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Neuroprotective properties of lifarizine compared with those of other agents in a mouse model of focal cerebral ischaemia

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1 Changes in the peripheral type benzodiazepine binding site density following middle cerebral artery occlusion in the mouse, have been used as a marker of neuronal damage. These sites can be identified using the selective ligand [^{3}H]-PK 11195 located on non neuronal cells, macrophages and astroglia, within the CNS. Glial cell proliferation and macrophage invasion is an unvoidable sequelae to cerebral ischaemic injury, secondary to neuronal loss. Following occlusion of the left middle cerebral artery (left MCA) a reproducible lesion was found in the parietal cortex within 7 days which gave rise to a significiant increase in [^{3}H]-PK 11195 binding.

2 Treatment of animals with the sodium channel blocker, lifarizine, significantly reduced the ischaemia-induced increase in [^{3}H]-PK 11195 binding when given either 30 min pre-ischaemia and three times daily for 7 days at 0.5 mg kg^{-1}, i.p. ($P<0.01$) or delayed until 15 min post-ischaemia and three times daily for 7 days at 0.5 mg kg^{-1}, i.p. ($P<0.001$). Lifarizine was an effective neuroprotective agent in this model of focal ischaemia in the mouse.

3 Lifarizine also showed a dose-related protection against the ischaemia-induced increase in [^{3}H]-PK 11195 binding with significant protection at doses of 0.1 mg kg^{-1}, i.p. ($P<0.05$), 0.25 mg kg^{-1}, i.p. ($P<0.01$) or 0.5 mg kg^{-1}, i.p. ($P<0.01$) 15 min post-ischaemia and b.i.d. for 7 days. No significant change is seen in the K_d for [^{3}H]-PK 11195. The first dose could be delayed for up to 4 h after cerebral artery cauterization and protection was maintained.

4 Phenytoin (28 mg kg^{-1}, i.v. 15 min and 24 h post-ischaemia) was also neuroprotective in this model ($P<0.01$). This agent is thought to interact with voltage-dependent sodium channels to effect its anti-convulsant actions and this mechanism may also underlie its neuroprotective actions in focal cerebral ischaemia.

5 Agents with other mechanisms of action were also shown to have significant neuroprotection in this model. The non-competitive NMDA antagonist, MK 801, showed significant neuroprotection in the model when given at 0.5 mg kg^{-1}, i.p. 30 min pre-ischaemia with t.i.d. dosing for 7 days ($P<0.001$). The dihydropyridine calcium antagonist, nimodipine was not protective when given using the same dosing protocol as MK 801, 0.5 mg kg^{-1} 30 min pre-occlusion and three times daily for 7 days but showed significant protection when given at 0.05 mg kg^{-1} 15 min post-ischaemia and three times daily for 7 days. The lipid peroxidation inhibitor, tirilazad (single dose 1 mg kg^{-1}, i.v.) showed significant neuroprotection when given 5 min post-ischaemia but not when the first dose was delayed for 4 h.

Keywords: Lifarizine; cerebral ischaemia; MK 801; tirilazad; nimodipine; phenytoin; neuroprotection; [^{3}H]-PK 11195

Introduction

Lifarizine is a centrally active neuronal sodium channel blocker which has shown potent neuroprotective activity in animal models of cerebral ischaemia; both focal, in the cat middle cerebral artery occlusion model (Kucharczyk *et al.*, 1991) and global, in the rat four-vessel occlusion model (Alps *et al.*, 1990). Lifarizine allosterically interacts with the toxin site 2 on the sodium channel (IC_{50} 55 nM, inhibition of batrachotoxin binding) and potently blocks sodium currents in N1E-115 neuroblastoma cells with an IC_{50} of 1.3 μM at a holding potential of -80 mV. The potency is modulated by changing the holding potential (7 μM at -100 mV; 0.3 μM at -60 mV) and has been shown to interact predominantly with the inactivated state of the channel (McGivern *et al.*, 1995). Since the inactivated state of the sodium channel will predominate in partially depolarized and depolarized neurones found in ischaemia, lifarizine will preferentially interact with sodium channels on these cells leaving the sodium channels on normal hyperpolarized neurones unaffected. Thus lifarizine might be expected to suppress recurrent anoxic depolarization induced by ischaemia and limit the damage to those salvageable cells in the ischaemic penumbra.

To assess the neuroprotective efficacy of lifarizine we have established a model of focal cerebral ischaemia in the mouse in which we have quantified ischaemic damage indirectly by use of the peripheral-type benzodiazepine ligand, [^{3}H]-PK 11195, which labels sites on non-neuronal cells such as microglia and macrophages (Anholt *et al.*, 1986), cell types which are elevated in brain tissues in response to ischaemia damage. This methodology has been shown to provide a reliable marker of ischaemic damage in forebrain ischaemia (Kenny *et al.*, 1990; 1992; Demerlé-Pallardy *et al.*, 1991) and focal ischaemia (Dubois *et al.*, 1988; Benavides *et al.*, 1990).

The protective effects of different classes of compounds active at the N-methyl-D-aspartate (NMDA) channel such as MK 801 (Park *et al.*, 1988; Gill *et al.*, 1991), AP-7 (Roman *et al.*, 1989), kynurenate (Germano *et al.*, 1988) and ifenprodil (Gotti *et al.*, 1988) have been shown to be effective in models of cerebral ischaemia and have further contributed to the excitotoxic theory of ischaemic damage. However, extensive attempts to limit cellular calcium entry following cerebral ischaemia with L-type calcium antagonists have, at best, proved inconclusive (Hossman, 1988; Spedding *et al.*, 1989). In this paper, we have assessed the potential neuroprotective properties of sodium channel blockers in the mouse middle cerebral artery-occlusion

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model and compared them with agents which act by alternative mechanisms, including the NMDA antagonist, MK 801, the L-type calcium channel blocker, nimodipine and the lipid peroxidase inhibitor, tirilazad.

Methods

Middle cerebral artery occlusion in the mouse

Adult male mice (Swiss CD-1, Charles River, U.K.) weighing 30–60 g were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}) intraperitoneally (i.p.) or 5% halothane in a 70% nitrous oxide: 30% oxygen gas mixture and the left middle cerebral artery (MCA) identified, essentially as described by Welsh *et al.* (1987). A skin incision was made between the orbit of the eye and the ear, the skin reflected to expose the superior pole of the parotid gland and temporalis muscle. These were retracted to expose the cranium overlying the distal course on the left MCA. A 1 mm burr hole craniectomy was performed with a fine dental drill to expose the left MCA which was then electro-coagulated. The burr hole was then sealed with bone wax and the temporalis muscle repaired with a single cat gut suture, the wound was then dusted with antibiotic powder and surgically closed. All surgical procedures including the onset to recovery were performed under operating lamps on a heated blanket to maintain constant body temperature. For the remainder of the recovery period, animals were housed singly with free access to food and water in a 12 h light-dark cycle.

Administration of drugs

A number of different dosing protocols were used which were chosen to suit the known bioavailability and tolerability of the compounds. The protocols were as follows:- (1) lifarizine (0.5 mg kg^{-1}), MK 801 (0.5 mg kg^{-1}) and nimodipine (0.5 mg kg^{-1}) i.p. 30 min pre-occlusion with i.p. dosing three times daily for 7 days; (2) lifarizine (0.05–0.5 mg kg^{-1}) i.p. 15 min post-ischaemia with i.p. dosing twice daily for 7 day; (3) lifarizine (0.5 mg kg^{-1}) and nimodipine (0.05 mg kg^{-1}) i.p. 15 min post-occlusion with i.p. dosing three times daily for 7 days; (4) tirilazad (1 mg kg^{-1}) single dose i.v. via a tail vein 5 min or 4 h post-ischaemia; (5) phenytoin (28 mg kg^{-1}) two doses i.v. via a tail vein 15 min and 24 h post-ischaemia.

Assay for cortical [^{3}H]-PK 1195 binding sites

After the appropriate recovery period (7 days) mice were decapitated and the brains rapidly removed over ice. A tissue sample from the territory of the left MCA, restricted to the ischaemic area within the parietal cortex, was dissected from the remaining brain tissue and frozen in liquid nitrogen. The corresponding area from the right, contralateral, parietal cortex was also dissected and frozen.

Tissues were rapidly thawed and homogenized in 20–25 vol 50 mM Tris HCl buffer (pH 7.4 at 4°C) with two 10 s bursts of a polytron tissue disruptor (Kinematica PT10) at maximal setting. The homogenate was spun at 48,000 *g* in a cooled (4°C) centrifuge. Tissue pellets were then washed twice more by homogenization and centrifugation. The final pellets were resuspended in 50 mM Tris HCl buffer (pH 7.4 at 25°C) and stored in liquid nitrogen until required for assay. Aliquots of tissue homogenate (50–80 μg protein/assay) were incubated in 50 mM Tris HCA buffer (pH 7.44 at 25°C) in a total volume of 0.5 ml. Assays were incubated for 30 min at 25°C and terminated by filtration over Whatman GF/B filters with two 5 ml washes of ice cold 50 mM Tris HCl buffer (pH 7.4 at 4°C) using a Brandel cell harvester. Non-specific binding was determined in the presence of 10 μM Ro 5-4864. Saturation binding isotherms were carried out with [^{3}H]-PK 11195 over the concentration-range 0.005–2 nM. In competition studies, [^{3}H]-PK 11195 was used at 0.2 nM with at least 12 concentrations of competing drug. Determination of assay protein was carried out with Pierce BCA protein assay kits and bovine serum albumin as standard.

Data analysis

Determination of the saturation binding parameters K_d (equilibrium dissociation constant) and B_{max} (receptor density) were calculated from analysis of saturation binding isotherms using the iterative non linear least square fitting programme LIGAND (Munson & Rodbard, 1980). Binding isotherms of competition data, in the form of displacement curves, were analyzed using a non linear least square curve fitting programme capable of iterative curve fitting to a single (with defined Hill slope) or two site model. The IC_{50} value (concentration of drug inhibiting 50% of specific binding) was converted to the inhibitory constant K_i by the equation of Cheng & Prusoff (1973) where $K_i = IC_{50}/1 + [L]/K_d$, ([L] is the concentration of radioligand employed). Statistical comparisons between groups of data were made using Student's *t* test for unpaired data and for multiple sample comparison, data were analyzed using one way analysis of variance with Dunnett's *t* test using the Statview application in an Apple Macintosh microcomputer. A level of statistical significance was assumed when $P < 0.05$.

Drugs and chemicals

The following drugs were used: lifarizine [1-{[2-(4-methyl-phenyl)-5-methyl]-1*H*-imidazol-4-yl-methyl}-4-diphenyl-methyl-piperazine] (Syntex); [^{3}H]-PK 11195 [1-(2-chlorophenyl-N-methyl-N-(1-methylpropyl) 3 isoquinoline carboamide] (86 Ci $mmol^{-1}$) NEN (DuPont U.K.). MK 801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo-[a,d]cyclo-hepten-5,10-imine] maleate RBI (Semat, U.K.), nimodipine (Bayer AG), phenytoin (Parke Davis) and dipyridamole (Boehringer). PK 11195, Ro 5-4864 (4'-chlorodiazepam) and tirilazad were synthesized by Syntex laboratories. All other drugs were obtained from Sigma (U.K.).

Drugs for *in vitro* binding studies were made up as stock solutions (1 mM) in either reverse osmosis distilled water or ethanol and diluted in assay buffer.

For *in vivo* administration lifarizine, tirilazad and nimodipine were made up in a minimal volume of ethanol and reverse osmosis distilled water (final ethanol concentration <2% v/v) and MK 801 was dissolved in distilled water. Stock concentrations of both compounds were made fresh daily, usually at 0.2 mg ml^{-1} for i.p. administration of 0.1–0.2 ml final volume. Phenytoin was made in the following vehicle: 40% polyethylene glycol, 10% ethanol, 50% ultra pure distilled water at a concentration of 15 mg kg^{-1} to ensure i.v. injection of drug or vehicle was not more than 0.1 ml.

Results

Pharmacological characterization of [^{3}H]-PK 1195 binding sites in mouse cortical membranes

Saturation analysis of [^{3}H]-PK 11195 binding to control non-ischaemic mouse cortical membranes indicated a single class of high affinity sites (K_d 0.2 ± 0.07; B_{max} 170 ± 28 fmol mg^{-1} protein). The density and affinity of these sites was similar to those measured in rat cortical membranes (Table 1). The selectivity of the [^{3}H]-PK 11195 binding site in mouse cortical membranes, in comparison to rats, was characterized by use of a range of compounds (Table 2). The peripheral type benzodiazepine binding site ligands, PK 11195 and Ro 5-4864 showed high affinity in both species whereas the centrally acting benzodiazepines showed lower affinity. The adenosine uptake blocker, dipyridamole, showed moderate affinity. The rank order and affinity of all compounds was similar in both, mouse and rat cortical membranes (PK 11195 > Ro 5-

Table 1 Saturation binding parameters of [^{3}H]-PK 11195 binding to control mouse and rat cortical membranes

Species	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Rat	0.21 ± 0.05	254 ± 21
Mouse	0.20 ± 0.07	170 ± 28

Saturation binding parameters for [^{3}H]-PK 11195 (0.005–2.0 nM) were determined by use of the iterative non-linear curve fitting programme LIGAND (Munson & Rodbard, 1980). Values represent mean ± s.e.mean for 4–5 separate determinations carried out in triplicate.

Table 2 Displacement of [^{3}H]-PK 11195 from rat and mouse cortical membranes

	Rat		*Mouse*	
Compound	pK_i	n_H	pK_i	n_H
PK 11195	9.28 ± 0.08	1.02	9.57 ± 0.04	0.98
RO 5-4864	8.48 ± 0.02	0.92	8.46 ± 0.07	0.79
Dipyridamole	7.19 ± 0.12	0.92	7.56 ± 0.05	1.14
Diazepam	7.25 ± 0.15	0.90	7.44 ± 0.17	0.88
Flunitrazepam	6.95 ± 0.09	0.93	7.12 ± 0.02	0.92

Inhibitory affinities (pK_i) of compounds were determined with 0.2 nM [^{3}H]-PK 11195 in the presence of at least 12 concentrations of competing drug. Values represent mean ± s.e.mean of 4–5 separate determinations carried out in duplicate.

4864 > dipyridamole > diazepam > flunitrazepam) and was consistent with the pharmacology of the peripheral type benzodiazepine binding site (LeFur *et al.*, 1983). The anti-ischaemic compounds (lifarizine, tirilazad, MK 801, nimodipine and phenytoin) were all devoid of affinity (pK_i < 5.5) for the [^{3}H]-PK 11195 binding site in the mouse cerebral cortex.

Post-ischaemic increases in [^{3}H]-PK 11195 binding density following left MCA occlusion

Changes in the density (and affinity) of [^{3}H]-PK 11195 binding to mouse cortical membranes at various time points post ischaemia are shown in Table 3 and Figure 1. Preliminary experiments indicated that short post-ischaemic periods (1–2 h) had no effect on the density of [^{3}H]-PK 11195 binding sites in mouse cortical samples. The increase at 24 h (n = 5) was small and non significant, whilst at 72 h the density of sites in the left cortical samples increased by 171% (P < 0.05, n = 6) the maximal increase was detected at 7 days and represented an

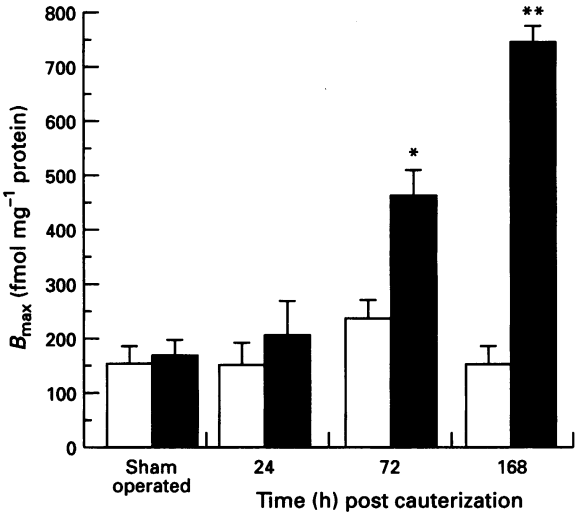

Figure 1 Post-ischaemic increases in [^{3}H]-PK 11195 binding density following focal cerebral ischaemia in the mouse: open columns, right cortex; solid columns, left cortex. Ischaemia was induced by cauterizing the left middle cerebral artery and the animals were allowed to recover for 1, 3 or 7 days. *P < 0.05, **P < 0.01 compared to corresponding right (non-ischaemic) hemisphere.

increase of 301% (P < 0.01, n = 5) compared to the density of sites in the corresponding right, contralateral, non-ischaemic, cortical samples. The density of sites in the non-ischaemic, right, cortical samples (from animals with the left MCA occlusion) was similar, and not significantly different, from the level in right or left cortical samples from sham-operated (non MCA occluded) animals. In subsequent experiments where attenuation of the ischaemia induced increase in [^{3}H]-PK 11195 binding was measured for various drug treatments; samples were assayed at 7 days post ischaemia in all cases.

The effect of lifarizine on the ischaemia induced increase in [^{3}H]-PK 11195 binding following left MCA occlusion

Treatment with lifarizine showed a significant reduction of the ischaemia-induced increase in [^{3}H]-PK 11195 binding when given 30 min pre-ischaemia followed by three times daily administration for 7 days at 0.5 mg kg^{-1}, i.p. (P < 0.01) and when given 15 min post-ischaemia and three times daily for 7 days (P < 0.001). Lifarizine also showed significant protection when given at 0.1 mg kg^{-1}, i.p. (P < 0.05), 0.25 mg kg^{-1}, i.p. (P < 0.01) or 0.5 mg kg^{-1}, i.p. (P < 0.01) 15 min post-ischaemia and twice daily for 7 days (Table 4 and Figure 2). No significant change was seen in the K_d for [^{3}H]-PK 11195 (Table 4).

Table 3 Post-ischaemic increase in [^{3}H]-PK 11195 binding density following focal cerebral ischaemia in the mouse

Left cortex (Ischaemic cortex)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Sham control	5	0.21 ± 0.05	170 ± 28
24 h	5	0.15 ± 0.13	208 ± 60
72 h	6	0.20 ± 0.08	462 ± 49*
168 h	5	0.21 ± 0.05	746 ± 28**
Right cortex (Non-ischaemic cortex)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Sham	5	0.20 ± 0.07	154 ± 32
24 h	5	0.27 ± 0.11	152 ± 42
72 h	6	0.20 ± 0.10	238 ± 34
168 h	5	0.20 ± 0.07	154 ± 32

[^{3}H]-PK 11195 binding (0.005–2.0 nM) was carried out on membrane homogenates from mouse cortex removed at various time points after left MCA-occlusion. Saturation binding parameters represent the mean ± s.e.mean for n separate determinations. *P < 0.05, **P < 0.01; (ANOVA, Dunnett's t-test compared to corresponding right, non-ischaemic control).

The first dose could be delayed for up to 4 h after cerebral artery cauterization and protection maintained (Figure 3). However, when treatment was delayed for 6, 12 or 24 h lifarizine did not confer significant protection.

The effect of MK 801 and nimodipine on the ischaemia-induced increase in [^{3}H]-PK 11195 binding following left MCA occlusion

To validate the quantification of the increase in [^{3}H]-PK 11195 binding as an index of neuronal damage, the effect of MK 801 was assessed. This compound has been shown to reduce ischaemic damage in several models of focal ischaemia (Park *et al.*, 1988; Gill *et al.*, 1991; McCulloch, 1991). The effect of MK 801 is shown in Table 4. As in the lifarizine study, vehicle control animals ($n=30$), indicated that occlusion of the left MCA caused an increase in the density of [^{3}H]-PK 11195 binding sites in left, ischaemic, cortical samples ($P<0.001$) with no corresponding increase in the right, contralateral, non-ischaemic, cortical samples. When MK 801 was administered to mice at 0.5 mg kg^{-1} 30 min pre-occlusion and three times daily for 7 days, the increase in [^{3}H]-PK 11195 binding in left, ischaemic, cortex was greatly reduced (Table 4, $P<0.001$) compared to the density in ischaemic cortical samples from vehicle control animals. Nimodipine, when administered under a similar dose regimen (0.5 mg kg^{-1}, i.p., 30 min pre-occlusion and twice daily for 7 days) did not afford any degree of protection and the density of [^{3}H]-PK 11195 sites in the left cortical samples from drug treated animals was not significantly different from control (Table 4). However, when nimodipine was administered at a lower dose (0.05 mg kg^{-1}, i.p.) at 15 min post occlusion and twice daily for 7 days there was a significant

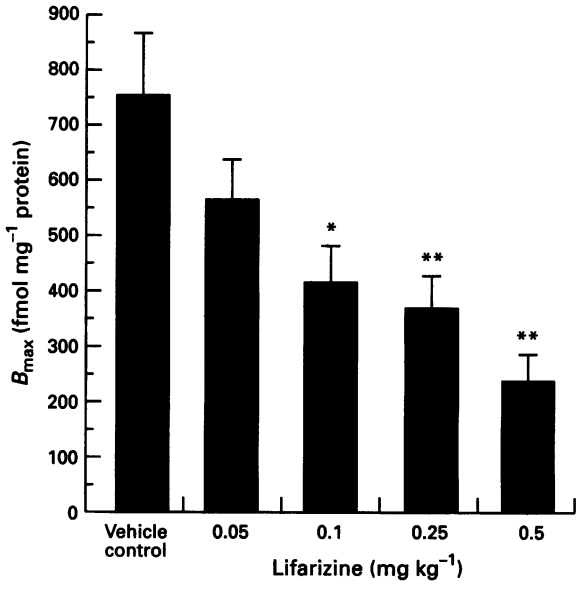

Figure 2 Efficacy of lifarizine in a mouse model cerebral artery-occlusion model. Ischaemia was induced by cauterizing the left middle cerebral artery and allowing the animals to recover for 7 days. Mice (30–60 g) were dosed 15 min post-ischaemia i.p. with placebo vehicle or the stated dose of lifarizine and then i.p. twice daily for 7 days. The animals were killed 6 h after the last dose. Data shown are the B_{max} values for [^{3}H]-PK 11195 binding in the ischaemic hemisphere. B_{max} values for the correspondig non-ischaemia hemisphere are shown in Table 4. $*P<0.05$, $**P<0.01$ compared to control group.

Table 4 The effect of different classes of anti-ischaemic agents on the ischaemia-induced increase in [^{3}H]-PK 11195 binding to mouse cortex

Treatment	n	K_d (nM)		B_{max} (fmol mg^{-1} protein)		*% damage*
		left	right	left	right	
Placebo (lifarizine)	9	0.19±0.07	0.21±0.06	752±113###	220±26	100
Lifarizine 0.5 mg kg^{-1} (1)	6	0.22±0.02	0.16±0.01	348±51**	242±26	20
Lifarizine 0.05 mg kg^{-1} (2)	10	0.22±0.01	0.23±0.04	564±72	257±21	58
Lifarizine 0.1 mg kg^{-1} (2)	10	0.21±0.06	0.22±0.06	418±65*	284±40	25
Lifarizine 0.25 mg kg^{-1} (2)	9	0.18±0.06	0.15±0.06	371±55**	268±41	20
Lifarizine 0.5 mg kg^{-1} (2)	10	0.17±0.04	0.15±0.03	241±46**	225±41	3
Lifarizine 0.5 mg kg^{-1} (3)	5	0.23±0.02	0.23±0.04	187±40***	185±49	0
Placebo (MK 801/Nim)	30	0.18±0.02	0.18±0.04	858±68###	263±21	100
MK 801 0.5 mg kg^{-1} (1)	6	0.11±0.04	0.14±0.01	302±21***	240±9	10
Nimodipine 0.5 mg kg^{-1} (1)	5	0.30±0.06	0.23±0.04	1229±224	325±71	152
Nimodipine 0.05 mg kg^{-1} (3)	5	0.31±0.06	0.31±0.06	373±68***	241±74	24
Placebo (tirilazad)	10	0.39±0.04	0.33±0.03	1120±134###	453±34	100
Tirilazad 1 mg kg^{-1} 5 min (4)	10	0.37±0.04	0.30±0.04	504±48**	285±28	33
Tirilazad 1 mg kg^{-1} 4 h (4)	9	0.29±0.03	0.37±0.03	1044±134	434±43	91
Placebo (phenytoin)	6	0.20±0.05	0.24±0.04	517±26###	147±17	100
Phenytoin 28 mg kg^{-1} (5)	6	0.21±0.03	0.20±0.03	325±15**	141±22	22

Ischaemia was induced by cauterizing the left middle cerebral artery and recovering the animals for 7 days. Mice (30–60 g) were dosed with the appropriate drug (at the stated dose) or placebo: (1) lifarizine, MK 801 and nimodipine i.p. 30 min pre-occulsion with i.p three times daily dosing for 7 days; (2) lifarizine i.p. 15 min post-ischaemia with i.p. twice daily dosing for 7 days; (3) lifarizine and nimodipine i.p. 15 min post-occlusion with i.p. three times daily for 7 days; (4) tirilazad single dose i.v. 5 min or 4 h post-ischaemia; (5) phenytoin two doses i.v. 15 min and 24 h post-ischaemia.

$$\% \text{ damage} = \frac{\text{left R1 (test)} - \text{right R1 (test)}}{\text{left R1 (placebo)} - \text{right R1 (placebo)}} \times 100$$

Statistical comparisons are made with the appropriate placebo group for each treatment group.
No statistical differences in the K_d values.
###$P<0.001$ (Student's t test) difference in placebo left (ischaemic) vs placebo right B_{max}. $*P<0.05$; $**P<0.01$; $***P<0.001$ (ANOVA, Dunnett's t test) difference in left treated vs placebo left B_{max}.

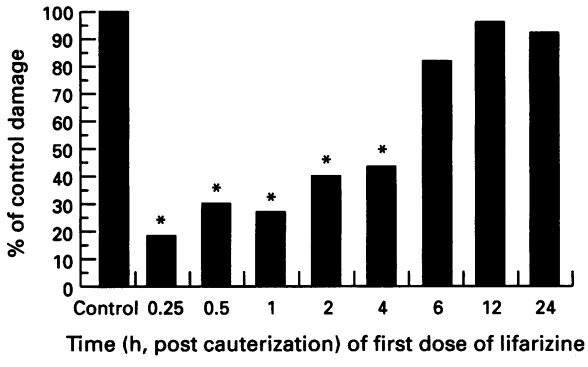

Figure 3 Effect of delaying the first dose of lifarizine on the ischaemia-induced increase in [^{3}H]-PK 11195 binding. Ischaemia was induced by cauterizing the left middle cerebral artery and allowing the animals to recover for 7 days. Mice (30–60 g) were dosed with lifarizine (0.5 mg kg^{-1}, i.p.) or placebo vehicle (0.1 ml i.p.) 15, 30 min, 1, 2, 4, 6, 12 or 24 h post-ischaemia and then i.p. twice daily for 7 days. The animals were killed 6 h after the last dose.

$$\% \text{ damage} = \frac{\text{left R1 (test)} - \text{right R1 (test)}}{\text{left R1 (placebo)} - \text{right R1 (placebo)}} \times 100$$

*$P<0.05$, compared to control group (statistics based on absolute values obtained for the control and treated groups at each time point).

($P<0.001$) decrease in the density of [^{3}H]-PK 11195 sites in left cortical tissue from nimodipine treated animals compared to untreated controls (Table 4).

The effect of tirilazad on the ischaemia-induced increase in [^{3}H]-PK 11195 binding following left MCA occlusion

Tirilazad, a 21-aminosteroid lipid peroxidation inhibitor, has been shown to reduce ischaemic damage in focal ischaemia (Unemura *et al.*, 1994). The effect of tirilazad is shown in Table 4. Vehicle control animals indicated that occlusion of the left MCA caused an increase in the density of [^{3}H]-PK 11195 binding sites in left, ischaemic, cortical samples ($P<0.001$). When tirilazad was administered to mice at 1 mg kg^{-1}, i.v. 5 min post-occlusion the increase in [^{3}H]-PK 11195 binding in left, ischaemic, cortex was reduced (Table 4, $P<0.01$). However, unlike lifarizine the dose could not be delayed and still provide significant protection. When the dose was given 4 h post-ischaemia no significant reduction in the ischaemia-induced increase in [^{3}H]-PK 11195 binding was seen (Table 4).

Protective effects of phenytoin following left MCA occlusion in the mouse

In this series of experiments, due to poor intraperitoneal absorption (McNamara *et al.*, 1989), phenytoin was administered as a single dose (28 mg kg^{-1}) via a tail vein, 15 min post-occlusion with one further dose (28 mg kg^{-1}) administered 24 h post-occlusion. In comparison to vehicle-treated animals, phenytoin caused a significant ($P<0.01$) decrease in the ischaemia-induced elevation of [^{3}H]-PK 11195 binding density in the left cortical tissues (Table 4).

Discussion

In this paper we have demonstrated the increase in cortical [^{3}H]-PK 11195 binding following MCA occlusion to be a sensitive, indirect, index of neuronal damage, which allows quantifiable assessment of potential neuroprotective agents. Whilst termed 'peripheral type' benzodiazepine binding sites, the sites also exist within the CNS but are associated with non-neuronal cells such as microglia and macrophages (Zavala & Lenfant, 1987; Benavides *et al.*, 1988). The post-ischaemic increase in peripheral type benzodiazepine binding sites demonstrated with [^{3}H]-PK 11195 in this paper is consistent with the temporal increase in non-neuronal cells such as reactive microglia and macrophages in response to ischaemic injury (Myers *et al.*, 1991; Morioka *et al.*, 1991). A similar temporal profile following focal ischaemia has been reported in another study in which this model has been characterised (Benavides *et al.*, 1990). However, in our studies we have restricted our analysis of [^{3}H]-PK 11195 binding to the ischaemic tissue within the parietal cortex and have achieved a much higher level of ischaemia-induced peripheral type benzodiazepine binding following MCA occlusion in comparison to that previously reported. This protocol therefore allows a more accurate quantification of drug-related neuroprotection. These experiments are further facilitated by the relatively low density of peripheral type benzodiazepine binding sites in control, non-ischaemic, mouse cortex since these sites show considerable species variation in density and their affinity for selective compounds (Awad & Gavish, 1987; Kenny *et al.*, 1990). Indeed, in species with high levels of control [^{3}H]-PK 11195 binding, the post-ischaemic increase in tissue peripheral type benzodiazepine binding is relatively less well-defined (Kenny *et al.*, 1990). However, the peripheral type benzodiazepine binding site in the mouse appears to be similar to that of the rat; relatively low density, displaying high affinity for Ro 5-4864.

The neuroprotection obtained with MK 801 is consistent with other findings in this model (Gotti *et al.*, 1990) and other models of focal ischaemia where the compound reduces infarct volume following MCA occlusion (Park *et al.*, 1988; Gill *et al.*, 1991). This finding adds to the substantial amount of evidence suggesting an excitotoxic role for glutamate in neuronal cell death. However, in the present work we have been able to show that other compounds including the sodium channel blocker, lifarizine, the anticonvulsant, phenytoin and the dihydropyridine calcium antagonist, nimodipine also protect against ischaemia damage indicating alternative neuroprotective mechanisms also exist.

Sodium and calcium overload is a critical factor in the initiation of the pathological conditions leading to cell death following cerebral ischaemia (Siesjo, 1981; 1988; Hass, 1983; Simon *et al.*, 1984; Gelmers, 1985; Spedding *et al.*, 1989; Pulsinelli, 1992). Lifarizine, through its block of neuronal sodium and calcium channels, has the potential to suppress ischaemia-induced recurrent depolarization, limit the cellular accumulation of Na^+ and Ca^{2+} and therefore reduce neurological damage following cerebral ischaemia. *In vitro* lifarizine has been shown to allosterically interact with the toxin site 2 on sodium channel (IC_{50} 55 nM, inhibition of [^{3}H]-batrachotoxin binding), protects against the toxicity induced by the sodium channel activator, veratridine in chick myocytes (Patmore *et al.*, 1991), and in primary cultures of cortical neurones (May *et al.*, 1995). Lifarizine also potently blocks sodium currents in N1E-115 neuroblastoma cells (K_i 0.2 μM; McGivern *et al.*, 1995).

In vivo lifarizine has been shown to reduce infarct size, attenuate cerebral oedema and stabilize energy reserves in brain tissue of cats subjected to occlusion of the middle cerebral artery (Kucharczyk *et al.*, 1991). The compound also reduced delayed neuronal cell death in the rat 4 vessel occlusion global ischaemia model (Alps *et al.*, 1990). The efficacy in the cat and rat focal models was associated with plasma levels of 3–13 ng ml^{-1} and 20–100 ng ml^{-1}, respectively and only minor decreases in mean arterial blood pressure (up to 10 mmHg). In the present study lifarizine was effective in a mouse focal model of cerebral ischaemia with protection at doses between (100–500 μg kg^{-1}). These data suggest that inhibition of the voltage-gated sodium channel in neuronal tissue may provide a powerful and alternative mechanism for neuroprotection following cerebral ischaemia. The observation of maintained efficacy when administration of the compound was delayed for up to 4 h after the insult also indicates that there may be a reasonable time window for intervention with such agents following cerebral infarction. This is in contrast to the lipid peroxidation inhibitor, tirilazad, which showed no significant protection

when the dose was delayed for 4 h. Indeed this may be the problem with this class of compounds where early loading of the damaged tissue may be crucial. Tirilazad is thought to attenuate the hydroxyl radical formation resulting from tissue acidosis and disruption of calcium homeostatis during cerebral ischaemia (Hall *et al.*, 1994) and has been shown to reduce ischaemic damage in animal models of focal ischaemia (Unemura *et al.*, 1994; Hall *et al.*, 1994). However, the actual mechanism and the degree to which free radicals participate in the cellular injury associated with brain ischaemia remains speculative because of the difficulty in measuring free radicals *in vivo*.

Phenytoin has been claimed to exert its anticonvulsant efficacy by reducing neuronal excitability through an interaction with voltage-dependent sodium channels (Willow *et al.*, 1985). In models of forebrian ischaemia, phenytoin protects hippocampal CA1 neurones from a period of temperature-regulated cerebral ischaemia (Taft *et al.*, 1989) and provides additive protection in the presence of other neuroprotective compounds (Hass & Alps, 1985). These protective effects appear to be related to the maintenance of ionic homeostasis and conservation of energy levels (Artru & Michenfelder, 1980; Kinouchi *et al.*, 1990). Phenytoin does not appear to have any vascular interactions and has no significant effects on cortical blood flow (Kennedy *et al.*, 1972) and does not interact with the NMDA receptor complex (Rogawski & Porter, 1990). However, the dose of phenytoin protective against ischaemic damage in the mouse MCA model (28 mg kg^{-1}) is consistent with its efficacy as an anticonvulsant (McNamara *et al.*, 1989). Furthermore, this dose has also been shown to reduce infarct volume following MCA occlusion in the rat (Boxer *et al.*, 1990). Extensive evidence indicates an interaction of phenytoin with voltage-dependent sodium channels; blockade has been shown to be frequency-dependent (Schwartz & Grigat, 1989) and to increase with the extent to which neurones are depolarized (Willow *et al.*, 1985). Blockade has been found to increase in the presence of raised extracellular K^+, a phenomenon well documented during cerebral ischaemia (Hansen, 1985).

The involvement of sodium channel activation in ischaemia neuronal damage is supported by a number of studies. Focal application of the sodium channel blocker, tetrodotoxin (TTX) protects hippocampal neurones from damage following four vessel occlusion (Yamasaki *et al.*, 1991). In hippocampal slices, TTX improves recovery and levels of ATP following hypoxia (Boening *et al.*, 1989), and delays the onset of hypoxic spreading depression (Ashton *et al.*, 1990). Under these conditions, the effect of reducing Na^+ entry would probably reduce the load on Na^+/K^+ ATPase and consequently reduce the build up of extracellular K^+ (Artru & Michenfelder, 1981). Similarly, phenytoin has been shown to delay the onset and severity of hypoxic depolarization when measured extracellulary in hippocampal slices and thus promote post hypoxic recovery (Kenny & Sheridan, 1992) and reduce epileptic burst firing (Ashton *et al.*, 1986). More recently, Vornov *et al.* (1994) described a protective affect of TTX against histologically-assessed damage produced by metabolic inhibition in organotypic hippocampal slice cultures. Systemically-administered lignocaine has been shown to limit infarct size in a cat middle cerebral artery occlusion model (Shokunbi *et al.*, 1990) and to promote the recovery of electrical activity in a rabbit model of incomplete global ischaemia (Rasool *et al.*, 1990). It is interesting to note that the non-selective sodium and calcium channel blocker, flunarizine, displays a similar profile to phenytoin in models of hypoxia *in vitro* (Ashton *et al.*, 1990; Pauwels *et al.*, 1991) and has a similar anticonvulsant profile *in vivo* (Binnie, 1988). Furthermore, the interaction of this compound with voltage-dependent sodium channels is thought to underlie its efficacy as a neuroprotective agent (reviewed in Pauwels *et al.*, 1991). Similarly riluzole, which binds to a site on the α subunit of the sodium channel, is thought to exert its anticonvulsant and anti-ischaemic effects by stabilization of the inactivated state of the sodium channel (Herbert *et al.*, 1994).

In previous studies with MK 801, neuroprotection has been found to be dose-dependent with less protection afforded with high doses, due to effects on cerebral blood flow and metabolism, such that drug imposed hypotensive effects increase infarct size following focal ischaemia (Osbourne *et al.*, 1987). In the present experiment, we found that protection with nimodipine was also apparent with the lower (0.05 mg kg^{-1}) rather than the higher (0.5 mg kg^{-1}) dose, an effect also probably related to its hypotensive properties and differential effect on cortical blood flow. Thus, extensive studies in global forebrain ischaemia have shown nimodipine to be ineffective (Hossman, 1988), whilst in focal ischaemia the majority of studies have attributed the beneficial effects of nimodipine to favourable increases in local cerebral blood flow (Wauquier *et al.*, 1988). Furthermore, other studies have indicated that nimodipine causes hypo-perfusion in some areas and hyper-perfusion in others (Smith *et al.*, 1983) and vasodilator effects of potent dihydropyridines can actually cause a decrease in blood flow in ischaemia areas in patients following ischaemia stroke, presumably by a steal effect. Consequently, nimodipine reduces ischaemic damage following MCA occlusion in the hands of some investigators/laboratories (Obana *et al.*, 1985; Germano *et al.*, 1987) but not others (Mohamed *et al.*, 1985; Kobayashi *et al.*, 1988). The dose-dependence of compounds whose underlying neuroprotective efficacy is based on benefical changes in localized cerebral blood flow is likely to compromise their use as potential stroke therapies. On this basis, a consideration of compounds interacting with voltage-dependent sodium channels may represent an alternative neuroprotective strategy.

Clinical data reported with lifarizine to date indicate that the compound is well tolerated in human subjects suffering acute ischaemic stroke (Squire *et al.*, 1994) and therefore suitable for study in larger populations of stroke patients. Further data from clinical trials regarding efficacy of sodium channel antagonists in stroke will be required to establish the value of this approach.

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