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European Journal of Pharmacology 511 (2005) 127–136

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In vivo neuroprotective effects of the novel imidazolyl nitrone free-radical scavenger (*Z*)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone (S34176)

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Received 24 June 2004; received in revised form 24 January 2005; accepted 28 January 2005
Available online 17 March 2005

Abstract

Herein, we report an extensive investigation of the neuroprotective effects of the compound (*Z*)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone (S34176) and the prototypic nitrone α-phenyl-*N*-*tert*-butylnitrone (PBN), in different in vivo paradigms of neuronal degeneration. Administration of S34176 (75 mg/kg i.p.) 30 min before transient (10 min) global ischaemia in Wistar rats significantly prevented delayed neuronal cell death in the hippocampal CA1 area 7 days post-ischaemia (24% vs. 73% in ischaemia control; $P<0.05$) whereas PBN was inactive under similar conditions. Furthermore, oral administration of S34176 (30 mg/kg) 60 min before and during (1×30 mg/kg p.o.) 6 days post-ischaemia, in combination with an acute post-ischaemia sub-protective dose (3×10 mg/kg i.p.) of the glutamate receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), resulted in an increased neuroprotective action (29% cell loss in drug-treated vs. 84% in ischaemia control $P<0.001$) compared to either compound alone. S34176 (20 mg/kg i.p.) also partially prevented kainic acid-induced neuronal cell death at 7 days post-exposure in the CA1 (41% in drug-treated vs. 74% for kainate-treated controls; $P<0.01$) and CA3 hippocampal region (22% vs. 53%; $P<0.01$). Under similar conditions, S34176 administered orally (40 mg/kg) produced a more marked protection against kainate-induced neuronal cell loss in the CA1 (13% in drug-treated vs. 82%; $P<0.001$) and CA3 areas (10% vs. 52%; $P<0.001$). Sub-chronic oral administration of S34176 (10 mg/kg) also partially reduced kainate-induced hippocampal cell death in the CA1 (53% vs. 77%; $P<0.01$) and CA3 (23% vs. 53%; $P<0.01$) areas. Dopamine depletion in the striatum of C57BL/6 mice induced by systemic D-methamphetamine injection was significantly reduced by S34176 (40±5% vs. 11.5±8%; $P<0.001$) (150 mg/kg i.p.) whereas PBN was inactive under similar conditions. S34176 represents a new centrally acting nitrone-based radical scavenger with neuroprotective properties in in vivo models of delayed neuronal cell death, and supports the therapeutic potential of this class of compound for the treatment of cerebral pathologies implicating chronic neurodegeneration.

Keywords: Neurodegeneration; Free radical scavenger; Ischaemia; Kainic acid; S34176; Nitrone

1. Introduction

Free radical-mediated cell damage has been suggested to play a pivotal role in both the ageing and neurodegenerative processes (Coyle and Puttfarcken, 1993; Reiter, 1995), and extensive efforts have been undertaken over the last 20 years to develop novel antioxidants capable of scavenging free radicals and/or preventing subsequent radical-mediated damage. Indeed, several different "classes" of antioxidants including the spin-trappers α-phenyl-*N*-*tert*-butylnitrone (PBN), *N*-*tert*-butyl-α-(2-sulphophenyl)-nitrone (S-PBN) and MDL101,002 or lipid peroxidation inhibitors such as U-78517F and edaravone 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

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doi:10.1016/j.ejphar.2005.01.043

(MPTP)-mediated dopaminergic dysfunction (Hall et al., 1991; Cao and Phillis, 1994; Watanabe et al., 1994; Schulz et al., 1995; Cappon et al., 1996; Yeh, 1999; Johnson et al., 1998). Paradoxically, only a limited number of centrally acting antioxidants, including ebselen and edaravone, have been proposed for clinical development for acute phase cerebral ischaemia but have demonstrated negligible or no clinical benefits (De Keyser et al., 1999). Consequently, the search for novel free radical scavengers with improved clinical efficacy and safety margins has continued as a potential therapeutic strategy for neurodegenerative disorders (Lapchak and Araujo, 2002). In this aim, the prototypic spin-trapping compound PBN, has attracted considerable attention over the last 10 years based on its protective effects in a multitude of pre-clinical paradigms based on oxidative-stress-mediated cell damage (for a review see: Floyd et al., 2002; Lapchak and Araujo, 2002). Consequently, additional nitrone-based analogues have been synthesised in an effort to develop novel compounds for the treatment of neurodegenerative disorders (Belayev et al., 2002; Durand et al., 2003; Thomas et al., 1997). A sulphonated analogue of PBN (disodium 4-(-[tert-butylimino)-methyl]benzene-1,3-disulphonate N-oxide; NXY-059) with an improved clinical safety profile (Lees et al., 2001) currently in active phase II trials for cerebral stroke, has demonstrated neuroprotective effects and improvement of functional scores in various preclinical models of stroke including; permanent focal ischaemia in the rat (Zhao et al., 2001; Kuroda et al., 1999; Sydserff et al., 2002) and marmoset monkey (Marshall et al., 2001). Interestingly, although NXY-059 has been shown not to pass the blood-brain barrier (Sydserff et al., 2002; Kuroda et al., 1999), a recent study has demonstrated that the permeability of NXY-059 is increased during prolonged ischaemia in vitro, probably resulting from a decreased integrity of this barrier (Dehouck et al., 2002).

Consequently, based on the wide-spectrum free radical scavenging properties of nitrone compounds and their efficacy in several in vivo models of neuronal degeneration, we developed a series of novel imidazolylnitrone compounds (Dhainaut et al., 2000) in order to evaluate their potential neuroprotective capacity. These studies permitted the selection of the derivative (*Z*)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone (S34176) (Fig. 1) with in vivo activities at equimolar doses to PBN (Dhainaut et al., 2000). Furthermore, preliminary studies demonstrated that S34176 and related imidazolylnitrones were able to trap superoxide and methyl radicals generated by chemical reactions in vitro (Lockhart et al., 2001a,b). In the present study, we report an extensive characterisation of the neuroprotective potential of S34176 in different in vivo paradigms of delayed neuronal cell death known to implicate oxidative stress (transient cerebral ischaemia, D-methamphetamine-induced dopaminergic dysfunction and kainic acid-induced hippocampal neurotoxicity) (Bruce and Baudry, 1995; Schulz et al., 1995; Cappon et al., 1996; Ueda et al., 1997). Preliminary results on the neuroprotective properties of S34176 were previously presented in abstract form (Lockhart et al., 2001a).

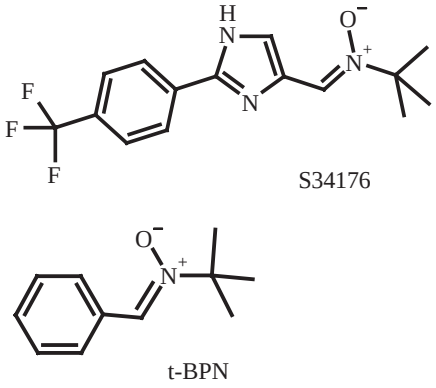

Fig. 1. Chemical structures of PBN (α-phenyl-*N*-*tert*-butylnitrone) and S34176 (Z)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone.

2. Materials and methods

2.1. Materials

Tert-butylphenylnitrone (PBN), kainic acid, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), were obtained from Sigma, France. D-methamphetamine was supplied by Calaire Chimie, France. S34176 [(*Z*)-α-[2-thiazol-2-yl)imidazol-4-yl]-*N*-*tert*-butylnitrone] was synthesised at Institut de Recherches Servier. Animals [Wistar rats, C57/BL6 mice] were housed in conditions conforming to EEC directive 86/609 (ILAR 1985). Environmental conditions were maintained as follows: air ventilation at 15 renewals per hour, 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 local ethical committee at Servier.

2.2. Methods

2.2.1. 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 either pre or post-ischaemia by i.p. route with the compound under study in Tween/saline (2 ml/kg) or with vehicle alone, 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 (Physitemp, Bat-12) 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. Statistical analyses was performed with a two-way analysis of variance (ANOVA) with a complementary Newman-Keuls test.

2.2.2. 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 day 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 in both hemispheres (from 3.8 to 4.1 mm anterior to I.A. line) in 7 μm hematoxylin-eosin stained coronal sections. Statistical analyses were performed with a two-way ANOVA with a complementary Newman-Keuls test.

2.2.3. 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 (MA) four times at two hour

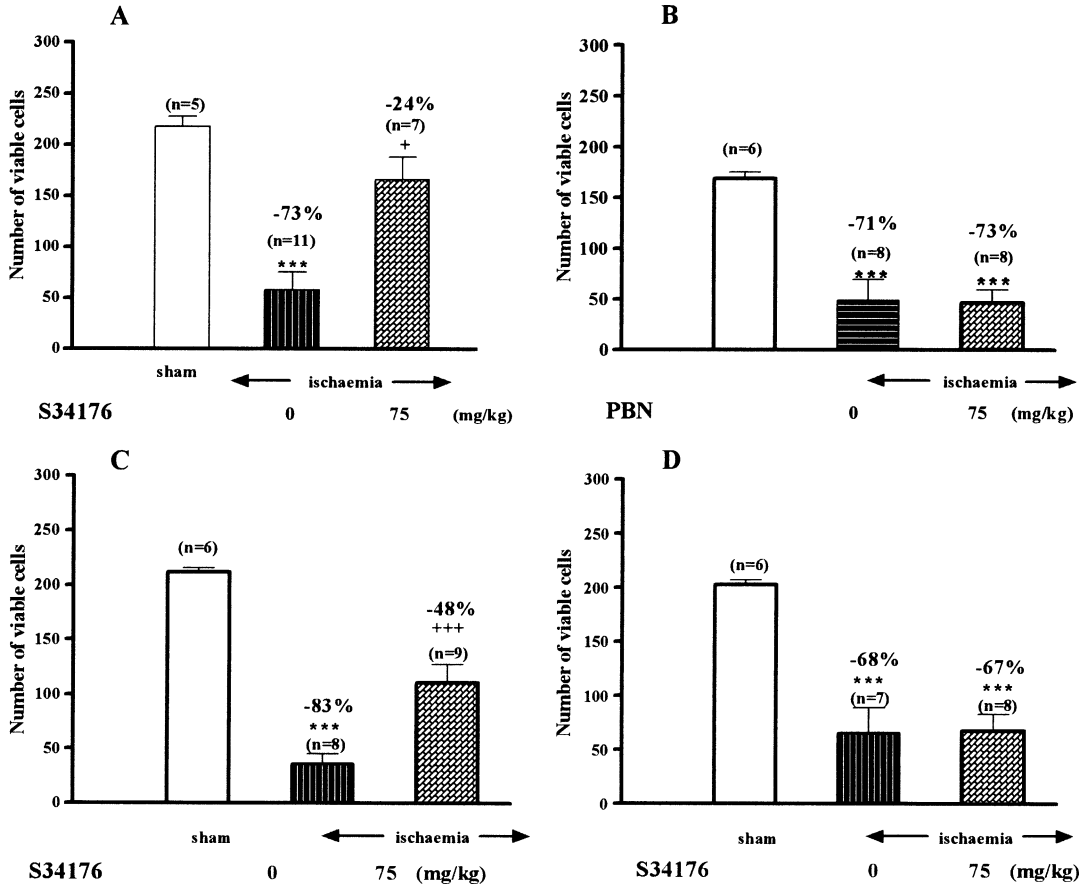

Fig. 2. Effect of S34176 and PBN on hippocampal neuronal death induced by transient global ischaemia. Male Wistar rats, were injected (75 mg/kg i.p.) with either S34176 or PBN or vehicle (Tween/saline) either 30 min before (A) and (B) a 10 min transient global ischaemia or in the case of S34176 5 min after (C) or 3 h after (D) transient global ischaemia. Seven days later the number of viable cells in the CA1 hippocampal field was counted in brain slices. Analysis: Student's *t*-test vs. sham-ischaemia with a two-way ANOVA (ischaemia×S34176) ***P<0.001, with complementary analysis (***P<0.001 vs. sham; ^{+}P<0.05, ^{+++}P<0.001 vs. ischaemia). Data: mean±S.E.M.; (n=number of animals/group). % Values indicate decrease in cell numbers vs. sham control.

intervals as previously described (Cosi et al., 1996). Compounds were injected (150 mg/kg i.p. in Tween/saline 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 15,000×*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. Statistical analyses was performed by a two-factor ANOVA with a complementary Newman-Keuls test.

3. Results

Pharmacokinetic parameters determined after low dose oral administration of S34176 (2 mg/kg) in the Wistar rat, indicated significant cerebral levels (measured by LC/MS) up to 8 h post-administration (0.86±0.14 μg/g brain; n=3), and at 16 h, levels in the order of 0.22 μg/ml were still observed (M. Bertrand, personal communication). Significant, circulating plasma levels of S34176 were detected (0.298±0.036 μg/ml) between 0.5 and 8 h post-administration. These data indicated that S34176 after low dose oral administration in the rat, had good oral bioavailability ($F_{calc.}$=86%) and a moderate $t_{1/2}$ value=2.5 h, with good blood brain barrier permeability.

S34176 also possessed no apparent affinity (IC_{50}>10,000 nM) for a panoply (~80) of different receptors, enzymes and uptake systems (CEREP, France). Monitoring of body temperature and gross behavioural parameters of Wistar rats treated with different doses (10–100 mg/kg p.o.) of S34176 indicated only a moderate (~0.5 °C) and transient (<60 min) decrease in body temperature at the dose of 60 mg/kg p.o. relative to vehicle-treated animals without any significant behavioural effects. At 100 mg/kg p.o. a more sustained but reversible hypothermic effect (−1.0 °C) was observed for up to 3 h post-administration without any significant behavioural manifestations apart from transient lethargy.

Induction of transient global ischaemia (10 min) by the four-vessels occlusion model in male Wistar rats resulted in a significant reduction (68–83% cell loss; P<0.001) in the number of viable neurones in the CA1 hippocampal region seven days post-ischaemia, compared to sham animals (Fig. 2A, D). Administration of S34176 (75 mg/kg i.p.), 30 min before ischaemia, prevented neuronal cell loss in the CA1 region (24% cell loss vs. 73% in ischaemia control; P<0.05) (Fig. 2A) whereas under similar experimental conditions PBN was inactive (73% cell loss vs. 71% in ischaemia control; P>0.05) (Fig. 2B). Furthermore, S34176 (75 mg/kg i.p.) injected 5 min after ischaemia also reduced cell loss in

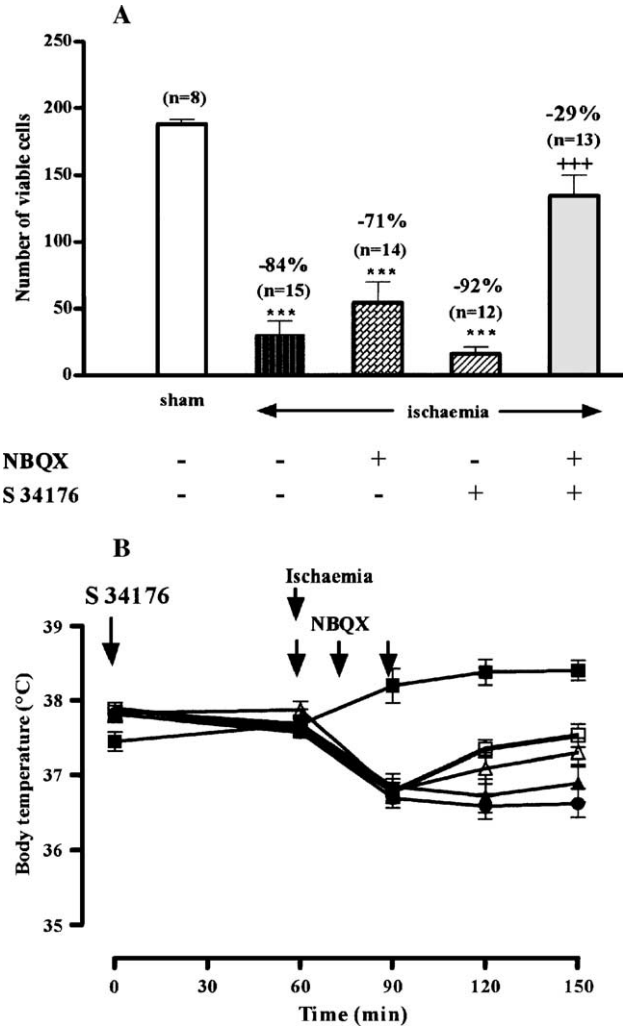

Fig. 3. Effect of combined S34176 and NBQX treatments on transient global ischaemia-mediated hippocampal cell loss. (A) Male Wistar rats subjected to a 10 min transient global ischeamia, were treated with either vehicle (Tween/distilled water) or reiterated sub-protective doses with NBQX (3×10 mg/kg i.p., at 0, 15, 30 min post-ischaemia) or by administration with S34176 (1×30 mg/kg p.o.) 60 min before, and during 6 days post-ischaemia at a similar dose or with a combination of the above treatments. Seven days later the number of viable cells in the CA1 hippocampal field was counted in brain slices. Analysis: Two-way ANOVA (ischemia×drug treatment) ***P<0.001, with complementary analysis (***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 (-△-), NBQX alone (-▲-); S34176 (-□-); NBQX/S34176 (-●-). Data: mean±S.E.M.; n=8–15 animals /group. % Values indicate decrease in cell numbers vs. sham control.

the CA1 hippocampal field (48% vs. 83% in ischaemia controls; $P<0.001$) (Fig. 2C), whereas no protective effect was observed when compound was administered 3 h post-ischaemia (67% cell loss vs. 68% in ischaemia controls; $P>0.05$) (Fig. 2D). In order to further evaluate the neuroprotective action of S34176 in this neurodegenerative paradigm we investigated the effect of a combination of a free radical scavenger with a glutamate receptor antagonist compared to either compound alone. In this aim, S34176 was administered 60 min before (1×30 mg/kg p.o.) and during 6 days post-ischaemia at a similar dose, in combination with acute reiterated sub-protective doses (3×10 mg/kg i.p., at 0, 15, 30 min post-ischaemia) of the AMPA receptor antagonist, NBQX. A significantly superior neuroprotective effect (29% cell loss in drugs-treated vs. 84% in ischaemia control $P<0.001$) compared to either compound alone; NBQX (71% cell loss $P>0.05$ vs. ischaemia-control) or S34176 (92% cell loss $P>0.05$ vs. ischaemia-control) was observed (Fig. 3A). Furthermore, a moderate decrease in rectal temperature (−1 °C) was obtained 30–60 min after ischaemia in the absence and presence of S34176 or different NBQX administrations (Fig. 3B).

The systemic administration of kainic acid (12 mg/kg s.c.) resulted in a significant reduction in the number of viable neurones in the CA1 (74–82% cell loss; $P<0.01$ vs. sham control) (Figs. 4A,C and 5B) and CA3 hippocampal areas (53% cell loss; $P<0.001$ vs. sham controls) at seven days post-injection (Fig. 4B,D). S34176 (1×20 mg/kg i.p.), administered 30 min before kainic acid injection, produced a highly significant reduction in neuronal cell loss in the CA1 hippocampal field at 7 days (41% cell loss for drug-treated vs. 74% for kainate-treated controls; $P<0.01$) (Figs. 4A and 5C). A comparable protective effect was also observed in the CA3 field (22% cell loss for drug-treated vs. 53% for kainate-treated controls; $P<0.01$) (Fig. 4B). Similarly, a single oral pre-administration (1×40 mg/kg) of S34176 produced a marked reduction in neuronal cell loss in the CA1 (13% cell loss for drug-treated vs. 82% for kainate-treated controls; $P<0.001$) and CA3 (10% cell loss for drug-treated vs 52% for kainate-treated controls; $P<0.001$) (Figs. 4C,D and 5D). Under similar experimental conditions no significant protection was observed for S34176 at 10 mg/kg p.o. compared to kainic-acid-treated controls (data not shown). However, a single administration of S34176 (10

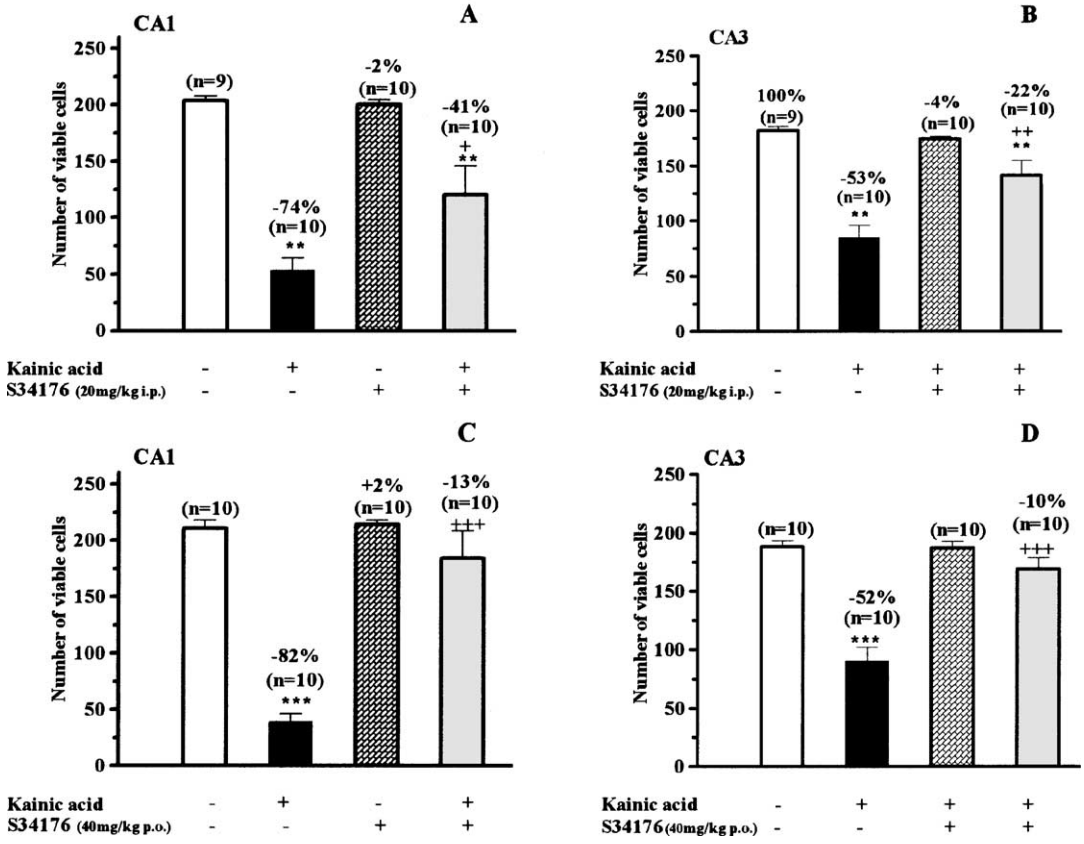

Fig. 4. Effect of S34176 on Kainic acid-mediated neuronal death. Wistar rats were injected with S34176 (20 mg/kg i.p.) (A) and (B) 30 min before a systemic injection of kainic acid (12 mg/kg s.c.) or administered by oral route with (C) and (D) S34176 (40 mg/kg p.o.) 1 h before kainic acid injection. The number of viable cells in the CA1 and CA3 hippocampal field were estimated in brain slices from both sets animals sacrificed on day 7. Analysis: Two-way ANOVA (Kainic acid×S34176) ***$P<0.001$, with complementary analysis (**$P<0.01$, vs. sham-control; $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ vs. kainic acid). Data: mean±S.E.M.; n=9–10 animals/group.

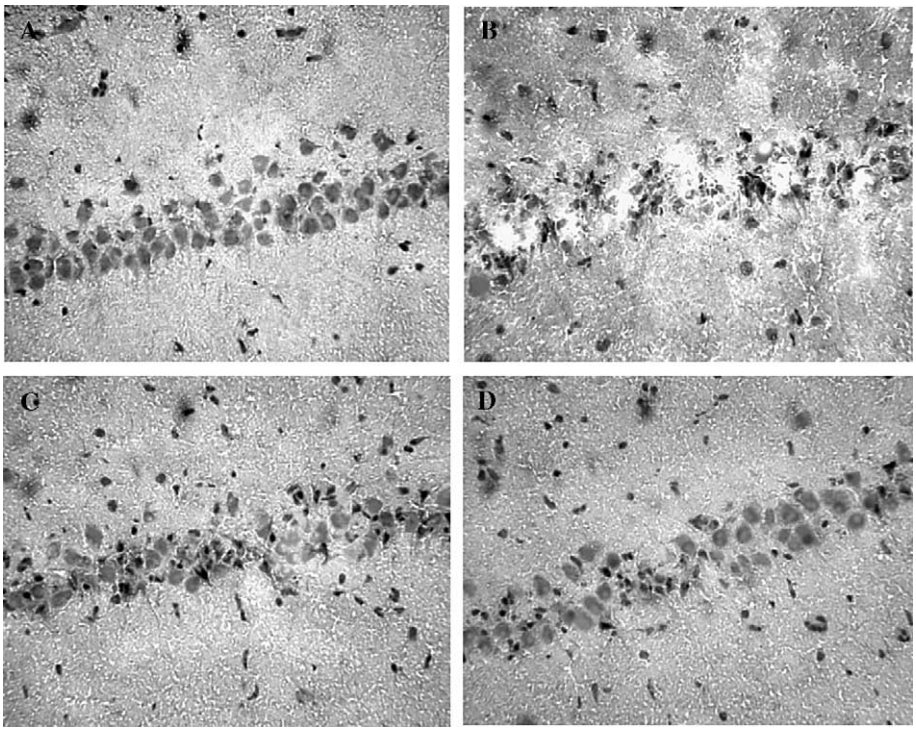

Fig. 5. Protective effect of S34176 against kainic acid-mediated cell loss in the CA1 hippocampal area. Representative photomicrographs of the CA1 hippocampal area of male Wistar rats on day 7 treated with (A) Vehicle, (B) Kainic acid (12 mg/kg s.c.), (C) Kainic acid (12 mg/kg s.c.)+S34176 (20 mg/kg i.p.) ~30% protection, (D) S34176 (40 mg/kg p.o.) ~70% protection.

mg/kg p.o.) 60 min before kainate injection, followed by a subchronic treatment with drug (1×10 mg/kg p.o. during 6 days) induced a partial abrogation of the hippocampal cell loss in the CA1 (53% cell loss for drug-treated vs. 77% for kainate-treated controls; $P<0.01$) and CA3 areas (33% cell loss for drug-treated vs. 53% for kainate-treated controls; $P<0.01$) (Fig. 6A,B). No protective effects were observed under similar experimental conditions with oral administration of S34176 at 2 mg/kg.

C57BL/6 mice administered with D-methamphetamine (5 mg/kg, i.p.) four times at 2-h intervals demonstrated a significant reduction (44%; $P<0.001$) in striatal dopamine levels compared to control animals at 3 days post-exposure (Fig. 7A). Furthermore, under these conditions D-methamphetamine induced a moderate hyperthermia in certain cases up to maximal levels of 2 °C following successive D-methamphetamine administrations, compared to control animals (Fig. 7B,D). S34176 or PBN alone (1×150 mg/kg i.p.), did not significantly modify striatal dopamine levels compared to control animals (Fig. 7A,C), although significant modification of rectal temperature were observed compared to control animals (Fig. 7B,D). At this dose, S 34176 markedly attenuated the D-methamphetamine-mediated depletion of striatal dopamine levels (11.5% decrease for drug-treated; vs. 40.1% for D-meth-controls; $P<0.01$) whereas PBN did not produce a significant protection (34.5% decrease for drug-treated vs. 44.7% for D-Meth-controls; $P>0.05$) (Fig. 7A,C).

4. Discussion

The neuroprotective action of S34176, and indeed related nitrones, may reside, but not necessarily exclusively, in their ability to trap reactive and thus potentially damaging, free radicals (Maples et al., 2001). We have demonstrated in preliminary studies that S34176 and related imidazolylnitrones had a propensity to trap chemically generated carbon and oxygen-centered radicals to produce a stable ESR-detectable radical adducts (Lockhart et al., 2001a). Furthermore, in the majority of the neurodegenerative paradigms used in the present study the neurotoxic mechanisms are to a great extent mediated by excessive OH° and $O_2^{-\circ}$ generation (Lancelot et al., 1995; Bruce and Baudry, 1995; Ueda et al., 1997), and consequently suggests that the protective mechanism of S34176 in these models, could be associated with an ability to efficiently scavenge these deleterious free radicals. Indeed, nitrones, unlike classic phenolic or tocopherol-based antioxidants nitrones are relatively weak inhibitors of lipid peroxidation (Thomas et al., 1997), with activities in the mM range, suggesting they do not possess potent radical chain-reaction breaking properties. Indeed, the structurally related imidazolylnitrone, S34176 or related analogues failed to prevent Fenton-mediated lipid peroxidation in mouse cortical membranes up to 2.5 mM concentrations (unpublished data).

The capacity of S34176, to prevent either kainic acid or ischaemia-reperfusion-mediated neuronal cell death, seven

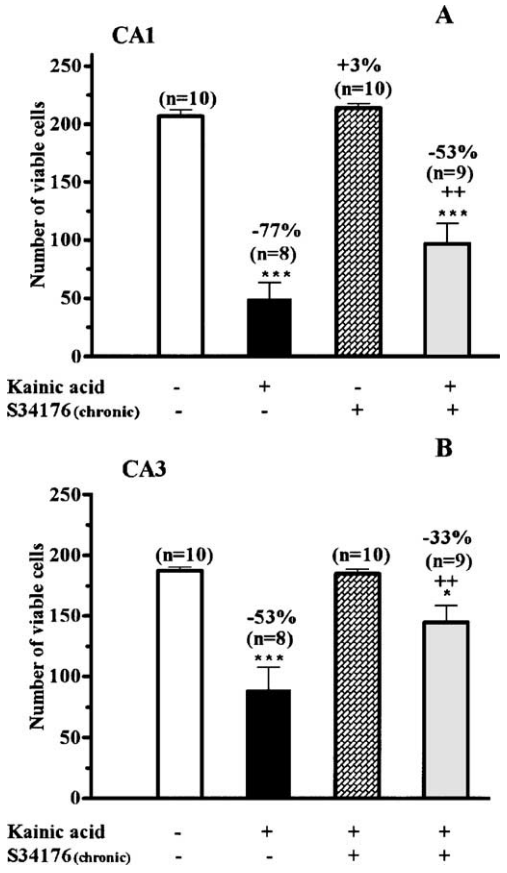

Fig. 6. Effect of chronic administration of S34176 on kainic-acid induced neurotoxicity. S34176 (10 mg/kg p.o.) was administered 60 min before kainate injection, followed by a subchronic oral treatment with drug (1×10 mg/kg/daily, during 6 days). The number of viable cells in the (A) CA1 and (B) CA3 hippocampal sub-fields were estimated in brain slices from animals sacrificed on day 7. Analysis: ANOVA with a post-hoc Newman-Keuls test: (*P<0.05, ***P<0.001, vs. sham-control; ^{++}P<0.01 vs. kainic acid). Data: mean±S.E.M.; n=8–10 animals/group.

days after a single oral pre-administration of compound at relatively low doses, 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. Indeed, the inability of S34176 to prevent ischaemia-mediated hippocampal damage when administered 3 h post-ischaemia is indicative of the limited therapeutic window within which radical scavengers could be beneficial. The over-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. Furthermore, previous studies have shown that kainate-mediated cell damage can be attenuated with radical scavengers (Miyamoto and Coyle, 1990; Bruce et al., 1992).

Previous reports have suggested that some protective actions of nitrone compounds, and in particular PBN, observed at relatively elevated doses (>100 mg/kg i.p.), may in part result from the mild hypothermic effect of this compound at these levels (Miller and O'Callaghan, 1994; Pazos et al., 1999). 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., 1990). It cannot be excluded, that the neuroprotective action of S34176 could also relate partly to its mild hypothermic effects at doses >60 mg/kg p.o., and consequently a capacity to modify the temperature alterations induced by the different experimental paradigms. However, S34176 at low doses (20–40 mg/kg p.o.), lacking any observable hypothermic effects, prevented kainate and global ischaemia-mediated hippocampal cell death. 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 S34176 in these different experimental paradigms. The contribution of mild hypothermic effects of S34176 cannot be excluded in all the investigated paradigms. Indeed, S34176 administered at the dose of 150 mg/kg i.p, induced a significant reduction in whole-body temperature in C57/BL mice, and was capable of D-methamphetamine-mediated striatal dopamine loss. These observations, are supported by previous reports indicating that alterations in body temperature can affect MA-mediated striatal dopamine depletion (Miller and O'Callaghan, 1994).

Despite the high cerebral and plasma levels observed with S34176 after low dose (2 mg/kg p.o.) administration, no neuroprotective effect was observed in the kainic acid model at these dose levels. These data suggest that relatively high circulating brain levels of S34176 are constantly required to counteract the neurodegenerative process initiated by kainic acid or indeed ischemic damage. These observations are not incoherent with the fact that a continuous formation of reactive oxygen radicals during reperfusion damage or following glutamate receptor activation requires a constant and high level of counteracting antioxidant species to abrogate the deleterious effects of radical damage. Clearly, such levels were probably obtained at dose levels of S34176 of 10 mg/kg p.o. during chronic administration, or at 40 mg/kg p.o. following acute administration in the kainic acid paradigm.

Previous reports have described anti-inflammatory activity for the prototypic nitrone PBN, based on its ability to inhibit the expression of pro-inflammatory genes in LPS-endotoxic shock models (Kotake et al., 1998; Sang et al., 1999). Consequently, we cannot as yet rule out the expression of similar mechanisms for S34176 in its neuroprotective action against kainate or ischaemia reperfusion injury, although we have demonstrated no direct inhibitory effect of S34176 on either cyclooxygenase-1, cyclooxygenase-2 or 5-lipoxygenase activity (unpublished data). Nevertheless, it is possible that a combination of radical scavenging, mild cerebral hypothermia and potential anti-inflammatory mechanisms may be contributing to the

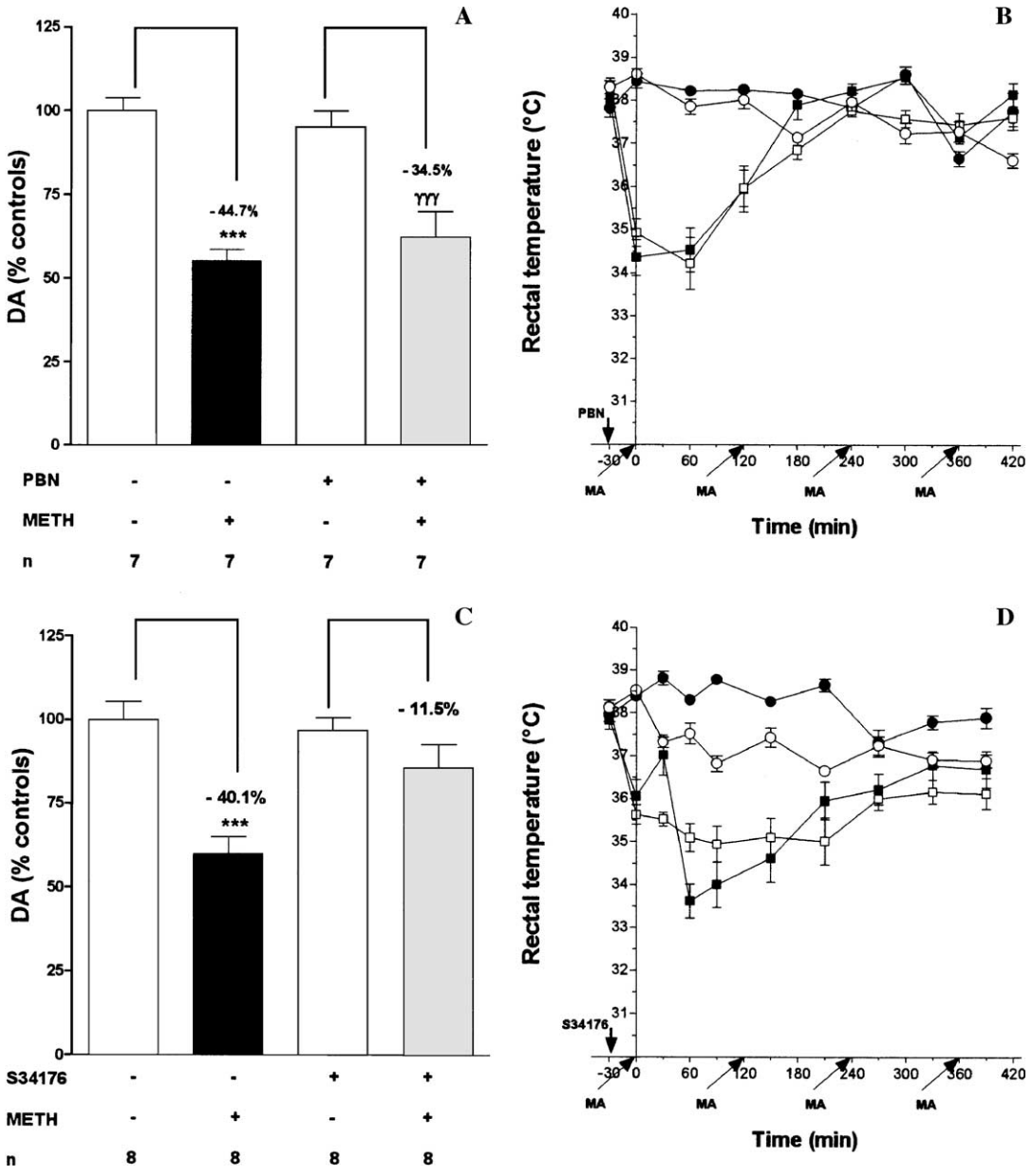

Fig. 7. Effect of S34176 and PBN on the d-methamphetamine-mediated reduction in striatal dopamine levels. Male C57BL/6 mice were administered with D-methamphetamine (5 mg/kg, i.p.) four times at 2-h intervals as previously described. (A) PBN or (B) S34176 were injected (150 mg/kg i.p.) 30 min before the first administration 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; $^{\gamma\gamma\gamma}P<0.001$ vs. compound alone). (B) and (D) Rectal temperature was regularly monitored during the period of drug administration. The different groups consisted of vehicle controls (-○-); S34176 or PBN alone (-□-); D-methamphetamine (-●-); S34176 or PBN/D-methamphetamine (-■-). Data: mean±S.E.M.; n=7–8 animals/group.

neuroprotective properties of this compound. Clearly, in the present study at equivalent doses S34176 demonstrated a superior neuroprotective potential compared to PBN in two paradigms of neuronal cell loss (ischaemia reperfusion and D-methamphetamine-mediated dopamine depletion). The superior activity of S34176 compared to PBN in these paradigms could reside in more optimal mechanistic action (radical adduct stability) (Lockhart et al., 2001a) and/or pharmacokinetic profile in terms of metabolic stability and blood brain barrier permeability (M. Bertrand, unpublished observations). Moreover, S34176 demonstrated more potent neuroprotective effects than the antioxidant 6-ethoxy-2,2-pentamethylen-1,2-dihydroquinoline (S33113), a potent lipid-peroxidation inhibitor, in the kainic acid and ischemic reperfusion models (Lockhart et al., 2001b).

Consequently, the ability of S34176, a free radical scavenger, to prevent neuronal cell death in these paradigms following a single pre-administered dose clearly demonstrates the fundamental role that early oxidative events play in the genesis of delayed neuronal degeneration. S34176 demonstrated limited efficacy when 3 h administered post-ischaemia, and was also ineffective in preventing focal

permanent -occlusion ischaemia (unpublished observations) and thus indicates that this compound alone may have a limited therapeutic benefit in the acute phase of cerebral ischaemia. On the other hand, our observations that S34176 in combination with a AMPA receptor antagonist, NBQX, demonstrated an improved outcome in terms of neuronal cell loss, suggests that combination therapy in the acute phase of ischaemia may be therapeutically relevant at a clinical level. Other groups have demonstrated that in different preclinical models of ischaemia that a combination of treatments with free radical scavengers and glutamate receptor antagonists or anti-thrombolytic agents can result in a synergistic improvement in functional or histopathological outcomes (Davis et al., 1997; Spinnewyn et al., 1999; Lapchak et al., 2002a,b; Lapchak and Ziviv, 2003). Furthermore, S34176 or related compounds with potentially improved clinical safety margins, combined with their proven neuroprotective efficacy in chronic degenerative disorders, could also have therapeutic benefit in the post-ischaemia phase to prevent the slow progressive neuronal cell loss that occurs in the ischemic penumbra following cerebral infarct.

Acknowledgements

We are grateful for Dr. M. Bertrand (Technology Servier) for providing data on the cerebral levels of S34176, C. de Montrion, M. Suc, and C. LeClanche for histology, L. Maioffiss-Dullin (Statistical Analysis Division) and also acknowledge the excellent assistance of C. Cuisinier, M-H. Gandon, A. Hugot and C. Morlat for secretarial assistance.

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