Effects of the 21-aminosteroid U74006F on experimental head injury in mice

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The ability of a novel non-glucocorticoid 21-aminosteroid U74006F to inhibit lipid peroxidation of central nervous system tissue in vitro and to enhance the early neurological recovery and survival of mice after a severe concussive head injury is described. In the in vitro studies, U74006F was found to be an extremely potent inhibitor of lipid peroxidation in an assay system where the glucocorticoid steroid methylprednisolone and the non-glucocorticoid steroids U72099E and U75718A were almost completely ineffective. In the head-injury studies, unanesthetized male CF-1 mice were subjected to a 900 gm-cm closed head injury produced by a 50-gm weight being dropped 18 cm. This concussive injury resulted in immediate unconsciousness (loss of righting reflex) in all animals and death in approximately 30%. Survivors received a tail vein injection of either vehicle or U74006F (0.001 to 30 mg/kg) within 5 minutes postinjury. Their neurological status was evaluated 1 hour later using a grip test. The grip-test score indicated that intravenous administration of a single dose of U74006F resulted in a significant improvement by as much as 168.6% in the neurological status 1 hour postinjury over a broad dose range (0.003 to 30 mg/kg). A 1-mg/kg dose given intravenously within 5 minutes and again at 1-½ hours after a severe injury, in addition to improving early recovery, also increased the 1-week survival rate to 78.6% compared to 27.3% in vehicle-treated mice (p < 0.02). The compound was also effective in enhancing early recovery after a more moderate injury. This study demonstrates that early treatment after severe concussive head injury with a potent inhibitor of iron-dependent lipid peroxidation can significantly benefit the injured brain in mice and promote both early neurological recovery and long-term survival.

KEY WORDS • 21-aminosteroid • U74006F • experimental head injury • lipid peroxidation • mouse

Previous work has demonstrated that, when administered in large (30- to 60-mg/kg) intravenous doses, the glucocorticoid steroid methylprednisolone sodium succinate (MP) can enhance the early neurological recovery of CF-1 mice following either a moderate or a severe concussive head injury. While few clinical data from controlled trials exist, one recent study has shown that high-dose treatment with MP (30 mg/kg initially, given intravenously) can significantly improve survival and recovery of speech in victims of severe head injury. At least one anecdotal report provides evidence for the possible efficacy of high-dose MP in clinical head injury.

In view of the extraordinarily large doses of MP required to exert a posttraumatic cerebroprotective effect, it has been hypothesized that this beneficial action is unrelated to classical glucocorticoid receptor activation which should be maximally affected at more conventional steroid doses. An earlier report from the present authors documented the ability of U72099E, a non-glucocorticoid analog of MP, to equal the efficacy and double the potency of MP in promoting early neurological recovery of severely head-injured mice. While multiple and perhaps interrelated mechanisms may underlie the beneficial effects on the injured brain of the glucocorticoid steroid MP or the non-glucocorticoid analog U72099E, a primary focus of our attention has been on the ability of these and certain other steroids to inhibit posttraumatic lipid peroxidation in central nervous system (CNS) tissue. For instance, MP has been shown repeatedly to inhibit lipid peroxidation in the injured spinal cord. More directly, a close correlation has been demonstrated between the efficacy and potency of the glucocorticoid steroids MP, prednisolone, and hydrocortisone as inhibitors of in vitro lipid peroxidation in rat brain synaptosomes and as promoters of recovery in head-injured mice. Moreover, the non-glucocorticoid steroid U72099E, in addition to being twice as potent as MP in improving early recovery of mice after severe head injury, is also
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twice as potent as MP as an inhibitor of lipid peroxidation in vitro. In the current investigation, U74006F (21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methyl-pregna-1,4,9(11)-triene-3,20-dione, monomethane sulfonate), a novel non-glucocorticoid 21-aminosteroid, is examined (Fig. 1). This agent is an extremely effective and potent inhibitor of iron-dependent lipid peroxidation in rat brain tissue. Consistent with this action, U74006F improves early neurological recovery and long-term survival times in mice after severe concussive head injury when administered over a broad range of intravenous doses.

Materials and Methods

Lipid Peroxidation Studies

The glucocorticoid MP (a sodium succinate), the non-glucocorticoid steroids U72099E and U75718A (also sodium succinates), and the 21-aminosteroid U74006F were compared for their ability to inhibit iron-dependent lipid peroxidation in CNS tissue in vitro. U75718A represents the steroid moiety of U74006F with a hemisuccinate ester (Na salt) in the 21 position in place of the complex amine (Fig. 1).

Rat brain homogenates (1:10, w/v) were prepared in Krebs buffer (15 mM N-2-hydroxy ethyl-piperazine-N'-2-methanesulfonic acid (HEPES), pH 7.4, 10 mM glucose, 140 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl₂, 1.4 mM KH₂PO₄, 0.7 mM MgSO₄) and used immediately. Incubations (100 μl) in Krebs buffer were at 37°C and contained 10 μl brain homogenate, 20% ethanol, and the concentration of compound indicated. Reactions were initiated by the addition of 200 μM Fe²⁺ freshly prepared as described elsewhere. After 20 minutes, the reaction was stopped by the addition of 500 μl ice-cold 12.5% trichloroacetic acid in 0.8 N HCl. Lipid peroxidation was assessed by the formation of thiobarbituric acid reactive products also as described previously.

Preparation of Compounds

Methylprednisolone, U72099E, and U75718A were prepared in H₂O. Although U74006F is soluble in 1 equivalent of HCl, at concentrations below 100 μM the compound binds avidly to glass and plastic. Therefore, all stock solutions and dilutions of U74006F were prepared in ethanol in which binding of the compound to glass was not a problem. U75718A was dissolved in 0.9% NaCl.

Mouse Head-Injury Studies

Male CF-1 mice, each weighing 17 to 22 gm, were used in this study. Water and food (Ralston Purina Laboratory Chow No. 5001) were provided ad libitum before the experiments. A diagram of the head-injury apparatus has been published previously. Each mouse was held by the dorsal skin of the neck, and its head was carefully and firmly placed on the metal baseplate of the injury apparatus. A Teflon impounder was lowered onto the center of the head. The striking surface of the impounder was flat and round and had a 6 mm diameter. A 50-gm stainless steel weight was released by a pin. The weight fell a distance of 18 cm along a stainless steel shaft and struck the impounder producing an approximated force of 900 gm-cm (50 gm x 18 cm). Anesthesia was not required since this injury consistently caused immediate unconsciousness as judged from the loss of righting reflex and the loss of any pain response.

At 1 hour after injury, the sensorimotor status of the head-injured mice was examined by means of a grip test. The mice were individually picked up by the tail and placed on a taut string 60 cm in length suspended between two upright metal bars 40 cm above a padded table. Care was taken that both front paws came in contact with the string, thus allowing each mouse an equal chance to grasp the string. The tail was gently released, at which time the mouse either fell, due to inability to hold on, or remained on the string. The length of time the mice could remain on the string in some manner (that is, using one to four paws, tail, or paws plus tail) was measured with a 30-second maximum. Groups of 20 mice were injured at one time in rapid succession.

The 1-hour neurological recovery data were evaluated in three ways. First, a mean grip-test score was calculated for each treatment group. This test consisted of an average of the time that all mice in the group...
remained on the string. Second, a determination was made of the percentage of mice that fell within 5 seconds ("severely impaired") versus the percentage that remained on the string for the full 30 seconds ("mildly impaired"). Third, the percentage of mice in each group that could not pull at least one hindlimb onto the string was noted as an index of hindlimb paraparesis.

An earlier report11 has documented that both immediate mortality and 1-hour neurological dysfunction of surviving mice are linearly related to injury force. However, subsequent studies (ED Hall, unpublished data) have also shown that the severity of the resulting injury (that is, acute mortality and 1-hour neurological deficit) at a given injury force (such as 900 gm-cm) varies with the size of the mice. For instance, mice in the 17- to 19-gm weight range typically display a higher acute mortality and a lower 1-hour postinjury grip-test score with a given injury than do mice weighing 20 to 22 gm. Accordingly, two injury severity levels were studied in the present investigation. The "severe" injury was produced by a 900 gm-cm injury in 17- to 19-gm mice and resulted in a 1-hour mean grip-test score of less than 7 seconds. In contrast, a "moderate" injury was generated with the same injury force in mice weighing 20 to 22 gm. The injection volume was held constant at 0.1 ml/mouse. Six groups of mice were used in each trial: one group received an injection of vehicle (0.05 N HCl) and five received injections of U74006F (in an amount ranging from 0.001 to 30 mg/kg). Each trial was conducted blindly in regard to which groups of mice received vehicle or U74006F. The drug solutions were prepared and then were relabeled in code by another person. After the 1-hour grip-test scoring, the mice were sacrificed and the treatment code was broken.

In the present study, data from multiple trials at most test doses have been combined to obtain a clearer picture of the potency and efficacy of U74006F in terms of the enhancement of early neurological recovery in the mice with "moderate" or "severe" head injury. The resulting mean grip-test scores at each dose were statistically compared to the mean score from vehicle-treated mice, specifically from the same trials, using a Student's t-test. In other words, each dose group was compared to its own control group of vehicle-treated mice. This was determined, followed by a second intravenous vehicle or U74006F dose at 1 to 5 hours after injury. The second postinjury grip test was measured at 2 to 4 hours, after which the mice were followed to determine survival over a period of 1 week.

### Analysis of Data

In the present study, data from multiple trials at most test doses have been combined to obtain a clearer picture of the potency and efficacy of U74006F in terms of the enhancement of early neurological recovery in the mice with "moderate" or "severe" head injury. The resulting mean grip-test scores at each dose were statistically compared to the mean score from vehicle-treated mice, specifically from the same trials, using a Student's t-test. In other words, each dose group was compared to its own control group of vehicle-treated mice. This
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In Vitro Lipid Peroxidation

The ability of U74006F to inhibit iron-dependent lipid peroxidation in rat brain homogenates is demonstrated in Table 1. This is an extremely potent compound which even inhibits lipid peroxidation at a 1 μM concentration. In this assay system, involving very intense lipid peroxidation, the glucocorticoid MP and the non-glucocorticoid steroids U72099E and U75718A were virtually inactive.

Early Neurological Recovery After Moderate or Severe Head Injury

Figure 2 displays the dose-response analysis showing the ability of U74006F to enhance the grip-test scores of male CF-1 mice following severe concussive head injury (mean 1 hour postinjury scores in vehicle-treated mice ranged from 3.5 to 6.7 seconds). The neurological status of the head-injured mice was significantly enhanced over a wide range of U74006F doses from 0.003 to 30 mg/kg. Interestingly, the dose-response curve was very flat and there was no significant difference in the degree of improvement between any two doses over this dose range. The percent increase in the 1-hour mean grip-test score ranged from 98.0% to 168.6% in relation to the paired vehicle mean scores. A 0.001 mg/kg (1 μg/kg) intravenous dose appeared to represent a threshold dose. At the other end of the dose-response curve, the effect falls off above 10 mg/kg.

Table 2 presents the effects of either a 1.0- or 3.0-mg/kg intravenous U74006F dose on the incidence of severe impairment, mild impairment, or paraparesis at 1 hour after either a moderate or a severe head injury. A significant improvement in all three categories was observed at both doses in the moderately and severely injured mice. The only exception was the incidence of paraparesis with a 1.0-mg/kg dose after the moderate injury (p < 0.1).

The dose-response curve for the non-glucocorticoid steroid U75718A appears in Fig. 3, showing improvement in the mean grip-test score 1 hour after a severe injury.

Results

Inhibition of In Vitro Lipid Peroxidation

Similarly, the incidence of “mildly impaired,” “severely impaired,” or paraparetic mice in the U74006F-treated versus the paired vehicle-treated mice from the same trials was compared using chi-square analysis. The difference in 1-week survival rates was also evaluated by chi-square analysis. In the case of U75718A, significant differences at a particular dose, in comparison to the paired vehicle-treated group, were assessed by the Duncan's multiple range test.

Fig. 2. Dose-response analysis of the effects of a single intravenous dose of U74006F administered 3 to 5 minutes after severe head injury on the 1-hour postinjury mean grip-test score. Values are means ± standard error of the means (S.E.) obtained from one to 14 accumulated trials. The number in each bar represents the total number of mice. The vehicle-treated mice that are compared to a particular U74006F dose are from the same trials. Asterisks indicate a significant (p < 0.05) effect by Student's t-test. i.v. = intravenous.
Fig. 3. Dose-response curve for the effects of a single intravenous dose of U75718A administered 3 to 5 minutes after severe head injury on the 1-hour postinjury mean grip-test score. Values are means ± standard error of the means (S.E.) obtained from the indicated number of mice. Asterisk indicates a significant (p < 0.05) effect by Duncan’s multiple range test. i.v. = intravenous.

head injury. This compound was also active in the mouse head-injury model. In contrast to U74006F, a 30-mg/kg dose of U75718A was required to improve neurological recovery significantly, with lower and higher doses having little or no effect. These findings are similar to those reported for the glucocorticoid MP11.13 and the 21-hemisuccinate non-glucocorticoid steroid U72099E.15 Interestingly, U75718A was also virtually inactive compared to U74006F in the rigorous lipid peroxidation assay reported here (Table 1). Thus, while the steroid moiety is sufficient for a cerebroprotective effect, the 21-amine substitution appears to be responsible for both the efficacy against lipid peroxidation and the in vivo cerebroprotective potency and broad therapeutic dose range of U74006F.

Improvement in Long-Term Survival After Severe Head Injury

Table 3 shows the results of an experiment in which the neurological recovery and 1-week survival after a severe concussive head injury was compared in vehicle-treated mice versus mice treated with U74006F (1 mg/kg intravenously at 5 minutes and 1½ hours after injury). There was a significant increase in the mean grip scores at 1, 2, and 4 hours. At 1 week, 51.3% more of the U74006F-treated mice had survived in comparison to vehicle-treated mice (p < 0.02).

Discussion

The results show that the 21-aminosteroid U74006F is remarkably potent and effective in promoting early neurological recovery of mice after either moderate or severe concussive head injury. In the severely injured mice, a single intravenous dose as low as 0.003 mg/kg (3 μg/kg) significantly improved the 1-hour postinjury grip-test score. However, the effective intravenous dose range extended as high as 30 mg/kg. The most extensively studied doses (1 or 3 mg/kg) significantly reduced the number of severely impaired mice that were unable to remain on the grip-test string more than 5 seconds or that were paraparetic, and increased the number of mice that could stay on the string for the full 30-second test period at 1 hour after injury (that is, mildly impaired). In addition to enhancing early recovery, repeated administration of a 1-mg/kg intravenous bolus was shown to nearly triple the 1-week survival rate.

As noted earlier, both the glucocorticoid steroid MP11.13 and the non-glucocorticoid MP analog U72099E13 also improved the early neurological recovery in the same murine head-injury model. However, intravenous doses of 30 to 60 mg/kg were required in order to be effective. Similarly, the steroid moiety of U74006F (U75718A) also improved the 1-hour grip-test score of severely injured mice, but a 30-mg/kg intravenous dose was needed for a significant effect. Thus, in the case of U74006F, while the steroid is perhaps partly responsible for the cerebroprotective effect, the 21-amine functional group would appear to account for the 10,000-fold increase in potency. Other studies have shown that U74006F is completely lacking in glucocorticoid receptor activity since concentrations as high as 10⁻⁷ M do not suppress adrenocorticotropic hormone (ACTH) release from cultured mouse AtT-20 pituitary tumor cells.1

The mechanism of the presently described cerebroprotective effect of U74006F, either at a molecular or a physiological level, is uncertain. The compound was designed as a potent inhibitor of iron-dependent lipid peroxidation8 and was selected for study on this basis. As shown in Table 1, U74006F is highly effective in this regard. A literal translation of in vitro concentrations to an in vivo effect is fraught with various assumptions. Nevertheless, if one considers a 1-mg/kg intravenous dose of U74006F (molecular weight 738.4) given to a 20-gm mouse, the peak concentration of the drug if distributed in total body water (58% of body weight or 1.2 ml) would be approximately 2.3 μM.

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<p>| TABLE 3 |
| Short-term recovery and long-term survival in severely head-injured mice treated with U74006F* |</p>
<table>
<thead>
<tr>
<th>Injectate No. of Tests</th>
<th>Grip Score (sec)</th>
<th>1 Hr</th>
<th>2 Hrs</th>
<th>4 Hrs</th>
<th>1-Wk Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>11</td>
<td>5.3 ± 2.0</td>
<td>9.1 ± 3.9</td>
<td>9.9 ± 3.5</td>
<td>27.3</td>
</tr>
<tr>
<td>U74006F</td>
<td>14</td>
<td>16.6 ± 3.3t</td>
<td>22.3 ± 2.8t</td>
<td>22.3 ± 2.9†</td>
<td>78.6†</td>
</tr>
</tbody>
</table>

* Effects of intravenous U74006F (1.0 mg/kg) given at 5 minutes and 1½ hours after severe head injury on early postinjury neurological recovery and 1-week survival in male CF-1 mice. Values are means ± standard error of the means.  
† p < 0.05 by Student’s t-test (two-tailed).  
‡ p < 0.02 by chi-square analysis.
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Since the compound is extremely lipophilic, the cell membrane concentration is probably much higher. Therefore, the probable in vivo brain-tissue concentrations are well within the concentration range necessary to inhibit iron-dependent lipid peroxidation in an in vitro assay where lipid peroxidation is very intense.

Actually, U74006F was chosen as one of the most active in a series of 21-aminosteroid compounds in which the ability to inhibit iron-dependent lipid peroxidation closely correlates with enhancement of early neurological recovery in head-injured mice. The precise nature of the anti-lipid peroxidation action of compounds in this series is thought to include a vitamin E-like membrane antioxidant action, a superoxide anion scavenging property, and possibly membrane-localized iron chelation.

At a more physiological level, U74006F has been shown in cats to antagonize the development of progressive spinal cord white matter ischemia after severe cord contusion injury. This pathophysiological phenomenon is believed to be due in large part to injury-induced microvasular lipid peroxidation. It is conceivable that a maintenance of cerebral blood flow may also contribute to the improved recovery and survival of mice after severe concussive head injury.

In addition to the inhibition of iron-dependent lipid peroxidation, U74006F may beneficially affect the injured nervous system by other mechanisms which remain to be explored. For instance, the list of demonstrated cerebroprotective mechanisms of MP (within the context of the injured spinal cord) includes inhibition of posttraumatic lipid peroxidation, prevention of neurofilament degradation, inhibition of arachidonic acid release and vasoactive prostaglandin F₂α, and thromboxane A₂ formation, and enhancement of neuronal excitability and synaptic transmission. Regarding one of these mechanisms, U74006F (10 μM concentration) has also been shown to inhibit either iron- or iodoacetate-induced arachidonic release from cultured cells.

Further studies are under way to determine the therapeutic mechanisms and potential of U74006F and related 21-aminosteroids in the acute treatment of CNS trauma, to both the head and the spine. Thus far, the ability of the compound to inhibit iron-dependent lipid peroxidation of CNS tissue in a highly potent and effective manner correlates with potency and efficacy in improving recovery and survival after experimental head injury.

References