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Protective effect of the antioxidant 6-ethoxy-2,2-pentamethylen-1,2-dihydroquinoline (S 33113) in models of cerebral neurodegeneration

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Abstract

In a previous study Dorey et al. [Bio. Org. Chem. Lett., 10 (2000) 935] a series of novel dihydroquinoline compounds were developed, based on the potent antioxidant 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin), and permitted the selection of the analogue 6-ethoxy-2,2-pentamethylen-1,2-dihydroquinoline (S 33113) lacking the hypothermic effects associated with ethoxyquin at equivalent doses. Herein, an extensive investigation of the neuroprotective capacity of S 33113 in different in vitro and in vivo paradigms of oxidative stress-mediated cellular degeneration was undertaken. In vitro S 33113 was a potent inhibitor ($IC_{50} = 0.29$ μM) of Fenton-reaction-induced lipid peroxidation in mouse cortical membranes. Administration of S 33113 either intraperitoneally (≤ 150 mg/kg i.p.) or orally (≤ 600 mg/kg p.o.) did not significantly modify body temperature in NMRI mice. Furthermore, S 33113 (150 mg/kg i.p. or 600 mg/kg p.o.) markedly reduced the lethality induced by an intracerebroventricular injection of *t*-butylhydroperoxide in NMRI (naval medical research institute) mice for up to 5 h. Oral administration of S 33113, significantly attenuated alloxan-mediated hyperglycaemia in NMRI mice at 400 and 600 mg/kg (60%; $P < 0.001$). Administration of S 33113 (150 mg/kg i.p.) 30 min before transient global ischaemia significantly prevented delayed neuronal cell death in the CA1 region of the rat hippocampal formation, 7 days post-ischaemia (33% cell loss vs. 88% in ischaemia controls; $P < 0.001$). Similarly, a single pre-administration of S 33113 (150 mg/kg i.p.) prevented kainic acid-induced cell death in the CA3 hippocampal region at 7 days post-exposure (17% cell loss vs. 52% in kainate-treated controls; $P < 0.01$). Furthermore, D-methamphetamine-mediated dopamine depletion in the striatum of C57BL/6 mice (39–46%) was significantly prevented with S 33113 administered at either (2 × 150mg/kg i.p.) (11%; $P < 0.01$) or (2 × 150 mg/kg p.o.) (17%; $P < 0.001$). In conclusion, S 33113 represents a novel dihydroquinoline compound with potential for the treatment of cerebral pathologies implicating chronic neurodegeneration. © 2001 Published by Elsevier Science B.V.

Keywords: Neurodegeneration; Antioxidant; Hypothermia; Ischaemia; S33113

1. Introduction

Considering the crucial role of free radicals in both ageing and neurodegenerative processes (Coyle and Puttfarcken, 1993; Reiter, 1995), extensive efforts have been undertaken in order to develop novel antioxidants capable of scavenging free radicals or preventing any subsequent radical-mediated damage. Indeed, several types of antioxidants including the spin-trappers *tert*-butylphenylnitrone (*t*-BPN) and *N-tert*-butyl-α-(2-sulphophenyl)-nitrone (S-PBN) or lipid peroxidation inhibitors [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] (U-78517F) and 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) are capable of preventing oxidative-mediated cell death in ischaemia-reperfusion injury, and D-methamphetamine, α-methylene-dioxymethamphetamine (MDMA) or *N*-methyl-1,2,3,6-tetrahydro-4-phenylpyridine (MPTP)-mediated neurogeneration (Hall et al., 1991; Cao and Phillis, 1994; Watanabe et al., 1994; Smith et al., 1995; Schulz et al., 1995; Cappon et al., 1996; Yeh, 1999). Chronic treatment of ageing rats with antioxidants including vitamin E and *tert*-butyl-phenylnitrone improves cognitive performance and survival (Socci et al., 1995; Sack et al., 1996). Paradoxically, only a limited number of centrally acting antioxidants, have been proposed as candi-

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dates for clinical development, and in all cases exclusively for the acute treatment of cerebral ischaemia (De Keyser et al., 1999).

Ethoxyquin, a synthetic molecule, has been described as a powerful antioxidant and potent lipid peroxidation inhibitor widely used in the agrochemical industry as a food preservative. Ethoxyquin also possesses anti-inflammatory activity (Levy, 1976; Spaethe et al., 1989) and acts as a dual cyclooxygenase/lipoxygenase inhibitor (Spaethe et al., 1989). The addition of ethoxyquin to the diet of male mice extended the average life-span (Comfort, 1971; Harman et al., 1976), and was paralleled by a decrease in the incidence of amyloidosis (Harman et al., 1976). Consequently, based on the potent antioxidant nature of ethoxyquin, a series of novel dihydroquinoline antioxidants were developed, in order to evaluate their potential neuroprotective capacity in several in vitro and in vivo paradigms implicating primarily neuronal cell death (Dorey et al., 2000). These studies permitted the selection of the derivative S 33113 with in vitro and in vivo activities equivalent to, but without the hypothermic effects associated with ethoxyquin at the same dose (Dorey et al., 2000). In the present study, we report an extensive characterisation of the activity of S 33113, in a series of in vitro (lipid peroxidation) and in vivo (*t*-butylhydroperoxide-mediated lethality, alloxan-mediated hyperglycaemia, global cerebral ischaemia and kainic acid-mediated neuronal cell loss and D-methamphetamine-induced dopaminergic dysfunction) paradigms implicating oxidative stress.

2. Materials and methods

2.1. Materials

Alloxan monohydrate, butylhydroperoxide, kainic acid were obtained from Sigma. D-methamphetamine was supplied by Calaire Chimie, France. S 33113 [6-ethoxy-2,2-pentamethylen-1,2-dihydroquinoline] was synthesised at Institut de Recherches Servier (Casara et al., 1999). Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinolene) (75% purity) purchased from Sigma, was converted to a hydrochloride salt and further purified (> 99% purity) in-house.

Animals [Wistar rats or NMRI (Naval Medical Research Institute)] were housed in conditions conforming to EEC directive 86/609 (ILAR 1985). Environmental conditions were maintained as follows: air ventilation at 15 renewals/h, light/dark cycle 12 h/12 h, temperature 21 ± 1°C, humidity 60 ± 5% and free access to food and water. All the experiments were carried out according to the guidelines of the European Community's Council for Animals experiments (DL 116/92) with the permission of the internal ethical committee at the Institut de Recherches Servier.

2.2. Methods

2.2.1. Lipid peroxidation in mouse cortical membranes

Mouse (male NMRI 20–30 g) cortical membranes (1 g/20 ml Tris–HCl, 20 mM, pH 7.4) were incubated for 15 min with the compound under study (0.01–10 μM) and then exposed for 15 min to $FeSO_4$ (0.1 mM)/ascorbic acid (1 mM)/H_2O_2 (1 mM). The reaction was stopped with the addition of trichloroacetic acid (20% v/v) at +4°C, centrifuged ($1500 \times g$ for 5 min) and any malondialdehyde

Table 1
In vitro receptor binding selectivity of S 33113 Assays performed by Cerep

System	Ligand	IC_{50} (nM)
Cyclooxygenase-1		> 10,000
Cyclooxygenase-2 (h)		~ 10,000
5-Lipoxygenase (h)		~ 10,000
TXA2/PGH2 (h)	[^{3}H]SQ29548	> 10,000
5-HT (r)	[^{3}H]5-HT	> 10,000
5-HT_{2A} (r)	[^{3}H]Ketanserin	> 10,000
5-HT_3 (m)	[^{3}H]BRL43694	> 10,000
AdenosineA_1 (h)	[^{3}H]DPCPX	> 10,000
Adenosine A_2 (r)	[^{3}H]CGS21680	> 10,000
5-HT uptake (r)	[^{3}H]Paroxetine	> 10,000
Dopamine uptake (r)	[^{3}H]BTCP	> 10,000
Noradrenaline uptake (r)	[^{3}H]nisoxetine	> 10,000
α_1-adrenergic (r)	[^{3}H]Prazosin	> 10,000
α_2-adrenergic (r)	[^{3}H]RX821002	> 10,000
CB_1-Cannabinoid (r)	[^{3}H]WIN55212-2	> 10,000
Dopamine-D1 (r)	[^{3}H]SCH23390	> 10,000
Dopamine-D2 (r)	[^{3}H]YM09151	> 10,000
Endothelin-ET_A (h)	[^{125}I]Endothelin-1	> 10,000
GABA (r)	[^{3}H]GABA	> 10,000
Histamine-H_1(gp)	[^{3}H]Pyrilamine	> 10,000
Histamine-H_2(gp)	[^{125}I]APT	> 10,000
L-type Ca^{2+} site (r)	[^{3}H]Diltiazem	> 10,000
K^+ ATP (r)	[^{3}H]Glibenclamide	> 10,000
K^+/volt(r)	[^{125}I]Dendrotoxin	> 10,000
Musacrinic M_1–M_5(r)	[^{3}H]QNB	> 10,000
Nicotinic (r)	[^{3}H]Cytisine	> 10,000
NMDA (r)	[^{3}H]CGP39653	> 10,000
AMPA (r)	[^{3}H]AMPA	> 10,000
NPY (r)	[^{3}H]NPY	> 10,000
Opiate (r)	[^{3}H]Naloxone	> 10,000
Sigma (r)	[^{3}H]DTG	> 10,000

Abbreviations: (r) rat brain; (h) human; (gp) guinea pig; DTG (1,3-di(2-tolyl)guanidine; NPY (neuropeptide Y); NMDA (*N*-methyl-D-aspartate; QNB (quinuclidinyl benzilate); GABA (γ-aminobutyric acid); AMPA (*a*-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid); BTCP (*N*-[1-(benzo[*b*]thien-2-ylcyclohexyl)]piperidine); DPCPX (1,3-dipropyl-8-cyclopentylxantine); TXA (thromboxane); SQ29548 ([1*S*-[1*a*,2*a*(*Z*), 3*a*4*a*]]-7-[3[[2-(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid); CGS21680 (2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamidoadenosine); SCH23390 (*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine); WIN55212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-3[(morpholinyl)-methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanonemesylate); BRL-43694 (endo-1-Methyl-*N*-(9-methyl-9-azabicyclo-[3.3.1]non-3-yl)-1*H*-indazole-3-carboxamide hydrochloride); RX821002 (2-(2-Methoxy-1,4-benzodioxan-2-yl)-2-imidazole); YM09151 ((±)-*cis*-*N*-(1-Benzyl-2-methyl-3-pyrrolidinyl)-5-chloro-4-(methylamino)-*o*-anisamide); CGP39653 (E-2-Amino-4-(phosphonomethyl)-3-heptenoic acid).

formed was converted into thiobarbituric acid reactive substances by the addition of an equivalent volume of thiobarbituric acid (0.67% w/v) to the supernatant, and heated at 100°C for 20 min. Samples were assayed at 532 nm and were expressed relative to a concentration curve of malondialdehyde (0–40 μM) obtained from a lipid-peroxidation kit (Oxis International).

2.2.2. t-Butylhydroperoxide-mediated lethality in NMRI mice

Based on the method previously described by Adams et al. (1993), male NMRI (28–35 g) mice ($n = 10$/group) were administered by intraperitoneal (i.p.) (150 mg/kg in 0.1% (w/v) Tween–saline; 20 ml/kg) or oral (p.o.) routes (600 mg/kg in Tween–water; 20 ml/kg) with the compound under study 30 or 60 min, respectively before an intracerebroventricular (i.c.v.) injection of *t*-butylhydroperoxide (1 μl of a 70% solution). Lethality was assessed 1, 2, 5 and 24 h after administration of *t*-butylhydroperoxide and was expressed as the percentage of survival relative to the lethality observed in *t*-butylhydroperoxide plus Tween/saline vehicle-treated animals.

2.2.3. Effect of compounds on body temperature in NMRI mice

At $t = 0$ h the rectal temperature of male NMRI mice was measured with a rectal probe (Physitemp, Bat-12). Animals were then treated with either vehicle (20 ml /kg) or the compound under study by either oral or intraperitoneal administration. Rectal temperature was assessed every 30 min for up to 2–3 h post-injection. Effect of compounds on the rectal temperature were estimated as the difference between control and treated groups at any given time.

2.2.4. Alloxan-induced hyperglycaemia in NMRI mice

Male NMRI (28–35 g) mice were administered by oral route with the compound under study (100–600 mg/kg in Tween–water; 20 ml/kg) 60 min before an intravenous (i.v.) injection of alloxan monohydrate (40 mg/kg). Ani-

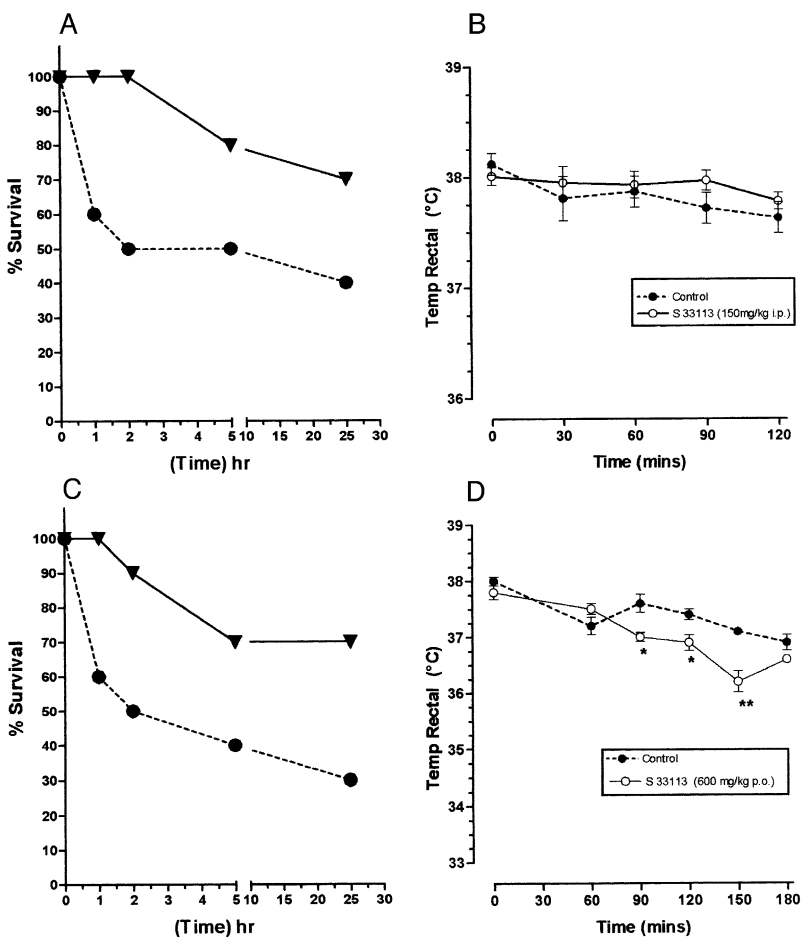

Fig. 1. Inhibition of *t*-butylhydroperoxide-mediated lethality. Male NMRI mice ($n = 10$/group) were injected (A) intraperitoneally (i.p.) (150 mg/kg) or (C) administered orally (600 mg/kg) with either vehicle (Tween–saline/water) (––●––) or S 33113 (–▼–) 30 or 60 min respectively, before an intracerebroventricular (i.c.v.) injection of *t*-butylhydroperoxide. Lethality was assessed and was expressed as the percentage survival. Data: median $n = 3$ experiments. The rectal temperature of male NMRI mice was measured at $t = 0$ h. Animals were then treated with either vehicle (––●––) or S 33113 (–○–) by either (B) intraperitoneal route (150 mg/kg) or (D) orally (600 mg/kg). Rectal temperature was assessed every 30 min for up to 2.5 h post-injection. Two-way analysis of variance (ANOVA) (treatment × time interaction) with a complementary Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$, vs. control at each time point). Data: mean ± S.E.M.; $n = 5$.

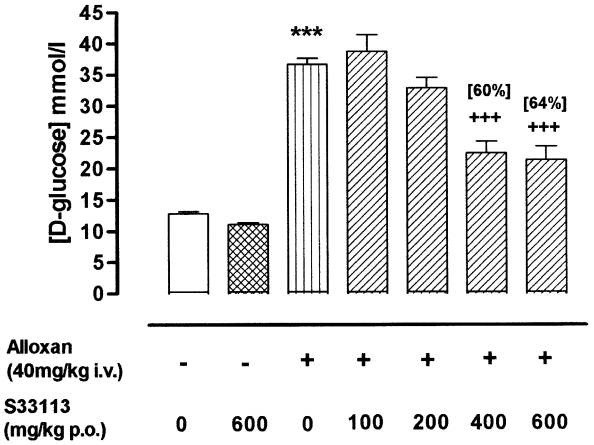

Fig. 2. Alloxan-mediated hyperglycaemia in NMRI mice. Male NMRI mice were administered by oral route with different doses (100–600 mg/kg) of S 33113; 60 min before an intravenous injection of alloxan (40 mg/kg). Animals were sacrificed at 24 h, the plasma was recovered and glucose levels were evaluated relative to standards. Data: mean ± S.E.M.; $n = 10$/group. One-way ANOVA with a complementary Newman–Keuls test: ($^{***}P < 0.001$ vs. vehicle; $^{+++}P < 0.05$ vs. alloxan group). Data in parenthesis represents the percentage inhibition compared to alloxan-treated controls.

mals were sacrificed at 24 h, the plasma recovered and D-glucose levels were evaluated by assay in a Roche Cobas-Fara Analyser.

2.2.5. Transient forebrain global ischaemia in Wistar rat

Transient forebrain ischaemia was induced by four-vessels occlusion according to the method of Pulsinelli and Brierley (1979). Male Wistar rats (280–320 g) were prepared for forebrain ischaemia under pentobarbital (60 mg/kg i.p.) anaesthesia. The vertebral arteries were definitively occluded by electrocauterisation and atraumatic clamps were placed around the carotid arteries without interrupting the arterial blood flow. The following day, animals were administered by i.p. route with the compound under study in Tween–saline (2 ml/kg) or with vehicle alone, and 30 min later cerebral ischaemia was induced in the unanaesthetised animal by tightening the clamps for 10 min. Carotid clamping results, within 1–2 min, in a loss of the righting reflex. Consequently, failure of animals to lose consciousness indicated that the ischaemia was not complete, and precluded the animal from the study. Body temperature was monitored within 2 h following administration of compound with a rectal temperature probe and animals were maintained (36.5–37.5°C) with heated lamps until recovery from ischaemia. Thereafter, animals were housed individually with free access to food and water. Seven days later animals were killed by decapitation, the brains were rapidly removed, and frozen at −30°C in isopentane and stored at −40°C until analysis. For each animal neuronal cell death was assessed in both hemispheres in three separate sections by counting viable cells in the CA1 field (1 mm^2) of the hippocampus (from 3.8 to 4.1 mm anterior to I.A. line) in 7 μm hematoxylin–eosin-stained brain sections.

2.2.6. Neuronal death induced by kainic acid in Wistar rat

Systemic administration of kainic acid induces seizures and neuropathological changes similar to those observed in human temporal lobe epilepsy (Ben-Ari , 1981). Male Wistar rats (140–160 g) were pre-treated by i.p. route with the compound under study or with vehicle (Tween/saline 2 ml/kg). Thirty minutes later, animals received a subcutaneous injection of kainic acid (12 mg/kg) and were individually housed and maintained under observation for altered behaviour. Rectal temperature was recorded every 30 min for 2 h following the drug injections. On days 3 or

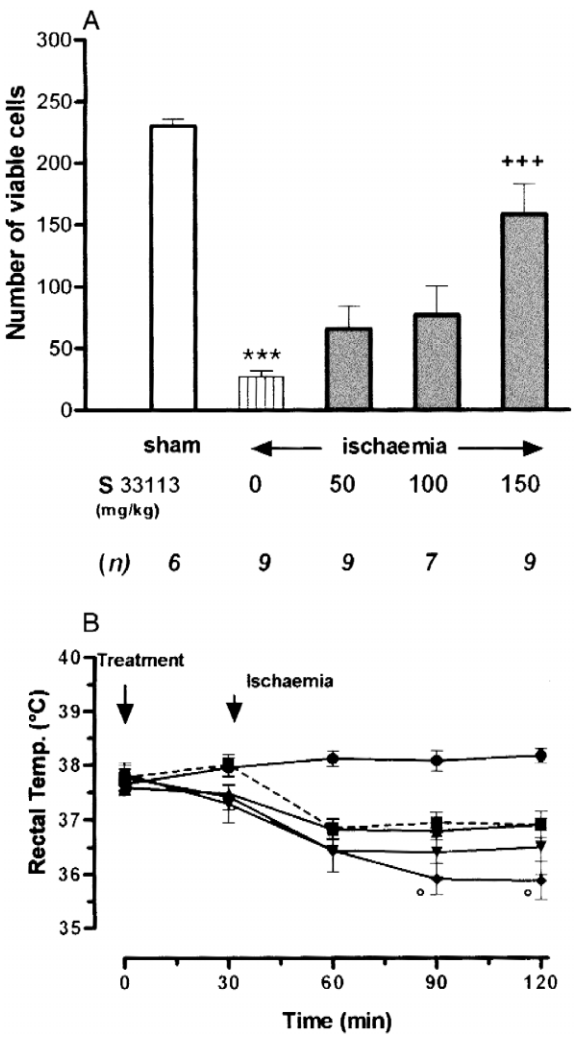

Fig. 3. Effect of S 33113 on neuronal death induced by transient global ischaemia. Male Wistar rats, were injected (50–150 mg/kg i.p.) with S 33113 or vehicle (Tween–saline), and 30 min later subjected to a transient global ischaemia. Seven days later (A) the number of viable cells in the CA1 hippocampal field were counted in brain slices. Analysis: One-way ANOVA with a complementary Newman–Keuls test ($^{***}P < 0.001$ vs. sham; $^{+++}P < 0.001$ vs. ischaemia). (B) Rectal temperature was monitored at the beginning of drug-treatment, and up to 1.5 h post-ischaemia. Sham-operated (–●–), vehicle-treated ischaemia (––■––), drug-treated ischaemia: 50 mg/kg (–▲–); 100 mg/kg (–▼–); 150 mg/kg (–◆–). Two-way ANOVA (treatment × time interaction) with a complementary Newman–Keuls test ($^{\circ}P < 0.05$; vs. ischaemic group); Data: mean ± S.E.M.; $n = 9$–12 animals/group.

7 post-exposure animals were sacrificed and the brains were rapidly removed, frozen at −30°C in isopentane and stored at −40°C until analysis. For each animal neuronal cell death was assessed in both hemispheres in three separate sections by counting viable cells in the CA1 and CA3 fields (1 mm^2) of the hippocampus (from 3.8 to 4.1 mm anterior to I.A. line) in 7 μm hematoxylin–eosin stained coronal sections.

2.2.7. Methamphetamine-induced dopamine depletion in striatum of C57BL/6 mice

C57BL/6 mice (20–25 g) were administered (5 mg/kg, i.p.) with D-methamphetamine four times at 2-h intervals as previously described (Cosi et al., 1996). Compounds were injected (150 mg/kg i.p. in Tween–saline or p.o. in Tween–water 20 ml/kg) 30 min before the first and third administrations of MA. Rectal temperature was regularly monitored during the period of drug administration. Three days later, mice were sacrificed by decapitation, brains were rapidly removed and both striata were collected, frozen in liquid nitrogen, and weighed. Striata were homogenised by sonication in 20 volumes of a perchloric acid solution (0.1 N) previously chilled on ice. Homogenates were centrifuged 20 min at 15000 × *g* (4°C) and supernatants were collected, frozen with liquid nitrogen and stored at −80°C until analysis. Striatal dopamine contents in supernatants were determined by high pressure liquid chromatography (HPLC) coupled to electrochemical detection. Monoamines were separated on a reverse phase column (MD150, ESA, EUROSEP, France). The mobile phase consisted of NaH_2PO_4 50 mM, 1-octane sulfonic acid 1.6 mM, acetonitrile 11%, adjusted to pH 3 with orthophosphoric acid and delivered at 0.35 ml/min flow rate. Monoamines were detected with a coulometric analytical cell (5011, ESA, EUROSEP, France) at +280 mV.

3. Results

The dihydroquinoline derivative S 33113 was previously shown by the present group to possess potent antioxidant activity (Dorey et al., 2000). Furthermore, the significant cerebral concentrations observed for S 33113 (26 μg/g brain) in untreated rat, 30 min after i.p. administra-

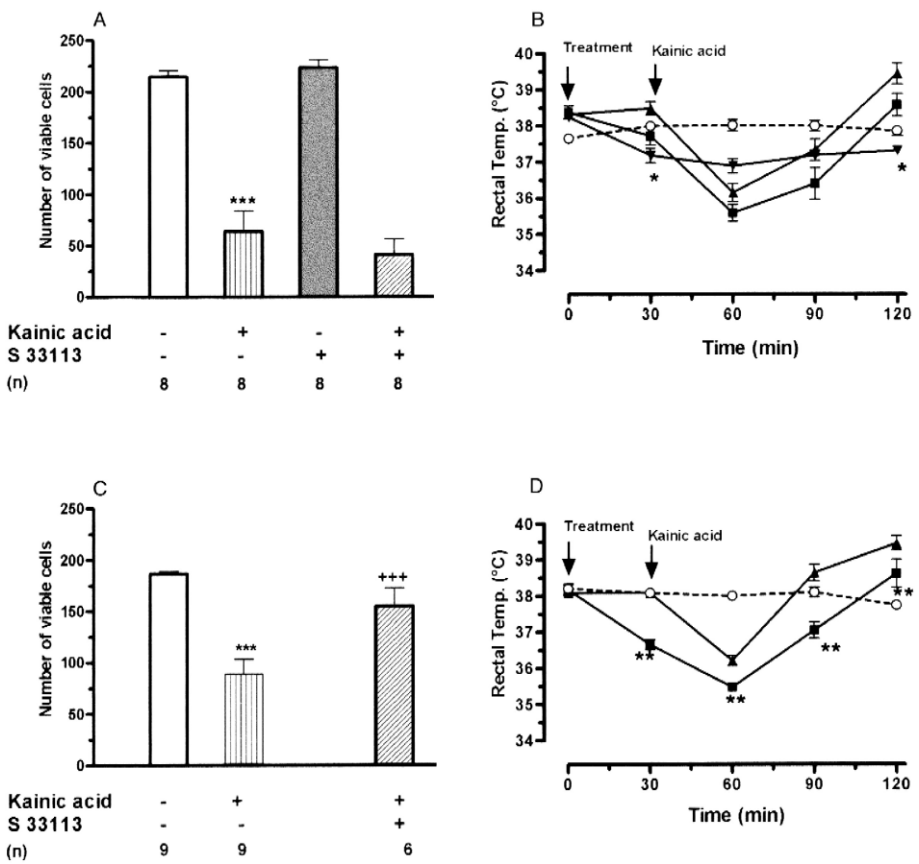

Fig. 4. Effect of S 33113 on Kainic acid-mediated neuronal death. Wistar rats were injected with S 33113 (150 mg/kg i.p.) 30 min before a systemic injection of kainic acid. The number of viable cells in the CA1 and CA3 hippocampal field were estimated in brain slices from animals sacrificed on (A) Group 1 day 3 (CA1); Two-way ANOVA with a post-hoc Newman–Keuls test: ($^{***}P < 0.001$ vs. control); or (C) Group 2 day 7 (CA3); One-way ANOVA with a post-hoc Newman–Keuls test: ($^{***}P < 0.001$ vs. control; $^{+++}P < 0.01$ vs. kainic acid). Rectal temperature was monitored at the beginning of drug-treatment, and up to 1.5 h following kainic acid injection. Vehicle controls (––○––); S 33113 (–▼–); kainic acid (–▲–) and S 33113/kainic acid-treated (–■–) groups. For reasons of clarity only the comparison of the groups kainate vs. kainate/S 33113 is presented ($^{*}P < 0.05$; $^{**}P < 0.01$). Data: mean ± S.E.M.; $n = 6–9$ animals/group.

tion (150 mg/kg), indicated that S 33113 was capable of passing the blood–brain barrier (M. Bertrand, unpublished observations). S 33113 possessed no apparent affinity (> 10000 nM) for a panoply of 28 different receptors as well as uptake systems, and demonstrated negligible inhibition of cyclooxygenase-1 (> 10 μM) with a slight inhibition of 5-lipoxygenase and cyclooxygenase-2 ($IC_{50} \sim 10$ μM) (Table 1). Moreover, S 33113 was a potent inhibitor of Fe^{2+}/H_2O_2/ascorbate-mediated lipid peroxidation in mouse cortical membranes with an EC_{50} value of 0.29 μM (0.20–0.38) [comparable to the EC_{50} value for ethoxyquin = 0.29 μM (0.15–0.33)].

Intracerebroventricular administration of the potent oxidant *t*-butylhydroperoxide in NMRI mice induced either instantaneously or within 5 min, seizures leading progressively (1–24 h) to respiratory failure and lethality for the majority of the treated animals (Fig. 1A and C). Pre-administration (30 min) with a single dose (150 mg/kg i.p.) of S 33113 resulted in a marked protection for up to 5 h post-exposure against the lethality induced by *t*-butylhydroperoxide (Fig. 1A). Although significant protection was also observed at 24 h, in general a high degree of variability was observed in terms of the percentage survival of control animals. Furthermore, in certain cases drug-treated animals at 24 h post-exposure, although not moribund demonstrated severe behavioural and motor deficits. Oral administration (600 mg/kg p.o.) of S 33113 60 min before injection of *t*-BHP also markedly reduced *t*-butylhydroperoxide-mediated lethality for up to 24 h (Fig. 1C). Monitoring of rectal temperature of NMRI mice treated with S 33113 indicated no significant effects on body temperature at the dose of 150 mg/kg i.p. in NMRI mice for up to 2 h post-injection compared to control animals (Fig. 1B). Furthermore, S 33113 (600mg/kg p.o.) did not significantly effect body temperature at 60 min post-injection whereas a significant but transient hypothermic effect (−0.4°C to −0.9°C) was observed between 90 and 150 min, relative to vehicle-treated animals (Fig. 1D).

Intravenous administration of alloxan (40 mg/kg i.v.) in NMRI mice resulted in a marked elevation (two- to three-fold) in glycaemia at 24 h post-exposure (Fig. 2). Pre-treatment (60 min) with S 33113 (100–600 mg/kg p.o.) produced a significant dose-dependent reduction in hyperglycaemia at 400 mg/kg (60%; $P < 0.001$) and 600 mg/kg (64%; $P < 0.001$) (Fig. 2). It must be noted that S 33113 alone, at 600 mg/kg p.o. only resulted in a moderate decrease of plasma glucose levels (14%; $P > 0.05$) in control animals.

Induction of transient global ischaemia by the four-vessels occlusion model in male Wistar rats resulted in a significant reduction (80–88% cell loss; $P < 0.001$) in the number of viable neurones in the CA1 hippocampal region 7 days post-ischaemia, compared to sham animals (Fig. 3A). A decrease in rectal temperature (−1°C) was ob-

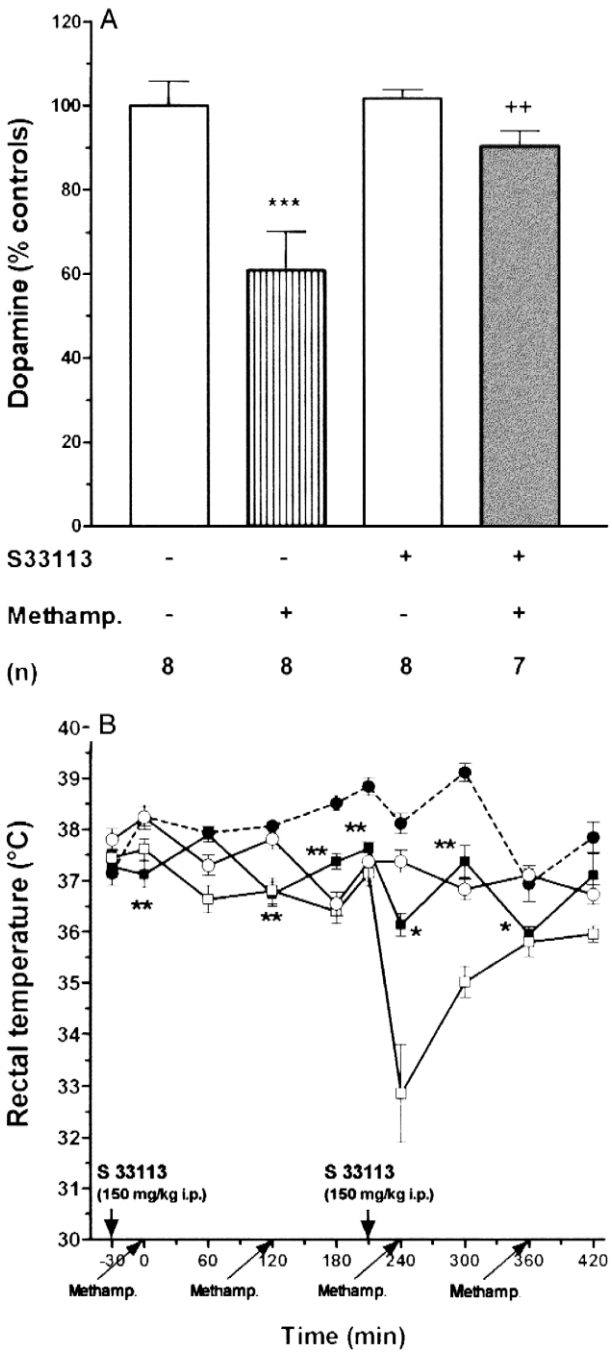

Fig. 5. Effect of S 33113 on the D-methamphetamine-mediated reduction in striatal dopamine levels. Male C57BL/6 mice were administered with D-methamphetamine (5 mg/kg, i.p.) four times at 2-h intervals. S 33113 was injected (150 mg/kg i.p.) 30 min before the first and third administrations of D-methamphetamine. Three days later, brains were analysed for striatal dopamine levels. Results are expressed as the percentage of control dopamine levels. Analysis: One-way ANOVA with a complementary Newman–Keuls test: ($^{***}P < 0.001$ vs. control; $^{++}P < 0.01$ vs. D-methamphetamine group). Rectal temperature was regularly monitored during the period of drug administration. The different groups consisted of vehicle controls (–○–); S 33113 alone (–□–); D-methamphetamine (––●––); S 33113/D-methamphetamine (–■–). One-way ANOVA with a complementary Newman–Keuls test at each time point. For reasons of clarity only the comparison of the groups D-methamphetamine vs. D-methamphetamine + S 33113 is presented ($^{*}P < 0.05$; $^{**}P < 0.01$). Data: mean ± S.E.M.; $n = 7–8$ animals/group.

served 30 min after ischaemia (Fig. 3B). Administration of S 33113, 30 min before ischaemia, prevented ischaemia-mediated neuronal cell loss in the CA1 region, partially at 50 mg/kg (71% cell loss; $P > 0.05$) and 100 mg/kg (69%; $P > 0.05$) and significantly at 150 mg/kg (33%; $P < 0.001$) (Fig. 3A). S 33113 (150 mg/kg i.p.)-treated animals exposed to global ischaemia also demonstrated a significant decrease (−1°C) in rectal temperatures relative to ischaemia-control animals, at 90 and 120 min post-ischaemia (Fig. 3B).

The systemic administration of kainic acid (12 mg/kg s.c.) resulted in a significant reduction (65% to 70%; $P < 0.001$) in the number of viable neurones in the CA1 hippocampal region at 3 days post-exposure (Fig. 4A) and in the CA3 hippocampal region (52%; $P < 0.001$) at seven days post-injection compared to untreated animals (Fig. 4C). Kainic acid induced a marked decrease (−1.9°C) in rectal temperatures in Wistar rats, 30 min post-injection, followed by an increase (+1.5°C) above control animal levels (Fig. 4B and D). S 33113 (150 mg/kg i.p.), administered 30 min before kainic acid injection, produced a highly significant reduction in neuronal cell loss in the CA3 hippocampal field at 7 days (17% cell loss; $P < 0.01$) (Fig. 4C) whereas no such effect (81%; $P > 0.05$) was observed in the CA1 field at 3 days post-exposure (Fig. 4A), compared to kainic-acid-treated controls. Administration of S 33113 alone, induced a moderate reduction (−1.0°C) in the rectal temperature of rats for up to 2 h post-exposure (Fig. 4B and D), whereas S 33113 tended to accentuate the kainic acid–mediated temperature decrease and correspondingly reduced ($P < 0.01$) kainic-acid-induced hyperthermic effects at specific time points (Fig. 4D).

C57BL/6 mice administered with D-methamphetamine (5 mg/kg, i.p.) four times at 2-h intervals demonstrated a significant reduction (39%; $P < 0.001$) in striatal dopamine levels compared to control animals at 3 days post-exposure (Fig. 5A). Furthermore, under these conditions D-methamphetamine induced a significant and maintained hyperthermia up to maximal levels of 2°C following successive D-methamphetamine administrations, compared to control animals (Fig. 5B). S 33113 alone, did not significantly modify striatal dopamine levels compared to control animals (Fig. 5A), and no significant modification of rectal temperature was observed after a single administration (150 mg/kg i.p.) of S 33113 compared to control animals (Fig. 5B). However, following the second administration of S 33113 a marked but transient decrease (−4.5°C) in rectal temperature was observed, (Fig. 5B). S 33113 markedly attenuated (11% decrease; $P < 0.01$) D-methamphetamine-mediated striatal dopamine depletion effects (Fig. 5A). Moreover, the hyperthermic effects of D-Methamphetamine MA were significantly ($P < 0.01$) at-

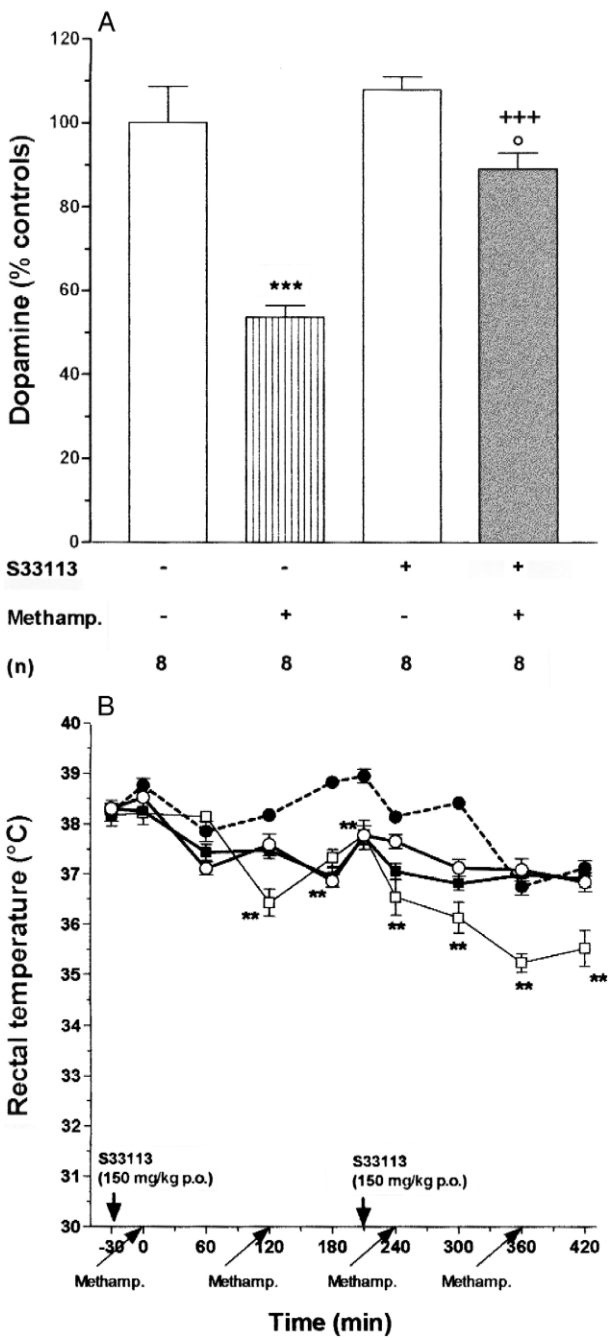

Fig. 6. Effect of oral administration of S 33113 on D-methamphetamine-mediated reduction in striatal dopamine levels. Male C57BL/6 mice were administered with D-methamphetamine (5 mg/kg, i.p.) four times at 2-h intervals. S 33113 (150 mg/kg p.o.) was administered 30 min before the first and third administrations of D-methamphetamine, and 3 days later brains were analysed for striatal dopamine levels. Results are expressed as the percentage of control dopamine levels. Analysis: One-way ANOVA with a complementary Newman–Keuls test: ($^{***}P <$ 0.001 vs. control; $^{\circ}P < 0.05$ vs. S 33113 group; $^{+++}P < 0.001$ vs. D-methamphetamine group). Rectal temperature was regularly monitored during the period of drug administration. The different groups consisted of vehicle controls (–○–); S 33113 alone (–□–); D-Methamphetamine (––●––) and S 33113/D-methamphetamine (–■–). One-way ANOVA with a complementary Newman–Keuls test at each time point. For reasons of clarity only the comparison of the groups D-methamphetamine vs. D-methamphetamine + S 33113 is presented ($^{**}P < 0.01$). Data: mean ± S.E.M.; $n = 8$ animals/group.

tenuated by administration of S 33113 (Fig. 5B). Under similar conditions D-methamphetamine-mediated striatal dopamine depletion (46.3% decrease: $P < 0.001$), oral administration of S 33113 (2×150 mg/kg p.o.) had no effect on control striatal levels of dopamine, but significantly attenuated (17% decrease; $P < 0.001$) D-methamphetamine-induced striatal dopamine depletion (Fig. 6A). Successive oral administrations of S 33113 alone, failed to significantly modify rectal temperature compared to control animals, whereas a significant ($P < 0.01$) and progressive decrease (up to -1.9°C) following the second administration of S 33113 in the presence of D-methamphetamine was observed, compared to D-methamphetamine-treated animals at specific time points (Fig. 6B).

4. Discussion

In the present study, we have carried out an extensive investigation of the neuroprotective potential of S 33113, a newly described dihydroquinoline antioxidant (Dorey et al., 2000), in different in vitro and in vivo paradigms of oxidative stress-mediated cellular degeneration. A previous study (Dorey et al., 2000) demonstrated that S 33113 potently inhibited L-homocysteic acid-mediated cell death (PC_{50} values = 0.18 μM) in HT-22 hippocampal neurones, whereas under similar conditions vitamin E, ebselen and MCI-186 possessed PC_{50} values of 0.8, 1.0 and 70.4 μM, respectively. The capacity of S 33113 to prevent L-homocysteic acid-mediated toxicity is likely to result from its ability to prevent lipid peroxidation propagation as S 33113 was a potent inhibitor ($IC_{50} = 0.29$ μM) of Fenton-induced lipid peroxidation in mouse cortical membranes. Indeed, under similar conditions other antioxidants such as ethoxyquin, butylated hydroxyanisole and propyl gallate prevented Fe^{2+}-mediated lipid peroxidation with IC_{50} values of 0.29, 2.8 and 13 μM, respectively. The considerable hydrophobicity of S 33113, based on calculated log P estimates (4.50) could also contribute to the potency in preventing lipid-peroxidation and L-homocysteic acid mediated toxicity.

In comparison with previous reports describing an anti-inflammatory activity of ethoxyquin based on cyclooxygenase/lipoxygenase inhibition (Spaethe et al., 1989), S 33113 demonstrated negligible inhibition of cyclooxygenase-1, cyclooxygenase-2 or 5-lipoxygenase activity. The radical scavenging activity of S 33113 is most likely of a similar mechanism as described for ethoxyquin (Thorisson et al., 1992; Taimir et al., 1993) and vitamin-E-related antioxidants, and is based primarily on the capacity of these molecules to react with peroxy radicals in order to interrupt the propagation of lipid peroxidation. Furthermore, ischaemic-mediated neuronal damage, alloxan-mediated destruction of the β-cells of the pancreas, and *t*-butylhydroperoxide-mediated cell damage are believed to be mediated by OH° generation (Adams et al., 1991, 1993; Heikkila et al., 1976; Lancelot et al., 1995), and consequently suggests that the protective mechanism of S 33113, could be associated with an ability to prevent OH°-dependent lipid peroxidation.

The capacity of S 33113, to prevent either kainic acid or ischaemia-reperfusion-mediated neuronal cell death, seven days after a single pre-administration of compound (150 mg/kg i.p.), illustrates the pivotal role of oxidative mechanisms immediately following glutamate receptor hyperactivity or reperfusion-ischaemia, respectively in the genesis of delayed neuronal death in the hippocampal region. In the present report, S 33113 only abrogated kainic acid-mediated cell death in the CA3 hippocampal region seven days post-injection, whereas no protective effect was observed in the CA1 region on day 3. The molecular and cellular events responsible for the selective vulnerability of hippocampal neurones to kainic acid are not yet fully understood, although the activation of kainate receptors, and subsequent free radical-mediated mechanisms of cell damage have been clearly established with in vitro (Bruce and Baudry, 1995) and in vivo (Ueda et al., 1997) systems. Previous studies have shown that kainate-mediated cell damage can be attenuated with radical scavengers (Miyamoto and Coyle, 1990; Bruce et al., 1992).

The neuroprotective action of S 33113 could also relate to its capacity to modify the temperature alterations induced by the different experimental paradigms. However, intraperitoneal administration of S 33113 in contrast to ethoxyquin significantly reduced butylhydroperoxide-mediated lethality (Dorey et al., 2000; this study), at doses (150 mg/kg i.p.) lacking any hypothermic effects in NMRI mice. Similarly, oral administration of S33113-1 (600 mg/kg) prevented butylhydroperoxide-mediated lethality at 1 h post-injection where no significant effects on body temperature were observed at this dose. S 33113 was also capable of attenuating alloxan-mediated hyperglycaemia at doses (400 mg/kg p.o.) lacking significant hypothermic effects (data not shown) in NMRI mice. In rat, on the other hand, S 33113 induced moderate effects on body temperature during the drug administration phase in both ischaemia-reperfusion and kainic acid protocols. Furthermore, S 33113 at doses lacking protective effects (50 and 100mg/kg i.p.) did not significantly modify ischaemia-induced hypothermia, and S 33113 (2×150 mg/kg i.p. or p.o.) was capable of significantly reducing D-methamphetamine-mediated hyperthermia. These observations, in contrast to a previous report (Cappon et al., 1996) do not appear to dissociate the attenuation of D-methamphetamine-mediated hyperthermia from neuroprotective activity, although Miller and O'Callaghan (1994) indicated that alterations in body temperature can affect D-methamphetamine-mediated striatal dopamine depletion.

The precise mechanisms underlying the moderate hypothermic action of S 33113, in mice and rat, at the doses tested in the present study is not fully understood. How-

ever, it is possible that the temperature effects may be exclusively peripheral, namely, dose-dependent metabolic effects as previously described for ethoxyquin (Burka et al., 1996) or as a result of interference with energy pathways, namely mitochondrial electron transport (Reyes et al., 1995) and/or ATPase activity (Hernandez et al., 1993). However, centrally mediated thalamic effects, which could influence peripheral temperature, may also be implicated. Nevertheless, although S 33113 produced considerably less effects on body temperature in mice relative to ethoxyquin at equivalent doses (Dorey et al., 2000), the present data does not permit a clear dissociation of the protective effects of S 33113 in the different paradigms from any effects on body temperature. However, it must be noted that in the present study only rectal temperatures were monitored, and that effects on core cerebral temperature may also be present, and thus contribute to the protective action of S 33113 in the different experimental paradigms. Consequently, in addition to the antioxidant activity of S 33113 it cannot be excluded that the protective effects of these compounds in the different experimental pathologies presented herein, may be associated with the hypothermic effects of these compounds. Although previous studies indicated that *t*-butyl-phenylnitrone attenuated neuronal cell loss in global ischaemia (Yue et al., 1992) and methamphetamine-mediated depletion of striatal dopamine (Cappon et al., 1996) at doses producing no significant peripheral hypothermia, in both these studies the effects on core cerebral temperatures were not evaluated. The potential implication, and indeed importance of brain hypothermia in abrogating neuronal cell damage in different pathological paradigms cannot be overstated. Hypothermia can reduce intracranial pressure after haemorrhage, preserve blood–brain barrier function, improve glucose utilisation, reduce brain metabolism, diminish free radical production and lipid peroxidation and attenuate neuronal cell death (for a review see: Dietrich et al., 1996). Indeed, hypothermia significantly reduces ischaemia-mediated free radical production and neuronal cell loss in ischaemia reperfusion models (Ginsberg et al., 1992; Dietrich et al., 1993; Kil et al., 1996) and drug-induced alterations in D-methamphetamine-mediated hyperthermia can attenuate D-methamphetamine-mediated depletion of striatal dopamine (Miller and O'Callaghan, 1994).

The present study, based on the neuroprotective action of S 33113 in *t*-butylhydroperoxide-mediated lethality, ischaemia-reperfusion, and kainic-acid-mediated cell death, argues in favour of drug penetration into the CNS. However, certain of the neurodegenerative paradigms used in the present study may jeopardise the integrity of the blood brain barrier (Dietrich et al., 1990), and consequently permit a non-selective entry of compounds into the cerebral tissue. Indeed, the invasive penetration of brain tissue and rupture of the blood brain barrier following i.c.v. administration of *t*-butylhydroperoxide, followed by any subsequent oxidative-mediated damage could contribute to the facility of S 33113 penetration into the brain in order to exert its protective effect. However, the significant cerebral concentrations observed for S 33113 (26 μg/g brain) in untreated rat, 30 min after intraperitoneal administration (150 mg/kg), provides direct evidence that S 33113 was capable of passing the intact blood brain barrier (M. Bertrand, unpublished observations).

Pre-administration of S 33113 in the different experimental paradigms implicating free radical-induced neuronal cell death, resulted in a significant abrogation of both acute and chronic oxidative stress-mediated cell damage. Further studies will be required to more fully assess the neuroprotective potential of S 33113, in both acute and chronic post-administration protocols. However, based on the present studies, S 33113 represents a novel orally active dihydroquinoline compound, possessing neuroprotective activity in vivo, with a potentially promising therapeutic profile for cerebral pathologies implicating oxidative stress.

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References

Adams, J.D., Klaidman, L.K., Huang, Y.M., Cheng, J.J., Wang, Z.J., Nguyen, M., Knusel, B., Kuda, A., 1991. The neuropathology of intracerebroventricular *t*-butylhydroperoxide. Mol. Chem. Neuropathol. 26, 95–106.

Adams, J.D., Wang, B., Klaidman, L.K., LeBel, C.P., Odunze, I.N., Shah, D., 1993. New aspects of brain oxidative stress induced by *tert*-butylhydroperoxide. Free Radical Biol. Med. 15, 195–202.

Ben-Ari, Y., 1981. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience 14, 375–403.

Bruce, A.J., Baudry, M., 1995. Oxygen-free radicals in rat limbic structures after kainic acid-induced seizures. Free Radical Biol. Med. 18, 993–1002.

Bruce, A.J., Najm, I., Malfroy, B., Baudry, M., 1992. Effects of desferoxamine/manganese complex, a superoxide dismutase-mimic, on kainate-induced pathology in rat brain. Neuroscience 13, 265–271.

Burka, L.T., Sanders, J.M., Matthews, H.B., 1996. Comparative metabolism and disposition of ethoxyquin in rat and mouse: II. Metabolism. Xenobiotica 26, 597–611.

Cao, X., Phillis, J.W., 1994. α-Phenyl-*tert*-butyl-nitrone reduces cortical infarct and edema in rats subjected to focal ischaemia. Brain Res. 644, 267–272.

Cappon, G.D., Broening, H.W., Pu, C., Morford, L., Vorhees, C.V., 1996. α-Phenyl-*N*-*tert*-Butyl-Nitrone attenuates methamphetamine-induced depletion of striatal dopamine without altering hyperthermia. Synapse 24, 173–181.

Casara, P., Dorey, G., Lestage, P., Lockhart, B., 1999. EP99402624.3-21014, ADIR and Compagnie, Courbevoie, France.

Comfort, A., 1971. Effect of ethoxyquin on the longevity of C3H mice. Nature 229, 254–255.

Cosi, C., Chopin, P., Marien, M., 1996. Benzamide, an inhibitor of poly(ADP–ribose) polymerase, attenuates methamphetamine-induced dopamine neurotoxicity in the C57BL/6 mouse. Brain Res. 735, 343–348.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689–695.

De Keyser, J., Sulter, G., Luiten, P.G., 1999. Clinical trials with neuroprotective drugs in acute ischaemic stroke: are we doing the right thing? Trends Neurosci. 22, 535–540.

Dietrich, W.D., Busto, R., Valdes, I., Halley, M., 1990. The importance of brain temperature in alterations of the blood–brain barrier during brain ischemia. J. Neuropathol. Exp. Neuro. 49, 486–497.

Dietrich, W.D., Busto, R., Alonso, O., Globus, M.Y.T., Ginsberg, M.D., 1993. Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. J. Cereb. Blood Flow. Metab. 13, 541–549.

Dietrich, W.D., Busto, R., Globus, M.Y.T., Ginsberg, M.D., 1996. Brain damage and temperature: cellular and molecular mechanisms. Adv. Neurol. 71, 177–194.

Dorey, G., Lockhart, B.P., Lestage, P., Casara, P., 2000. New quinolinic derivatives as centrally active antioxidants. Bio. Org. Chem. Lett. 10, 935–939.

Ginsberg, M.D., Sternau, L.L., Globus, M.Y.T., Dietrich, W.D., Busto, R., 1992. Therapeutic modulation of brain temperature: relevance to ischemic brain injury. Cerebrovasc. Brain Metab. Rev. 4, 189–225.

Hall, E.D., Braughler, J.M., Yonkers, P.A., Smith, S.L., Linseman, K.L., Means, E.D., Scherch, H.M., Von, V.P., Lahti, R.A., Jacobsen, E.J., 1991. U-78517F: a potent inhibitor of lipid peroxidation with activity in experimental brain injury and ischaemia. J. Pharmacol. Exp. Ther. 258, 688–694.

Harman, D., Eddy, D.E., Noffsinger, J., 1976. Free radical theory of ageing: inhibition of amyloidosis in mice by antioxidants; possible mechanism. J. Am. Geriatr. Soc. 24, 203–210.

Hernandez, M.E., Reyes, J.L., Gomez-Lojero, C., Sayavedra, M.S., Melendez, E., 1993. Inhibition of renal uptake of *p*-aminohippurate and tetraethylammonium by the antioxidant ethoxyquin in the rat. Food Chem. Toxicol. 5, 363–367.

Heikkila, R., Winston, B., Cohen, G., 1976. Alloxan-induced diabetes: evidence for hydroxyl radical as a cytotoxic intermediate. Biochem. Pharmacol. 25, 1085–1092.

Kil, H.Y., Zhang, J., Piantadosi, C.A., 1996. Brain temperature alters hydroxyl radical production during cerebral ischemia/reperfusion in rats. J. Cereb. Blood Flow. Metab. 16, 100–106.

Lancelot, E., Callebert, J., Revaud, M.L., Boulu, R.G., Plontkine, M., 1995. Detection of hydroxyl radicals in rat striatum during transient focal ischaemia: possible implication in tissue damage. Neurosci. Lett. 19, 785–788.

Levy, L., 1976. The anti-inflammatory action of some compounds with antioxidant properties. Inflammation 1, 333–345.

Miller, D.B., O'Callaghan, J.P., 1994. Environment, drug- and stress-induced alterations in body temperature affect the neurotoxicity of substituted amphetamines in the C57BL/6J mouse. J. Pharmacol. Exp. Ther. 270, 752–760.

Miyamoto, M., Coyle, J.T., 1990. Idebenone attenuates neuronal degeneration induced by intrastriatal injections of excitotoxins. Exp. Neurol. 108, 38–45.

Pulsinelli, W.A., Brierley, J.B., 1979. A new model of bilateral hemispheric ischaemia in the unanaesthetised rat. Stroke 10, 267–272.

Reiter, R.J., 1995. Oxidative processes and antioxidative defence mechanisms in the aging brain. FASEB J. 9, 526–533.

Reyes, J.L., Hernandez, M.E., Melendez, E., Gomez-Lojero, C., 1995. Inhibitory effect of the antioxidant ethoxyquin on electron transport in the mitochondrial respiratory chain. Biochem. Pharmacol. 49, 283–289.

Sack, C.A., Socci, D.J., Crandall, B.M., Arendash, G.W., 1996. Antioxidant treatment with phenyl-α-*tert*-butyl nitrone (PBN) improves the cognitive performance and survival of ageing rats. Neurosci. Lett. 205, 181–184.

Schulz, J.B., Matthews, R.T., Jenkins, B.G., Brar, P., Flint Beal, M., 1995. Improved therapeutic window for treatment of histotoxic hypoxia with a free radical spin trap. J. Cereb. Blood Flow Metabol. 15, 948–952.

Smith, M.L., Zhao, Q., Pahlmark, K., Siesjö, K., 1995. Delayed treatment with a spin trap reduces infarct size in focal ischemia. J. Cereb. Blood Flow Metab. suppl. 15, S53.

Socci, D., Crandall, B., Andorn, A., 1995. Chronic antioxidant treatment improves the cognitive performance of aged rats. Brain Res. 693, 88–94.

Spaethe, S.M., Hsueh, W., Needleman, P., 1989. Pharmacological manipulation of inflammation in rabbit hydroephrosis: effects of combined cyclooxygenase/lipoxygenase inhibitor ethoxyquin, a thromboxane synthase inhibitor RS-5156 and a PAF antagonist L-659,989. J. Pharmacol. Exp. Ther. 248, 1308–1316.

Taimir, L., Smelhausova, M., Prusikova, M., 1993. The reaction of 1-cyano-1-methylethyl radical with antidegradant ethoxyquin and its aminyl and nitroxide derivatives. Die Angew. Makromol. Chem. 206, 199–207.

Thorisson, S., Gunstone, F.D., Hardy, R., 1992. Some oxidation products of ethoxyquin including those found in autoxidising systems. Chem. Phys. Lipids 60, 263–271.

Ueda, Y., Yokoyama, H., Niwa, R., Konaka, R., Ohya-Nishiguchi, H., Kamada, H., 1997. Generation of lipid radicals in the hippocampal extracellular space during kainic acid-induced seizures in rats. Epilepsy Res. 26 (2), 329–333.

Watanabe, T., Satoshi, Y., Egawa, M., Nishi, H., 1994. Protective effects of MCI-186 on cerebral ischaemia: possible involvement of free radical scavenging and antioxidant actions. J. Pharmacol. Exp. Ther. 268, 1597–1604.

Yeh, S.Y., 1999. *N-tert*-Butyl-alpha-phenylnitrone protects against α,′-Methylene-dioxymethamphetamine-induced depletion of serotonin in rats. Synapse 31, 169–177.

Yue, T.L., Gu, J.L., Lysko, P.G., Cheng, H.Y., Barone, F.C., Feuerstein, G., 1992. Neuroprotective effects of phenyl-*t*-butyl-nitrone in gerbil global brain ischemia and in cultured rat cerebellar neurones. Brain Res. 574, 193–197.