Pyrrolopyrimidines: Novel Brain-Penetrating Antioxidants with Neuroprotective Activity in Brain Injury and Ischemia Models

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ABSTRACT

A novel group of antioxidant compounds, the pyrrolopyrimidines, has been discovered recently. Many of these possess significantly improved oral bioavailability (56–70% in rats), increased efficacy and potency in protecting cultured neurons against iron-induced lipid peroxidative injury and as much as a 5-fold increase in brain uptake compared with the 21-aminosteroid antioxidant compound, tirilazad mesylate (U-74006F), described earlier. They appear to quench lipid peroxidation reactions by electron-donating and/or radical-trapping mechanisms. Several compounds in the series, such as U-101033E and U-104067F, demonstrate greater ability than tirilazad to protect the hippocampal CA1 region in the gerbil transient (5-min) forebrain ischemia model. Delaying treatment until 4 hr after the ischemic insult still results in significant CA1 neuronal protection. U-101033E is still effective in salvaging a portion of

There is now a significant amount of information that supports a role of oxygen radical-induced LP in the pathophysiology of acute CNS injury and ischemia (Braughler and Hall, 1989; Hall and Braughler, 1989; Siesjo et al., 1989). The 21-aminosteroid (lazaroid), tirilazad, has been demonstrated to be a potent inhibitor of LP that acts by a combination of chemical radical scavenging and membrane stabilization mechanisms. It has been shown to reduce traumatic and ischemic damage in several experimental models, and a correlation has been demonstrated between attenuation of oxygen radical levels and/or LP and the neuroprotective effect in several instances (see review by Hall et al., 1994). Currently, tirilazad is being actively investigated in phase III clinical trials in head and spinal cord injury, ischemic stroke and SAH. Results from a multinational European/Australian/ New Zealand trial in SAH have demonstrated a highly significant reduction in 3-month mortality and improvement in the incidence of "Good" recovery (Glasgow Outcome Scale) in patients treated with tirilazad (Kassell et al., 1996).

Received for publication September 16, 1996.

the CA1 neuronal population when the ischemic duration is extended to 10 min. In addition, U-101033E has been found to be protective in the context of focal cerebral ischemia, reducing infarct size in the mouse permanent middle cerebral artery occlusion model, in contrast to tirilazad which is minimally effective. These results suggest that antioxidant compounds with improved brain parenchymal penetration are better able to limit certain types of ischemic brain damage than those which are localized in the cerebral microvasculature. However, the activity of U-101033E in improving early post-traumatic recovery in mice subjected to severe concussive head injury is similar to that of tirilazad. Last, the oral bioavailability of many pyrrolopyrimidines suggests that they may be useful for certain chronic neurodegenerative disorders in which lipid peroxidation plays a role.

Tirilazad appears to act, in large part, on the CNS microvascular endothelium (Audus et al., 1991; Raub et al., 1993; Hall et al., 1994) and consequently has been shown to protect the BBB, to maintain cerebral or spinal cord blood flow autoregulatory mechanisms and/or to reduce delayed vasospasm in multiple models (Hall et al., 1994). Therefore, its ability to protect neural tissue from traumatic or ischemic insult in many models may be largely indirect. Indeed, tirilazad, most likely because of its limited penetration into brain parenchyma, has generally failed to affect delayed neuronal damage in the selectively vulnerable hippocampal CA1 and striatal regions (Beck and Bielenburg, 1990; Buchan et al., 1992; Sutherland et al., 1993), although it has some ability to protect cortical neurons (Sutherland et al., 1993). Moreover, in models of permanent focal ischemia in which microvascular effects may be less important than in temporary ischemia paradigms, the compound's ability to affect infarct size, although demonstrated in some experiments (Beck and Bielenburg, 1991; Park and Hall, 1994), has been inconsistent (Xue et al., 1992).

ABBREVIATIONS: LP, lipid peroxidation; SAH, subarachnoid hemorrhage; BBB, blood-brain barrier; MCA, middle cerebral artery; PBN, N-*tert*-butyl- α -phenylnitrone; HPLC, high-performance liquid chromatography; CNS, central nervous system; ³H-AIB, [³H]- α (methylamino)isobutyric acid.

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Thus, we reasoned that LP-inhibiting (antioxidant) compounds with improved brain penetration might possess certain advantages over the microvascularly localized tirilazad in certain CNS injury situations. Recently, we have discovered a new group of compounds, the pyrrolopyrimidines (fig. 1; Bundy *et al.*, 1995), which are equal or better antioxidants than tirilazad, but with significantly improved ability to penetrate the BBB and gain direct access to neural tissue. The present report details the effects of these in regard to inhibition of iron-dependent lipid peroxidative injury to cultured mouse spinal cord neurons and neuroprotective activity in



Pyrrolopyrimidines



U-94430



U-104067



U-89843



U-101033



U-106311



models of focal and global cerebral ischemia and concussive head injury.

Materials and Methods

All experiments received prior approval by the Institutional Animal Use and Care Committee of Pharmacia & Upjohn, Inc. to ensure that they were performed in strict compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Ani*mals.

Iron-dependent lipid peroxidative injury to cultured fetal mouse spinal neurons. Fetal mouse spinal neurons were cultured

> Fig. 1. Chemical structures of the 21-aminosteroid or "lazaroid" tirilazad (U-74006) and selected pyrrolopyrimidines. Tirilazad is a nonglucocorticoid 21-aminosteroid. The primary chemical antioxidant portion of the molecule is the amino moiety bound to the 21 position of the steroid side chain. Physicochemical studies indicate that the highly lipophilic (i.e., hydrophobic) steroid moiety orients itself within the fatty acid core of the membrane and is largely responsible for the high affinity of the compound for cell (e.g., endothelium) membranes (Hall et al., 1994). The more hydrophilic amino substitution exists closer to the surface in juxtaposition to the phosphate head groups of the phospholipids. In contrast, the pyrrolopyrimidines lack the highly lipophilic steroid moiety, but bear some structural resemblance to the bis-pyrrolidinylpyrimidinyl piperazine 21amino substitution of tirilazad. Lack of the steroid serves to lessen the high affinity for and retention in lipid bilayers.

as described previously (Hall *et al.*, 1991). Spinal neurons were dissected from 13- to 15-day-old embryos of CD-1 virus-free mice. The tissue was chemically and mechanically dissociated. The cells were resuspended in minimum essential media plus additional nutrients and transferred to 24-well, Falcon Primaria culture plates. The cells were plated at a density of approximately 6×10^5 /ml (total volume in well, 1 ml). Plates were maintained at 35° C in $8\% \text{ CO}_2/95\%$ humidity. Cytosine arabinoside was added at a final concentration of 5 μ M on day 6 and removed on day 8. Assays were run on day 15.

Test compounds were dissolved in dimethyl sulfoxide in a 10 mM concentration. They were then diluted to a concentration of 100 μ M in a solution of fatty acid-free bovine serum albumin in phosphatebuffered saline (30 mg/ml). With use of this stock solution, the compounds were added to the cultures to obtain final concentrations ranging from 0.3 to 100 μ M. After 1 hr, the medium containing the test compound was removed and replaced with degassed, argonpurged Krebs' buffer containing 5.55 mM glucose and 200 μ M ferrous ammonium sulfate. After 40 min of exposure to the iron-containing medium, viability of the neurons was assessed by uptake of [³H]- α -(methylamino)isobutyric acid as described previously (Buxser and Bonventre, 1981; Hall *et al.*, 1991). The data represent the uptake (cpm) in iron-exposed cultures as a mean % of parallel noniron-exposed cultures. All the determinations were run in triplicate wells for each drug concentration.

Determination of oxidation potential. Electrochemical oxidation followed by HPLC with UV detection was used to study the oxidation potential of the test compounds. The mobile phase consisted of 89% water, 10% methanol and 1% ammonium acetate. A 10- μ l solution (0.3 μ g/ μ l) was injected onto an HPLC system. At 1 ml/min, the mobile phase flowed into an ESA (Millipore Corp.; Milford, MA) guard cell. The guard cell was set at electrochemical potentials ranging between 0 and 1000 mV. Next, the mobile phase flowed into a Waters (Chelmsford, MA) C8 symmetry column where oxidative by-products could be separated. Flow carried column effluent into a UV detector that monitored light absorption at 230 nm. For each sample, 1 ng of pyrrolopyrimidine was injected onto the column. One minute after injection, the electrochemical potential across the guard cell was turned off until the next injection. During this 1-min period of electrochemical oxidation, electrical current output was observed to increase rapidly and then diminish to base line. The magnitude of current output was directly proportional to the electrochemical potential setting. This suggests that the pyrrolopyrimidines were oxidized in proportion to voltage across the guard cell. Quantitation of the test compound was based on the area of peaks which co-eluted with a nonoxidized sample of the test compound. Peak areas associated with oxidation were normalized to a peak that was measured under conditions where the test compound was not oxidized. The potential that resulted in a 50% loss of starting material was determined.

Determination of brain uptake in mice. Female CF-1 mice (Charles River; Portage, MI) were injected via a tail vein with 23 μ mol/kg (approximately 10 mg/kg) of test compound in propylene glycol (5 mg/ml). At either 5 or 60 min after the dose, the mice were deeply anesthetized with methoxyflurane followed by intracardiac perfusion with 10 ml of 0.9% saline. The brain samples were placed in a volume of acetonitrile equal to five times the brain weight and kept on ice during processing. The samples were sonicated for 20 sec, followed by centrifugation at 2060 \times g for 15 min at 4°C. The supernatants were analyzed by HPLC on a reverse phase column (Zorbax SB-CN, 250×4.6 mm, 5 μ m particle size, Dupont, Chadds Ford, PA) with an autoinjector (Perkin Elmer ISS 100) with 100-µl injections and a flow rate of 1 ml/min. The isocratic mobile phase used consisted of various proportions of 100:1 (v/v) acetonitrile/trifluoroacetic acid and 100:0.115 (v/v) water/trifluoroacetic acid. UV detection (Applied Biosystems Spectroflow 783 Foster City, CA) was used for all compounds. However, fluorescence detection (Applied Biosystems 980) was found to be more sensitive for quantifying some of the pyrrolopyrimidine compounds, with excitation set at 320 nm and an emission cutoff filter at 389 nm. For others that were not fluorescent, electrochemical detection (Waters 460 with a glassy carbon electrode and Ag/AgCl reference electrode) was used at 700 mV.

Other mice were sacrificed, but not perfused. In these, the harvested, unperfused brains were bisected medially and fixed in 10% buffered formalin for 10 to 20 min at 25°C. The tissues were then frozen and 7- μ m-thick cryosections were quickly dried, coverslipped and viewed by confocal scanning laser microscopy at 4°C. Sections excited with a 100-mW laser at 352 to 358 nm emitted fluorescence between 457 and 600 nm. The fluorescence intensity was transformed into a pseudo-color image.

Determination of oral bioavailability in rats. Male Sprague-Dawley rats (Charles River; Portage, MI) weighing 250 to 300 g were surgically implanted with a dosing/sampling cannula in their superior vena cava and allowed a 1-week recovery period. The animals had access to water *ad libitum* and were fasted overnight before and for 4 hr after each treatment. Three animals were used for the determination of oral bioavailability. They were administered approximately a 10 mg/kg i.v. bolus and a 25 mg/kg oral solution by gavage in a cross-over design, with a minimum of a week between doses. Serial blood samples (250 μ l) were obtained at defined time points from before dosing through 48 hr postdosing.

After centrifugation $(14,000 \times g \text{ for } 3-4 \min)$ of the blood samples, a 100- μ l aliquot of plasma was transferred to a 1.5-ml tube and plasma proteins precipitated with 0.5 ml of acetonitrile containing an internal standard. After vortexing and recentrifugation, the supernatant was transferred to an injection vial and 100 μ l injected into the HPLC. The prepared samples were chromatographed on a Zorbax RX-C8 reversed phase column (4.6 mm inside diameter imes 250mm, 5- μ m particle size) with a Brownlee RP-8 Newguard (3.2 mm inside diameter \times 15 mm, 7- μ m particle size) guard column. The mobile phase consisted of 65 to 72% (v/v) acetonitrile and 35 to 28%water containing 0.3% (v/v) triethylamine and adjusted to pH 5 with glacial acetic acid, and was delivered at a flow rate of 1 ml/min. The column effluent was monitored by UV detection at a wavelength of 250 nm. Concentration of the test compound was determined by calculating the ratio of the peak height for the test compound to that for an internal standard and by comparing the ratio to a linear standard curve.

Oral bioavailability was calculated by dividing the integrated area under the 48-hr plasma elimination curve after oral dosing by the area under the curve after i.v. dosing, correcting first for the fact that a 2.5 times higher dose was used for the oral administration.

Gerbil forebrain ischemia model. Male Mongolian gerbils (Tumblebrook Farms; West Brookfield, MA) weighing 45 to 55 g were anesthetized with methoxyflurane. A 1- to 2-cm midline throat incision provided access to both carotid arteries, which were occluded with microaneurysm clips. After 5 min of near-complete ischemia, the clips were removed and reperfusion allowed for 5 days. The animals were placed in a warming box with an ambient temperature maintained at 37°C during the ischemic insult and then until the animals regained their righting reflex after reperfusion. Previous studies from this laboratory have demonstrated that this warming procedure successfully maintains both rectal and brain temperatures of the animals at 35.5°C while the animals are kept in the chamber (Hall et al., 1993). Typically, the test compound was administered p.o. (3, 10 or 30 mg/kg) 30 min before induction of ischemia and again at 2 hr after reperfusion, followed by additional single oral doses at 24, 48 and 72 hr. The concentration of drug in the vehicle (40% hydroxypropyl cyclodextrin) was adjusted such that the oral administration volume was held constant at 0.1 ml.

After 5 days, the animals were deeply re-anesthetized with methoxyflurane and perfused intracardially with phosphate-buffered saline (pH 7.2) until the effluent was cleared of blood (2 min), followed by perfusion with 10% formaldehyde, 10% acetic acid and 80% methanol (FAM). The brains were removed and stored overnight in FAM. They were then blocked and embedded in paraffin. Fivemicron-thick cross-sections were taken through the dorsal hippocampus (1.4–3.0 mm posterior to Bregma), mounted on gelatin-coated slides and stained with cresyl violet (Hall and Pazara, 1988). The number of hippocampal CA1 neurons was counted under light microscopy (magnification \times 320). All normal-appearing CA1 pyramidal neurons in a 315- μ length of the CA1 region were counted bilaterally and averaged. Two sections were examined per animal and the counts averaged. Animals which showed significant asymmetry in regard to the CA1 neuronal counts (>50% difference between hemispheres) were discarded. All analyses were carried out in a blinded fashion in relation to the identification of vehicle *versus* pyrrolopyrimidine-treated gerbils.

All data were expressed as mean \pm standard error. Statistical evaluation was performed by an analysis of variance, followed by a Student's *t* test with Bonferroni correction for multiple comparisons (*i.e.*, three dose groups or three treatment initiation times).

Mouse focal cerebral ischemia model. Male CD-1 mice (Charles River; Portage, MI), weighing 18 to 22 g, were used. They had access to water and chow *ad libitum*. Under methoxyflurane anesthesia, an incision was made over the temporoparietal region, and the skull was exposed by retraction of the musculature and parietal gland. A small burr hole exposed the MCA. The MCA was cauterized in two places 2 mm apart and then cut, just above the origin of the lenticulostriate arteries by use of bipolar diathermy.

At 5 min and again at 60 min postocclusion, the mice received an i.v. (tail vein) injection of vehicle (0.02 M citric acid) or test compound (0.03, 0.1, 0.3, 1.0 or 3.0 mg/kg). The injection volume was kept constant at 0.05 ml.

At 6 hr postocclusion, the mice were re-anesthetized and perfused intracardially with 5 ml of a 4% solution of 2,3,5-triphenyltetrazolium chloride and brains removed and fixed overnight in 10% buffered formalin at room temperature in the dark. After the overnight fixation, the brains were cross-sectioned into eight 2-mm slices. The most anterior and posterior sections were discarded. The remaining six sections were placed in a 6-well culture dish and each covered with distilled water to prevent drying. 2,3,5-Triphenyltetrazolium chloride reacts with mitochondrial cytochrome oxidases in viable tissue and produces a deep red color, whereas the infarcted, dead tissue does not stain. With an Image 1 Analysis System (Universal Imaging Corporation; West Chester, PA), the infarct area of each brain slice was quantitated and expressed as a percent of the total area. The percent infarct areas were averaged to give an estimate of the total infarct volume per brain.

Differences between infarct size were compared between vehicleand U-101033E-treated mice by analysis of variance, followed by Student's t test (two-tailed).

Mouse head injury model. Details of the mouse head injury model have been published in detail elsewhere (Hall *et al.*, 1988). Male CF-1 mice (Charles River; Portage, MI) weighing 17 to 20 g were used. Each mouse that survived the injury received vehicle (0.05 N HCl) or test drug in a 0.1-ml volume as an i.v. bolus within 5 min postinjury. Groups of 20 to 25 mice were injured in rapid succession. Each trial was carried out in a blinded fashion, with a vehicle group and three groups receiving either 0.1, 1 or 10 mg/kg of test compound.

At 1 hr postinjury, the neurological status of the injured mice was blindly evaluated by a grip test. The mean time that the injured mice could remain on the grip string was measured (30 sec maximum), and comparisons between vehicle- and drug-treated mice made with an analysis of variance followed by Student's t test (two-tailed).

Results

Comparison of inhibition of iron-dependent lipid peroxidative neuronal injury. Table 1 shows the IC_{50} values and maximum percent protection of cultured fetal

TABLE 1

Comparison of the potency and efficacy of U-74006F and selected pyrrolopyrimidines in regard to protection against lipid peroxidative impairment of viability (*i.e.*, attenuation of ³H-AIB uptake) in cultured fetal mouse spinal neurons by 200 μ M ferrous ammonium sulfate application for 40 min

Cultures were pretreated with test compound in varying concentrations for 1 hr. The medium containing the compound was then removed, and the treated cultures were exposed to 200 μM ferrous ammonium sulfate for 1 hr, followed by determination of ³H-AIB uptake. Potentials required to oxidize 50% of the test compound were determined as described under "Methods."

Compound	IC ₅₀	Maximum Protection	Potential Required to Oxidize 50% of Test Compound
	μM	% control	mV
U-74006F	25.4	60.5	540
U-87663E	1.3	100	530
U-89843E	7.0	83.4	423
U-94430E	0.3	75.8	369
U-101033E	1.1	75.8	408
U-104067E	10.6	80.6	894
U-106311E	4.5	100	125

mouse spinal neurons from iron-induced lipid peroxidative injury (system described in detail elsewhere; Hall *et al.*, 1991; Zhang et al., 1996) for tirilazad and the pyrrolopyrimidines. Protection of cellular viability was measured in terms of preservation of amino acid uptake (*i.e.*, uptake of ³H-aminoisobutyric acid) as described and validated by Buxser and Bonventre (1981). As seen, the pyrrolopyrimidines are consistently more potent and efficacious in this *in vitro* model. Figure 2 shows full concentration-response graphs for the pyrrolopyrimidines U-89843E and U-101033E versus tirilazad (U-74006F). U-89843D, and even more so U-101033E, are more potent and more efficacious than U-74006F. Figure 3 shows similar graphs for U-104067E and its para-hydroxylated metabolite U-106311E. U-106311E is significantly more potent and efficacious than U-104067E, which has a concentration-response relationship quite similar to that of U-74006F (compare with fig. 2). It should be noted that neither tirilazad nor any of the pyrrolopyrimidines affected the base-line level of ³H-aminoisobutyric acid uptake (*i.e.*, in the absence of iron; data not shown).

Figure 4 presents the hypothetical antioxidant (i.e., LPinhibiting) mechanisms of the pyrrolopyrimidines. Mechanism I, relating to U-87663, U-89843, U-94430 and U-101033, involves a scenario of initial electron donation and consequent quenching of either a lipid alkoxyl (LO), lipid peroxyl (LOO) or hydroxyl (OH) radical followed by peroxyl radical trapping. Circumstantial evidence for the intermediacy of radical cations I and II in the reaction of the pyrrolopyrimidines with lipid peroxides is provided by preparative scale electrochemical experiments. Electrochemical oxidation of U-87663 under very mild conditions, in either methanol or acetic acid, affords the chemically labile 5-methoxy and 5-acetoxy derivatives, respectively, in good yield (data not shown). These products are therefore rationalized by invoking solvent capture of radical cations I and II. Further evidence is provided by the finding that chemical oxidation of U-87663 under radical cation-producing conditions (e.g., benzoyl peroxide) provides the corresponding 5-benzoyloxy derivative, presumably *via* a similar mechanism. In contrast, mechanism II requires the metabolic para-hydroxylation of U-104067 to U-106311, which can then act as a phenolic-like peroxyl radical scavenger. The significant, albeit less potent



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Fig. 2. Protection of the viability (uptake of ³H-AIB) of cultured fetal mouse spinal cord neurons against iron-induced lipid peroxidative injury by tirilazad (U-74006F) or the three pyrrolopyrimi-Ú-89843E, U-94430F dines or U-101033E. Values are means ± standard error for triplicate determinations. Cultures were pretreated with test compound in varying concentrations for 1 hr. The medium containing the compound was then removed and the treated cultures exposed to 200 µM ferrous ammonium sulfate for 40 min, followed by determination of ³H-AIB uptake. The absolute level of uptake-related radioac-

65 to

Fig. 3. Protection of the viability (uptake of ³H-AIB) cultured fetal mouse spinal cord neurons against iron-induced lipid peroxidative injury by the pyrrolopyrimidines U-104067F or U-106311E. Values are means \pm standard error for triplicate determinations. Cultures were pretreated with test compound in varying concentrations for 1 hr. The medium containing the compound was then removed and the treated cultures were exposed to 200 µM ferrous ammonium sulfate for 40 min, followed by determination of ³H-AIB uptake. The absolute level of uptake-related radioactivity in iron-exposed, non-drug-treated cultures ranged from 483 \pm 30 to 3629 \pm 987 cpm. CON, control.

and less efficacious, antioxidant neuroprotective action of the parent compound, U-104067, is likely caused by a physicochemical membrane-stabilizing mechanism that is perhaps similar to that demonstrated for U-74006F (Hall et al., 1994).

Comparison of brain uptake. The comparative brain uptake of tirilazad (U-74006F) and the pyrrolopyrimidines in mice after i.v. administration of molar equivalent doses (approximately 10 mg/kg) is shown in figure 5. Each of the pyrrolopyrimidines produced significantly higher brain levels 5 min after injection compared with tirilazad. For and U-87663E, U-89843E, U-94430E, U-101033E U-104067F, the brain levels at the initial time point were 4.7, 3.2, 3.7, 2.8 and 5.1 times higher, respectively, than the levels of tirilazad. For U-87663E, the brain levels were still 2.3 times higher than tirilazad at 60 min postinjection. It is also clear that the brain levels seem to fall off faster for the pyrrolopyrimidines as a further reflection of their greater

membrane permeabilities (Sawada et al., 1995a, b). In other words, they diffuse into the brain and, in the absence of repeated dosing, they may diffuse out of the brain more quickly than tirilazad. Additionally, we have taken advantage of the intrinsic fluorescent properties of U-87663E to demonstrate unequivocally by fluorescence microscopy that this prototype pyrrolopyrimidine efficiently penetrates the BBB in mice after i.v. dosing (fig. 6). Moreover, it concentrates in brain parenchyma and not the cerebrovascular endothelium (fig. 6, inset).

Comparison of oral bioavailability. Table 2 shows the comparative oral bioavailability and terminal plasma halflives of tirilazad (U-74006F) versus several pyrrolopyrimidines. The bioavailability of the included pyrrolopyrimidines ranges from a low of 20.7% (U-106311E) to a high of 84.6%(U-89843E). The terminal half-lives range from 2.7 hr (U-106311E) to 22.1 hr (U-87663E). These values are considerably higher than those for the 21-aminosteroid U-74006F.

Antioxidant Mechanism I: U-87663, U-89843, U-94430, U-101033



Fig. 4. Probable antioxidant mechanisms of the pyrrolopyrimidines. Antioxidant mechanism I, which is relevant to U-87663, U-89843, U-94430 and U-101033, involves reaction of an alkoxyl (LO·), peroxyl (LOO·) or hydroxyl (OH) radical in which the pyrrolidine nitrogen quenches the radical species via the transfer of an electron. The pyrrolopyrimidine, in turn, becomes a radical cation. The cationic center is transferred to the 5-position of the pyrrolopyrimidine which can then react with (i.e., trap) a second radical species, most likely a peroxyl radical. This particular scenario is unlikely in U-104067. Rather, its antioxidant capacity is probably dependent on para-hydroxylation of the aromatic ring, resulting in a phenolic-type electron-donating antioxidant (U-106311). Indeed, U-106311 has a much lower oxidation potential than U-104067 (see table 1).

Pyrrolopyrimidine neuronal protection in transient forebrain ischemia. As noted above, tirilazad has been demonstrated to have very limited ability to attenuate selective hippocampal CA1 vulnerability in models of transient forebrain ischemia (Beck and Bielenburg, 1990; Buchan *et al.*, 1992; Sutherland *et al.*, 1993). This is most likely caused by its limited BBB penetration in the context of models where BBB permeability is minimally compromised. In contrast, figure 7 illustrates that oral preischemic treatment (plus repeated postreperfusion treatment) with any of the BBB-permeable pyrrolopyrimidines (U-94430E, U-101033E and U-104067F) produces significantly more CA1 neuronal protection than that observed in vehicle-treated animals. Figure 8 shows the dose-response curve for the ability of U-101033E to protect the CA1 region. Dose levels of 10 or 30 mg/kg (\times 5) are significantly effective, but doses as low as 1 and 3 mg/kg appear to have some effect. Figure 9 displays the therapeutic window for the efficacy of U-101033E in regard to CA1 protection in the gerbil 5-min forebrain ischemia model. The initiation of dosing 30 min before ischemia (plus repeated postreperfusion dosing) is the most effective. However, a delay in dosing to 4 hr after reperfusion still provides a statistically significant neuroprotective effect.

Pyrrolopyrimidine neuroprotection in permanent focal ischemia. U-101033E has also been examined for the ability to limit brain infarct volume in mice subjected to permanent MCA occlusion. Mice received two doses of test compound, one at 5 min and a second at 60 min postocclusion.



Fig. 5. Comparison of the mouse brain levels of U-74006F and selected pyrrolopyrimidines at 5 or 60 min after a 23 μ M/kg (approximately 10 mg/kg) i.v. bolus. Mice were perfused with saline at sacrifice to eliminate any contribution of blood to the measured levels. Values are means \pm standard error for n = 3. Asterisk indicates P < .05 versus U-74006F at the same time point.



Fig. 6. Low magnification fluorescence photomicrograph of a slice of mouse cerebral cortex, with dura mater intact, showing penetration of the pyrrolopyrimidine U-87663 into brain parenchyma after a 23 μ M/kg (approximate 10 mg/kg) i.v. bolus. The color scale shows relative drug-associated fluorescence pseudocolor intensity (white = highest concentration). The black holes represent brain microvessels. The circular structure on the right is a pial artery. Note that vessel walls show relatively low drug-related fluorescence compared to parenchyma (higher magnification inset).

TABLE 2

Oral bioavailability and terminal plasma half-lives of tirilazad (U-74006F) and pyrrolopyrimidines in Sprague-Dawley rats

,,,,		
Compound	Oral Bioavailability	Terminal Half-life
	%	hr
U-74006F	14.6	2.9
U-87663E	55.9 ± 4.5	22.1 ± 2.4
U-89843E	84.6 ± 19.5	10.9 ± 3.4
U-94430E	70.2 ± 3.1	8.9 ± 1.0
U-101033E	78.3 ± 10.9	11.2 ± 0.6
U-104067F	69.9 ± 14.2	15.7 ± 2.8
U-106311E	20.7 ± 3.3	2.7 ± 1.0

Values are means ± standard error.

As observed in figure 10, U-101033E potently reduced infarct volume by as much as 27% at a dose of only 0.1 mg/kg (×2) i.v. In addition, U-87663E and U-89843E have also been found to be significantly effective in the mouse permanent MCA occlusion model, whereas tirilazad (0.1–3.0 mg/kg × 2)



Fig. 7. Attenuation of hippocampal CA1 neuronal loss of the pyrrolopyrimidines U-94430E, U-101033E and U-104067F at 5 days after a 5-min episode of bilateral carotid occlusion in male Mongolian gerbils. n = 10 animals/group. Gerbils were dosed with 30 mg/kg p.o. preischemia plus 2 hr after reperfusion and once daily on days 2, 3 and 4. Asterisk indicates P < .05 *versus* the vehicle-treated group (Veh).



Fig. 8. Dose response for the effect of U-101033E to salvage hippocampal CA1 neurons at 5 days after a 5-min episode of bilateral carotid occlusion in male Mongolian gerbils. n = 10 animals/group. Gerbils were dosed with each dose level p.o. preischemia plus 2 hr after reperfusion and once daily on days 2, 3 and 4. Asterisk indicates P < .05 *versus* the vehicle-treated group (Veh).

has shown only nonsignificant trends toward infarct reduction (data not shown).

Pyrrolopyrimidine enhancement of early neurological recovery in brain-injured mice. Figure 11 demonstrates the ability of acutely administered (5 min postinjury) intravenous U-101033E to enhance the early (1 hr) neurological recovery of male CF-1 mice subjected to a severe concussive head injury. A dose-related effect is seen over the range of 0.1 to 10 mg/kg. A similar magnitude of effect has been reported for several other pyrrolopyrimidines (Bundy *et al.*, 1995) and for tirilazad (Hall *et al.*, 1988, 1992).

Discussion

This report describes a novel group of potent inhibitors of iron-dependent LP in neural tissue, the pyrrolopyrimidines (e.g., U-87663E, U-89843D, U-101033E). Members of the series have also been reported to inhibit *in vitro* neuronal injury by peroxynitrite (Fici *et al.*, 1996). With both iron and peroxynitrite-induced cellular injury, the pyrrolopyrimidines show increased potency and efficacy compared with the 21aminosteroid tirilazad. In addition, the pyrrolopyrimidines possess improved BBB permeability and brain parenchymal



Fig. 9. Therapeutic window for the effect of U-101033E to salvage hippocampal CA1 neurons at 5 days after a 5-min episode of bilateral carotid occlusion in male Mongolian gerbils. n = 10 animals/group. Gerbils were dosed beginning at each time point with 30 mg/kg p.o. plus 2 hr later and on the subsequent days. Asterisk indicates P < .05 *versus* the vehicle-treated group (Veh).



Fig. 10. Dose-response for U-101033E reduction of 6 hr infarct size in male CF-1 mice subjected to permanent unilateral occlusion of the right MCA. The mice received one dose of compound i.v. at 5 min postocclusion and a second dose at 1 hr. Values are percent reduction in mean infarct volume (summation of eight cross-sections through the infarct) compared with a vehicle-treated group of animals. n = 8 to 13 animals/group. Asterisk indicates P < .05 *versus* vehicle.

penetration compared with tirilazad which remains largely localized in the cerebral microvascular endothelium (Raub et al., 1993; Hall et al., 1994). In previously reported experiments, the comparative brain uptake of tirilazad and selected pyrrolopyrimidines has also been evaluated in rats in terms of first-pass extraction of radiolabeled compounds after intracarotid injection (Sawada et al., 1995b; Hall et al., 1995). With this technique, tirilazad is only 6% extracted, which is not much better than the extraction of sucrose or thiourea to which the BBB is essentially nonpermeable. In contrast, the pyrrolopyrimidine U-87663E is 83% extracted on the first pass through the cerebral circulation, and U-89843E is 88% extracted. These extraction values are very near that measured for the freely BBB-diffusible butanol. Thus, the pyrrolopyrimidines appear to permeate the BBB quite readily. Confocal laser microscopy has also been used to show that U-87663E readily gains access to the intracellular space of cultured cells (Sawada et al., 1995a, b). As a result of the



Fig. 11. Dose-response for the effect of U-101033E to improve the early (1 hr) neurological recovery of male CF-1 mice after a severe concussive head injury. n = 6 to 9 mice in the U-101033E-treated groups and 23 mice in the vehicle-treated group (V). The data are presented as percent decrease in infarct size. However, the statistical analysis was actually performed using the raw percent infarct size values.

greater brain and perhaps intracellular penetration, compounds like U-101033E have neuroprotective efficacy in attenuating selective neuronal damage in highly vulnerable regions, such as the CA1 area of the hippocampus, after a transient episode of forebrain ischemia. In contrast, tirilazad is largely ineffective in salvaging CA1 neurons in rodent forebrain ischemia paradigms (Beck and Bielenburg, 1990; Buchan *et al.*, 1992; Sutherland *et al.*, 1993; Hall *et al.*, 1995).

Other purportedly brain-penetrable antioxidants have been described previously with neuroprotective efficacy in transient forebrain ischemia models in either gerbils or rats, including LY-178002 (Clemens et al., 1991), PBN (Phillis and Clough-Helfman, 1990) and dimethylthiourea (Pahlmark et al., 1993). However, much higher doses of all of these compounds are required to achieve neuroprotection, whereas U-101033E is significantly effective at oral dose levels as low as 10 mg/kg. This suggests that these earlier described and studied compounds either may not be as brain-penetrable as thought or perhaps they are not as effective in attenuating oxygen radical-induced, iron-catalyzed LP as the pyrrolopyrimidines. In addition, U-101033E has been shown to have at least a 4-hr postischemic therapeutic window. In contrast, PBN's ability to protect CA1 neurons in the identical gerbil forebrain ischemia model, which requires higher mg/kg doses (100 mg/kg) than those presently used, is lost by 2 hr after reperfusion (Phillis and Clough-Helfman, 1990). Nevertheless, further study of U-101033E and other pyrrolopyrimidines is necessary before an exact assessment of their neuroprotective potency and efficacy can be established in comparison with the earlier described antioxidant compounds.

The pyrrolopyrimidines similarly outperform tirilazad in the context of permanent focal cerebral ischemia. In the face of permanent vascular occlusion, a successful neuroprotective compound must intuitively be able to penetrate the underperfused ischemic penumbral zone to be optimally effective in salvaging the still viable, but potentially doomed, neural tissue. Although not directly determined in the current study, it is likely that U-101033E is able to penetrate the ischemic penumbra more effectively than the microvascularly localized tirilazad. Although tirilazad has been reported to reduce infarct volume in the setting of permanent MCA occlusion in Sprague-Dawley (Park and Hall, 1994) and Fischer (Beck and Bielenburg, 1991) rats, it has not been shown to be efficacious in the same model in the spontaneously hypertensive rat strain (Xue *et al.*, 1992). Likewise, tirilazad is only marginally effective in the mouse permanent MCA model (Hall *et al.*, 1995). Similarly, another antioxidant, dihydrolipoate, has not shown activity in the mouse permanent MCA occlusion model (Prehn *et al.*, 1992). In contrast, the nitrone spin-trapping agent PBN is effective in reducing infarct size in the rat permanent MCA occlusion model although high doses (100 mg/kg i.p.) are required (Cao and Phillis, 1994). However, PBN's protective efficacy in the mouse model has not been evaluated.

A greater efficacy of the pyrrolopyrimidines has also been seen in the context of temporary focal ischemia. Rats subjected to 90 min of MCA occlusion showed a 47% smaller brain infarct 7 days later when pretreated with a 3 mg/kg i.v. dose of U-101033E, plus 3 mg/kg i.v. 15 min before and 1 hr after reperfusion. Equivalent dosing with the 21-aminosteroid U-74389G (16-desmethyl tirilazad) only achieved a 17% mean infarct size reduction, which was not statistically significant. U-101033E, but not U-74389G, also improved early postreperfusion neurological recovery (Schmid-Elsaesser *et al.*, 1996). Similarly, PBN has been reported to reduce infarct size significantly in the rat temporary MCA occlusion model (Zhao *et al.*, 1994).

Interestingly, in regards to severe concussive brain injury, the degree of acute neurological recovery enhancement observed with U-101033E is similar to, but not greater than, that reported for tirilazad (Hall et al., 1988, 1992). However, in the mouse concussive injury model, significant BBB disruption is observed as early as 5 min postinjury (Hall et al., 1992). Thus, tirilazad is better able to penetrate into brain parenchyma and therefore gain access to the endangered central neurons, perhaps as well as U-101033E. Indeed, the uptake of tirilazad is greater in injured than in noninjured mouse brain (Hall et al., 1992). Nevertheless, despite this greater posttraumatic BBB penetration, much of tirilazad's neuroprotective action may still be mediated at the microvascular level (Hall et al., 1994). Secondary microvascular damage in brain injury, and its potential attenuation by the microvascularly localized tirilazad, may be as important as parenchymal neuronal injury mechanisms which would be most efficiently countered by the brain-penetrable pyrrolopyrimidines. In any event, it will be interesting to investigate the combination of tirilazad, which is largely localized in the brain microvascular endothelium (Audus et al., 1991; Raub et al., 1993; Hall et al., 1994), with U-101033E, which permeates the vascular wall and exerts direct neuroprotection. Although it is likely that, within the overall spectrum of traumatic and ischemic CNS insults, antioxidant compounds that localize in brain microvasculature or that penetrate the brain parenchyma will have specific therapeutic roles to play, in some cases they may be complementary.

It has been increasingly recognized that oxygen radicalinduced neuronal damage may also have an important role in the pathogenesis of the major chronic neurodegenerative diseases. These include amyotrophic lateral sclerosis (Rosen *et al.*, 1993; Gurney *et al.*, 1996), Parkinson's disease (Cohen, 1986; Jenner *et al.*, 1992; Youdim *et al.*, 1993) and Alzheimer's disease (Subbarao *et al.*, 1990; Smith *et al.*, 1994). Thus, the pyrrolopyrimidines may be useful in slowing the progression of these disorders by virtue of their excellent oral bioavailability, brain penetrability and antioxidant neuroprotective efficacy.

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