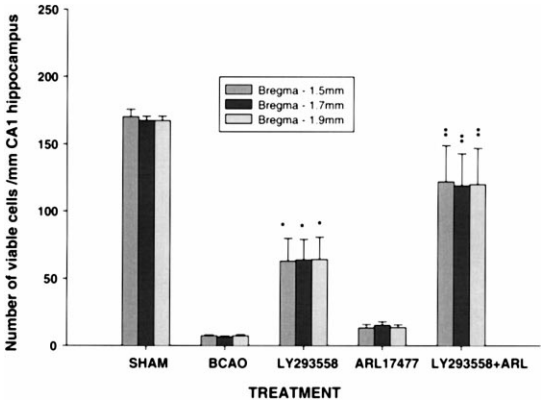

Fig. 4. The effects of LY293558 administered at 20 mg/kg i.p. 30 min before and at 10 mg/kg at 2.5 and 5.5 h after, ARL17477 (25 mg/kg i.p.) 0 and 3 h after 5 min BCAO or the combination of LY293558 and ARL17477 on the neuronal density in the CA1 region of the hippocampus 5 days after surgery. Histological results are expressed as mean ± S.E.M. viable cells/mm CA1 hippocampal region ($n = 8$ animals per group). Five minutes BCAO produced a severe loss in neurones in the CA1 region ($P < 0.0001$). LY293558 alone provided a significant ($P < 0.05$) degree of neuroprotection against the ischaemia-induced cell death in the hippocampus. ARL17477 (25 mg/kg i.p.) provided a small protection, but this failed to reach significance. The combination of LY293558 and ARL17477 produced a much greater degree of protection than either compound alone ($P < 0.001$). Student's *t*-test.

pyramidal cell density was counted at three different stereotaxic levels in the CA1 region of the hippocampus and the results expressed as mean ± S.E.M. neuronal density per 1 mm CA1.

In the first two experiments, we evaluated the effects of MK-801 administered at 2.5 mg/kg i.p. 30 min prior to occlusion alone or in combination with either 7-nitroindazole 25 mg/kg i.p. or ARL17477 25 mg/kg i.p. administered immediately and 3-h post-occlusion. The results indicated that MK-801 provided significant protection (18% and 26%) in both experiments. 7-Nitroindazole provided some evidence of neuroprotection (10%–18%), however, this result did not reach statistical significance. When dosed in combination, MK-801 and 7-nitroindazole provided an enhanced (49%) neuroprotective effect (Fig. 1). When dosed alone, ARL17477 failed to produce any neuroprotective effect. However, when dosed in combination, ARL17477 and MK-801 together provided a significantly increased degree (78%) of neuroprotection (Fig. 2).

In a subsequent set of experiments, we evaluated the effects of LY293558 alone or in combination with either 7-nitroindazole or ARL17477. LY293558 20 mg/kg was administered by the i.p. route 30 min prior to occlusion followed by two further treatments at 3-h intervals, where the dose was reduced to 10 mg/kg. As previously, 7-nitroindazole 25 mg/kg i.p. and ARL17477 25 mg/kg i.p. were administered immediately and again 3-h post-occlusion. Firstly, LY293558 provided significant neuroprotection (20%) and 7-nitroindazole produced a small (10%) degree of protection, which failed to reach significance. However, when dosed in combination, LY293558 and 7-nitroindazole provided a significantly increased (44.5%) neuroprotection (Fig. 3). In the second study, LY293558 provided more marked degree of neuroprotection (37%) when dosed alone, while ARL17477 showed no effect, as seen previously. However, when LY293558 and ARL17477 were dosed in combination the degree of neuroprotection observed was significantly greater (71%) than the calculated additive effects of the individual treatments (Fig. 4).

It is clear that the combination of MK-801 or LY293558 with 7-nitroindazole or ARL17477 provide synergstic neuroprotection and this summarised in Table 1.

4. Discussion

In the present studies, we carried out four experiments to evaluate the effects of the combination of an excitatory amino acid antagonist (MK-801 or LY293558) and a NOS inhibitor (7-nitroindazole or ARL17477) in the gerbil model of cerebral ischaemia. The results indicate that both MK-801 and LY293558 provided a significant ($P < 0.05$) degree of neuroprotection. We noticed that LY293558 provided a greater degree of protection than MK-801 and this is in agreement with previous studies in our laboratory (Hicks et al., 1999) and other reports that indicate that AMPA receptor antagonists provide greater protection than NMDA receptor antagonists in global cerebral ischaemia (Sheardown et al., 1993). It has also been reported that delayed treatment with AMPA receptor antagonists, but not NMDA receptor antagonists reduces neocortical infarction in rats (Xue et al., 1994). We also evaluated the effects of 7-nitroindazole (25 mg/kg i.p.) and ARL17477

Table 1
The % protection with MK-801 or LY293558 and 7-nitroindazole or ARL17477 alone or in combination in the gerbil model of cerebral ischaemia
Data is expressed as % neuroprotection at 1.7 mm caudal to bregma. $n = 8$ animals per group.

Experiment no.	% Protection with MK-801	% Protection with LY293558	% Protection with 7-NI	% Protection with ARL17477	Predicted % protection of combination	Actual % protection of combination
Exp. 1	10	–	18	–	28	49
Exp. 2	26	–	–	8	34	78
Exp. 3	–	20	10	–	30	44.5
Exp. 4	–	37	–	9	46	71

(25 mg/kg i.p.) alone in the model and found that 7-nitroindazole provided some neuroprotection, but this did not reach significance. Similarly, ARL17477 provided only a minimal degree of protection in the current studies. We have previously shown that higher doses of 7-nitroindazole (40 and 50 mg/kg) provide a significant degree of protection in this model (O'Neill et al., 1996) and we have also carried out studies indicating that ARL17477 provides good neuroprotection at 50 mg/kg i.p. (O'Neill et al., unpublished results). We used low doses of both compounds in the current experiments as we were looking for increased neuroprotection.

In the first series of experiments, we found that the combination of MK-801 and 7-nitroindazole provided greater protection than either alone (44.5% as compared with 20% and 10% for either alone). Likewise, the combination of MK-801 and ARL17477 provided 78% protection, while MK-801 or ARL17477 alone provided 26% and 8% protection, respectively. In the second series of studies, we found that the combination of LY293558 and 7-nitroindazole provided greater (45%) protection compared with LY293558 (20%) or 7-nitroindazole (10%) alone. Similar results were observed with the combination of LY293558 and ARL17477 and the percent protection were as follows: LY293558 (26%), ARL17477 (8%) and the combination (78%). The results appear to indicated that the combination of an NMDA or AMPA receptor antagonist with a neuronal NOS inhibitor provides a synergistic neuroprotective effect. However, the synergistic effect appeared more marked with ARL17477 than 7-nitroindazole in each case (Table 1).

Some earlier studies evaluated the effects of combining various neuroprotectants in a model of cerebral ischaemia and it was reported that the combination of MK-801 (1 mg/kg i.p.) and the γ-aminobutyric acid agonist, muscimol (1 mg/kg i.p.) was neuroprotective in a rat model of focal cerebral ischaemia, but neither agent alone provided any protection (Lyden and Lonzo, 1994). In another study, it was reported that the combination of MK-801 (5 mg/kg i.p.) and nicardipine (1 mg kg^{-1} day^{-1} for 3 days) produced an additive neuroprotective effect in the gerbil model of global cerebral ischaemia (Hewitt and Corbett, 1992). Önal et al. (1997) have reported synergistic effects of citicoline and MK-801 in a rat model of focal ischaemia.

Several recent studies have implicated apoptotic mechanisms in ischaemic situations. Therefore, some newer studies reporting the effects of NMDA receptor antagonists in combination with anti-apoptotic strategies have been reported. Du et al. (1996) demonstrated an additive neuroprotective effect using dextrorphan and cyclohexamide in rats subjected to transient focal cerebral ischaemia. More recently, it has been shown that there is a synergistic effect by combining a subthreshold dose of MK-801 with caspase inhibitors (z-VAD.FMK or z-DEVD.FMK). The authors also demonstrated that pre-treatment with a subthreshold dose of MK-801 extended the time window for a high dose of z-DVED.FMK from 1 to 3 h post-occlusion (Ma et al., 1998). Other recent studies have reported greater neuroprotection by combining two antioxidants (Schmid-Elsaesser et al., 1999) and synergistic effects by combining a low-dose of basic fibroblast growth factor and citocoline (Schäibtz et al., 1999) in rats models of transient focal ischaemia. In the current studies, we have observed a synergistic effect using excitatory amino acid antagonists in combination with a NOS inhibitor. Ca^{2+} entry through AMPA receptors under ischaemic conditions removes the magnesium block of NMDA receptor allowing further calcium entry. NOS is a calcium dependent enzyme and activation of the NMDA receptor is known to activate NOS. It appears that by attenuating calcium entry and then further blocking NO production (i.e., inhibiting the pathway at two points) has a much larger effect than either alone. This may also have other benefits as the inhibition required at each step is not as great as either alone and therefore lower doses can be used. MK-801 and other NMDA receptor antagonists are known to cause side effects (Koek et al., 1988) and at higher doses can be neurotoxic in rat brain (Olney et al., 1990). In the present studies, we cannot rule out the effects of drug interactions on pharmokinetics, blood pressure or blood flow and indeed these effects could influence the degree of neuroprotection observed. However, our studies have shown the benefit of inhibiting several steps that contribute to ischaemic damage and medicinal chemistry may allow both activities to be combined in a single molecule. Many of the recent clinical trials with NMDA receptor antagonists in acute stroke have failed to provide efficacy, and in many cases the dose was limited due to adverse side-effects (Lees, 1997). Conducting clinical trials using combinations of compounds which may interact may also prove difficult, but if single compounds with dual action can be used this may be successful.

In conclusion, the present studies indicated a synergistic degree of protection using the combination of either an NMDA receptor antagonist or an AMPA receptor antagonist in combination with a NOS inhibitor. These results suggested that multiple mechanisms contribute to ischaemia-induced cell death and that combination therapy provides greater protection and should be considered in the future as a possible treatment for neurodegenerative disorders.

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