Protective effects of tirilazad mesylate and metabolite U-89678 against blood-brain barrier damage after subarachnoid hemorrhage and lipid peroxidative neuronal injury

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The 21-aminosteroid lipid-peroxidation inhibitor, tirilazad mesylate (U-74006F), recently was shown in a large multinational Phase III clinical trial to decrease mortality and improve neurological recovery in patients 3 months after onset of aneurysmal subarachnoid hemorrhage (SAH). A major tirilazad metabolite in animals and man, U-89678 is formed when the 4–5 double bond in the A-ring is reduced and has been postulated to contribute significantly to tirilazad’s neuroprotective effects. In the first experiment of the present study, the authors compared the effects of tirilazad and U-89678 on acute blood-brain barrier (BBB) damage in rats subjected to SAH via injection of 300 μl of autologous nonheparinized blood under the dura of the left cortex. The rats were treated by intravenous administration of either 0.3 or 1.0 mg/kg of tirilazad or U-89678 10 minutes before and 2 hours after SAH, and BBB damage was quantified according to the extravasation of the protein-bound Evans’ blue dye into the injured cortex 3 hours post-SAH. The results revealed that 0.3 and 1.0 mg/kg tirilazad significantly reduced SAH-induced BBB damage 35.2% (p < 0.05) and 60.6% (p < 0.0001), respectively, in comparison to treatment with vehicle. The 0.3- and 1.0-mg/kg doses of U-89678 also decreased injury by 39.1% (p < 0.05) and 21.3% (not significant), respectively. In the second experiment, the investigators assessed the relative abilities of tirilazad and U-89678 to protect cultured neurons from iron-induced lipid peroxidative injury. Fetal mouse spinal cord cells were pretreated with 3, 10, or 30 μM tirilazad or U-89678 for 1 hour and then exposed to 200 μM ferrous ammonium sulfate (FAS) for 40 minutes. Cell viability was measured in terms of the uptake of [3H]methylaminoisobutyric acid 45 minutes after the FAS treatment. Both compounds enhanced neuronal survival in a concentration-dependent fashion. Although the two were equally efficacious, U-89678 was slightly more potent than its parent. On the basis of these findings, the authors conclude that the tirilazad metabolite, U-89678, possesses vaso- and neuroprotective properties that are essentially equivalent to the parent 21-aminosteroid. Hence, U-89678 probably contributes to the protective effects of tirilazad in SAH and other insults to the central nervous system.

KEY WORDS • tirilazad • U-89678 • blood-brain barrier • 21-aminosteroid • subarachnoid hemorrhage • lipid peroxidation • neuron

Materials and Methods

Subarachnoid Hemorrhage

All experiments received prior approval from the Corporate Animal Welfare Committee of Upjohn Laboratories to ensure that they were performed in strict compliance with the National Institutes of Health guidelines concerning laboratory animals.
Animals. Sixty-one male Sprague-Dawley rats, weighing 245 to 315 g, were used in this experiment. Animals were housed four to a cage and given ad lib access to commercially available rodent food and tap water throughout the study. A 12-hour light–dark cycle was maintained in the colony, with lights on from 6:00 a.m. to 6:00 p.m.

Procedure. The rats were initially anesthetized with a subcutaneous injection of 0.24 ml/100 g KAX (a mixture of 25 mg/ml ketamine, 1.7 mg/ml xylazine, and 0.25 mg/ml acepromazine; Butler Co., Brighton, MD). The right femoral vein was catheterized with PE-50 tubing and locked with a 1:200 heparin/saline solution. A burr hole, 2.7 mm in diameter, was drilled over the left cortex 3 to 4 mm posterior to the bregma and 3 mm left of the sagittal suture. A small hole was made in the dura with a 26-gauge needle, and a piece of PE-10 tubing was slipped under the dura and advanced 4 to 5 mm anteriorly. Ten minutes before SAH was induced, a 0.3- or 1.0-mg/kg dose of drug or an equal volume of vehicle (2 ml/kg) was injected into each rat intravenously. Subarachnoid hemorrhage was induced by injecting 300 μl of autologous nonheparinized arterial blood under the dura at a rate of 50 μl/30 seconds, as previously described by Zuccarello and Anderson.22 The blood was obtained from separate donor rats, each providing blood for 3 to 4 hemorrhages. One hour later, animals received intravenous administration of 50 mg/kg of a 25 mg/ml filtered solution of Evans’ blue dye in 0.9% saline. Two hours post-SAH, the rats were given a second dose of drug or vehicle. Three hours post-SAH, the rats were sacrificed: the animals were reanesthetized with 0.24 ml/100 g KAX and perfused with 60 ml saline at 100 mm Hg. The rat brains were removed and the left cortex was isolated. Tissue stained with Evans’ blue dye was extracted in 3 ml formamide at room temperature for 72 to 96 hours, then quantified fluorometrically (SIM AMINCO SPF-500 Spectrofluorimeter; EX = 625, slit =10; EM = 675, slit = 20). The brains were dried for 2.5 days at 100°C so that cortical Evans’ blue dye content could be expressed as nanograms per milligram of dry weight.

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Treatment groups consisted of rats receiving either 0.3 or 1.0 mg/kg tirilazad or U-89678. Whereas the tirilazad was delivered in citric acid, U-89678 was delivered in a vehicle containing citric acid plus 50 μl/ml 1 N HCl. Each group contained eight to 10 animals. To quantitate edema caused by direct mechanical damage (such as catheter insertion) in addition to damage secondary to an increase in intracranial pressure produced during the infusion of a 300-μl volume of blood, a sham control group was included. In this control group of four animals, 300 μl of autologous blood plasma, rather than whole blood, was injected underneath the dura. Last, it was necessary to quantify background Evans’ blue dye levels in rats not subjected to SAH. Hence, six rats were injected intravenously first with vehicle, followed by Evans’ blue dye, and then with vehicle once again.

Data Analysis. The data were analyzed using separate one-way analyses of variance, each comparing the mean cortical levels of Evans’ blue dye from groups treated with tirilazad or U-89678 with those of rats treated with vehicle. Because the first analysis using tirilazad violated the assumption of homogeneity of variance (Ftest), a log transformation was performed on these data and the analysis repeated. Each analysis was followed by appropriate independent t-test comparisons (two-tailed) between groups.

Iron-Induced Injury to Cultured Spinal Cord Neurons

Cell Culture. Fetal mouse spinal cord cells were cultured as previously described.23 Briefly, spinal cords were dissected from 13- to 15-day-old embryos of CD-1, virus-free mice. The tissue was chemically and mechanically dissociated and the cells were resuspended in minimum essential media plus additional nutrients, including 10% horse serum, and transferred to 24-well tissue culture plates. The cells were plated at a density of approximately 6 × 105 cells/ml (total volume in well = 1 ml) and the plates were maintained at 37°C in a 10% CO2 atmosphere with 95% humidity. Arabinosylcytosine was added at a final concentration of 5 μM on Day 6 of culture and removed on Day 8. Cells were fed three times per week. Experiments were performed on Day 15.

Drug Treatment. Stock solutions of tirilazad and U-89678 (10 mM each) were prepared in dimethylsulfoxide (DMSO) and further diluted with DMSO to concentrations of 0.3, 1, and 3 mM. Final dilutions of 3, 10, and 30 μM were made in modified Eagles’ medium (MEM) containing 5% horse serum and 0.001% pluronic acid. Pluronic acid moderately increases the permeability of the cell membrane to allow drug entry. Medium containing the test compound was added to the cell culture well. After a 60-minute pretreatment at 37°C, the medium containing the test compound was replaced with 1 ml Kreb’s buffer, and 200 μM ferrous ammonium sulfate (FAS) was added for an additional 40 minutes. The FAS (20 mM) was prepared in degassed, argon-purged Kreb’s buffer, and diluted 1:100 in 1 ml Kreb’s buffer in the cell well.

Viability. The [%H]α-(methyl)-aminoisobutyric acid (AIB; New England Nuclear, Boston, MA) was prepared in Kreb’s buffer at 1 μCi/ml concentration.4 Buffer containing FAS was removed and the cells were incubated for 45 minutes with AIB solution, rinsed twice with 0.5 ml Kreb’s buffer, and lysed with 0.5 ml of a 0.5% aqueous solution of Triton-X 100 prior to addition of 5 ml of scintillation cocktail. Results were normalized to control cultures and expressed as the percent of control ± standard error of the mean in four experiments.

Data Analysis. Groups treated with tirilazad and U-89678 were compared, on the basis of AIB uptake, in two separate one-way analyses of variance. Each analysis was followed by individual t-test comparisons (two-tailed).

Results
Effects of Tirilazad and U-89678 on Subarachnoid Hemorrhage–Induced Blood-Brain Barrier Damage

The amount of Evans’ blue dye extracted from the cortices of rats treated with citric acid (39.8 ± 9.9 ng/mg; five animals) and citric acid plus HCl (47.7 ± 9.4 ng/mg; five

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Animals) did not differ significantly. Consequently, these two groups were combined (43.7 ± 6.2 ng/mg). Background levels of Evans’ blue dye were low (4.8 ± 1.6 ng/mg), demonstrating the nontoxic nature of this dye. Evans’ blue extravasation in sham controls was also low (7.8 ± 1.3 ng/mg), indicating that there was very little brain injury caused by catheter insertion or by the increase in intracranial pressure that occurred during the SAH procedure.

Figure 2 shows a comparison of Evans’ blue dye content extracted from the left cortex of rats treated both pre- and post-SAH with 0.3 or 1.0 mg/kg tirilazad or U-89678 with that extracted from vehicle-treated and sham-hemorrhage groups. It is apparent that Evans’ blue dye extravasation was markedly increased 3 hours post-SAH in comparison to that found in the sham-hemorrhage group (p < 0.0005), indicating that the SAH produced significant BBB damage. The extravasation of Evans’ blue dye in the injured cortex was reduced 35.2% (p < 0.05) and 60.6% (p < 0.0001) in groups treated with 0.3 and 1.0 mg/kg tirilazad, respectively, in comparison to groups treated with vehicle. In addition, the difference between the protection afforded by these two doses was statistically significant (p < 0.05), indicating that the higher 1.0 mg/kg dose was more effective in reducing post-SAH BBB damage. The 0.3 and 1.0 mg/kg doses of U-89678 also reduced cortical levels of Evans’ blue dye by 39.1% (p < 0.05) and 21.3% (not significant), respectively.

In Vitro Protection of Spinal Cord Neurons by Tirilazad and U-89678

Figure 3 displays a comparison of the effectiveness of tirilazad and U-89678 in protecting murine neuronal cultures from iron-induced lipid peroxidative injury. Neuronal viability, expressed as the percent of AIB uptake by noninjured cells, is presented as a function of drug concentration. Cell viability was preserved in a dose-dependent fashion after exposure to 3 to 30 μM concentrations of both tirilazad and U-89678 (p < 0.0001 for each). Statistical comparisons revealed that cell survival was significantly enhanced in cultures treated with 3, 10, and 30 μM concentrations of both tirilazad and U-89678 in comparison to untreated injured controls (all p < 0.005). At 10 μM, cell viability was significantly better preserved in cultures treated with U-89678 compared to those treated with tirilazad (p < 0.01); however, at 30 μM, the two compounds were equally effective. These results lead us to suggest that although the maximum efficacy of tirilazad and its metabolite U-89678 are the same, the metabolite with the partially reduced A-ring is perhaps a half-log more potent than the parent.

Discussion

It is well known that the presence of blood in the subarachnoid space causes an increase in BBB permeability. Post-SAH cerebral edema and an associated rise in intracranial pressure occurs as a consequence of the permeability disruption. Indeed, the results from the present study in rats confirm that SAH very rapidly disrupts BBB permeability. This was evidenced by the marked increase in cortical uptake of Evans’ blue dye observed in vehicle-treated rats 3 hours post-SAH in comparison to both background and sham-hemorrhage controls. In sham-treated controls from the present study, placement of the catheter and subarachnoid injection of blood plasma, rather than whole blood, elevated cortical Evans’ blue dye levels only slightly above that of background controls. Therefore, plasma alone does not trigger BBB damage. Rather, hemoglobin or some other whole blood constituent must mediate the injury. Evidence to date indicates that oxygen radicals may play a significant role in the genesis of BBB damage after CNS
trauma by triggering the peroxidation of membrane polyunsaturated fatty acids within the vascular endothelium.7,11,12,15,23 For instance, we have shown that the progressive increase in BBB permeability, observed after controlled cortical impact injury in the rat, is preceded by a burst of \( \cdot \)OH formation and a linear increase in the concentration of lipid hydroperoxides in the injured cortex.21

Treatment with 1.0 mg/kg tirilazad 10 minutes before and 2 hours after SAH attenuated the SAH-induced increase in BBB permeability by 60.6%. In addition, an even lower dose of tirilazad (0.3 mg/kg) significantly reduced SAH-induced BBB damage by 35.2%. These results are in line with numerous studies showing that tirilazad consistently attenuates BBB damage in multiple models. For instance, the compound has been shown to reduce BBB permeability after subarachnoid injection of FeCl\(_3\), arachidonic acid, or whole blood in the rat.24,25

Tirilazad has also been shown to decrease the posttraumatic increase in BBB permeability observed after head injury in the rat26 and mouse.8,11 Consistent with its ability to maintain posttraumatic BBB integrity, the drug is also effective in reducing brain edema following experimental SAH10 and focal cerebral ischemia.13,23 Recent in vivo and in vitro data indicate that tirilazad does not readily cross the BBB but rather is largely localized within the vascular endothelium.1,10 Therefore, as a microvascursively localized antioxidant, its particular efficacy in protecting BBB integrity is not surprising.

A principal human metabolite of tirilazad, U-89678, is formed when the 4–5 double bond of the A-ring is reduced. It has been suggested that U-89678 might contribute significantly to the overall efficacy of tirilazad in clinical SAH.8 Indeed, because of the structural similarity between these two 21-aminosteroids, one would expect U-89678 to localize within the vascular endothelium and exert the same kind of vasoprotective action as observed with tirilazad. In fact, the results from our SAH experiment demonstrate that equivalent doses of tirilazad and its metabolite U-89678 produce a similar reduction in BBB permeability (35.2% and 39.1% reduction, respectively, at 0.3 mg/kg). Experiments showed that U-89678 was less effective than tirilazad at the 1 mg/kg intravenous dose level. However, this difference in efficacy may be due to the fact that U-89678 was studied in its less water-soluble free-base (nonacid salt) state. Hence, drug solubility and delivery to the vascular endothelium may have been less than optimal at the higher dose.

Although much of the beneficial effect of tirilazad and U-89678 may be mediated through a vasoprotective action, the question of their relative direct neuroprotective activities should be addressed. This is due to the belief that some tirilazad (and probably U-89678) can penetrate into the brain parenchyma and that this penetration is enhanced after SAH or other CNS injuries as a consequence of BBB disruption. Thus, it is relevant to note that in the present study both tirilazad and U-89678 significantly enhanced survival of cultured neurons against iron-induced lipid peroxidative injury. Whereas U-89678 was slightly more potent than tirilazad, the two compounds appeared to possess the same maximum antioxidant neuroprotective efficacy. The role of iron in the initiation and propagation of oxygen radical–induced lipid peroxidative injury is well documented.3,17 In models of SAH, free iron released from hemoglobin and ferritin/transferrin stores may play a particularly important role in the development of BBB as well as direct neuronal damage.24,25

In summary, the results from the present study lead us to suggest that tirilazad and its metabolite, U-89678, possess roughly equivalent vasogenic and neuroprotective properties. As noted above, initial Phase III clinical results show that tirilazad dosing can decrease mortality and improve neurological recovery at 3 months post-SAH.15 Interestingly, the beneficial effect of tirilazad administration in clinical SAH has thus far been demonstrated only in men. The blunted response in women is paralleled by lower blood levels of tirilazad, and in particular, lower levels of U-89678 (by as much as 60%) compared to those measured in men. This is consistent with the probable important role of this metabolite in tirilazad’s overall neuroprotective action.

References

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