Neuroprotection (Focal Ischemia) and Neurotoxicity (Electroencephalographic) Studies in Rats with AHN649, a 3-Amino Analog of Dextromethorphan and Low-Affinity N-Methyl-d-Aspartate Antagonist1

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ABSTRACT

AHN649, an analog of dextromethorphan (DM) and a relatively selective low-affinity N-methyl-d-aspartate antagonist, was evaluated for neuroprotective effects using the rat intraluminal filament model of temporary middle cerebral artery occlusion. Rats were subjected to 2 h of focal ischemia followed by 72 h of reperfusion. In vehicle-treated rats, middle cerebral artery occlusion resulted in neurological deficits and severe infarction measuring 232 ± 25 mm³, representing approximately 25% contralateral hemispheric infarction. Post-treatment with AHN649 (0.156–20 mg/kg i.v.) or DM (0.156–10 mg/kg i.v.) significantly reduced cortical infarct volume by 40 to 60% compared with vehicle-control treatments. AHN649 neuroprotection was linear and dose dependent (ED50 = 0.80 mg/kg), whereas DM neuroprotection (ED50 = 1.25 mg/kg) was nonlinear and less effective at the higher doses (2.5–10 mg/kg). Although impaired neurological function scores improved in all groups by 24 to 72 h, the most dramatic improvement was associated with AHN649 treatments. In a rat electroencephalographic model of brain function, separate neurotoxicity experiments revealed that acute i.v. doses of DM caused seizures (ED50 = 19 mg/kg) and death (LD50 = 27 mg/kg). In contrast, AHN649 failed to induce seizure activity at doses up to 100 mg/kg (LD50 = 79 mg/kg). Collectively, AHN649 is described as a potent, efficacious neuroprotective agent devoid of serious central nervous system neurotoxicity and possessing potential therapeutic value as antistroke treatment. Furthermore, the feasibility of targeting low-affinity N-methyl-d-aspartate-site ligands as postinjury therapy for ischemic brain injury has been confirmed.

Patent anticonvulsant and neuroprotective actions associated with high-affinity blockers of glutamate receptors, especially those coupled to the noncompetitive N-methyl-d-aspartate (NMDA)/phencyclidine (PCP) recognition site of the channel complex, have been firmly established (Rogawski, 1992; Muir and Lees, 1995). However, increasing awareness of the potential neurotoxicity associated with these ligands, including neuropathology, motor dysfunction, seizures, and possible hallucinogenic and/or psychotomimetic actions, may limit their development as clinical drugs. Because hyperactivity of glutamate synapses remains one of the most significant pathological consequences of cerebral ischemia, mechanisms targeting attenuation of glutamatergic excitotoxicity at the molecular, receptor, and/or ion channel level continue to be explored for therapeutic implications (Sztatkowski and Attwell, 1994; Muir and Lees, 1995; Whetsell, 1996).

Consistent with excitatory amino acid mechanisms underlying clinical episodes of severe pathological depolarization or impaired metabolism (i.e., cellular ischemia), low-affinity NMDA- and/or PCP-site ligands have been synthesized and developed with the intent of providing therapeutic efficacy with limited toxicity (Rogawski, 1992, 1993; Cregan et al., 1997). In principle, a relatively selective low-affinity NMDA/PCP ligand would 1) retain significant anticonvulsant or neuroprotective properties by virtue of use-dependent/non-competitive and/or voltage-dependent mechanisms without 2) the unwanted side effects associated with potent activation of the high-affinity NMDA/PCP receptor complex. PCP analogs have been synthesized that fit this receptor affinity and pharmacodynamic profile (Rogawski, 1992), and non-PCP compounds possessing micromolar channel-blocking af-

ABBREVIATIONS: NMDA, N-methyl-d-aspartate; MCAo, middle cerebral artery occlusion; EEG, electroencephalogram; NS, neurological scores; AHN649, 3-amino-17-methyl morphinan; DM, dextromethorphan; PCP, phencyclidine; SWS, slow-wave sleep; TTC, 2,3,5-triphenyltetrazolium chloride; TI, therapeutic index.
finity at the noncompetitive NMDA/PCP-binding site have also been demonstrated as safe and effective anticonvulsant and neuroprotective agents in animal studies (Rogawski, 1993; Palmer et al., 1995; Cregan et al., 1997).

We recently described the anticonvulsant and neuroprotective properties of an analog of dextromethorphan (DM), namely AHN649 (3-amino-17-methyl morphinan), which is a relatively selective ligand for low-affinity NMDA/PCP binding sites ($K_i = 9.9 \mu M$; Newman et al., 1996). In rat models of experimental epilepsy, including maximal electroshock-, fluoroethyl-, and NMDA-induced convulsions, AHN649 is a potent anticonvulsant devoid of neurobehavioral toxicity (Tortella et al., 1994). In subsequent studies, AHN649 has been shown to prevent glutamate-induced cell death and the associated changes in excitotoxic intracellular calcium dynamics in neuronal cultures (Tortella et al., 1995) and to possess in vivo neuroprotective efficacy similar to those of other NMDA antagonists in a rat model of global forebrain ischemia (Tortella et al., 1997a).

The primary clinical target for an anti-ischemia neuroprotective drug is the treatment of stroke in humans. Regardless of etiology, focal disturbance of blood flow and subsequent disruption of neuronal metabolism leading to cell death characterizes this ischemic insult. Because most clinical strokes are acute focal ischemic episodes, animal models mimicking focal ischemia to produce brain infarction, such as that occurring after occlusion of the middle cerebral artery occlusion (MCAo), appear to have significant relevance (Hunter et al., 1995). The MCAo filament procedure was first introduced by Koizumi et al. (1986) and Longa et al. (1989) as an extracerebral “noninvasive” model of experimental stroke in rats. In general, permanent MCAo produces a focal ischemic injury marked by a core area of hemispheric infarction localized within the immediate borders of the MCA territory, namely the striatum and overlying parietal/temporal cortical tissues. A penumbral area of pathologically compromised neuronal tissue surrounds the core infarction and is located more distant from the MCA. The intraluminal filament model using a 2-h transient occlusion reproduces this injury, yet permits reflow of the MCA territory. This more closely models the clinical event of ischemic stroke and reflow that may occur in more than 80% of patients experiencing acute embolic occlusion of the MCA trunk (Ringelstein et al., 1992). In addition, the incorporation of experimental reperfusion is critical because although primate studies have shown that cerebral reperfusion may be beneficial to neuronal survival (Crowell et al., 1970), it has also been suggested that restoration of blood flow may actually worsen outcome by further promoting brain edema (Kuroiwa et al., 1988) and blood-brain barrier disruption (Yang et al., 1994) and physiologically promoting cellular mechanisms of delayed neuronal injury (Hallenbeck and Dutka, 1990; Halsey et al., 1991). Therefore, by experimentally mimicking this injury process, one ensures a pathophysiological complexity consistent with the observed clinical disorder.

In the present study, we examined AHN649 (in comparison with DM) in a rat model of focal cerebral ischemia and reperfusion and conducted a comprehensive dose-effect analysis of functional neurotoxicity using spontaneous cortical electroencephalographic (EEG) activity in unanesthetized rats.

Materials and Methods

Animal Preparation. For MCAo experiments, 77 male Sprague-Dawley rats (270–330 g; Charles River Labs, Raleigh, VA) were implanted with a chronic indwelling jugular vein catheter and prepared for temporary focal ischemia using the filament method of MCAo and reperfusion (Britton et al., 1997). Anesthesia was induced with 5% halothane and maintained with 2% halothane delivered in oxygen. Body temperature was maintained normothermic (37 ± 1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Briefly, the right external carotid artery was exposed, and its branches were coagulated. A 3-0 uncoated monofilament nylon suture with rounded tip was introduced into the internal carotid artery via an arteriotomy in the external carotid artery and advanced (approximately 22 mm from the carotid bifurcation) until it lodged in the proximal region of the anterior cerebral artery, thus occluding the origin of the MCA. The endovascular suture remained in place for 2 h and then was retracted to allow reperfusion. After surgery, animals were placed in recovery cages with ambient temperature maintained at 22°C. During the 2-h ischemia period and the initial 6-h posts ischemia period, 75-W warming lamps were also positioned directly over the tops of each cage to maintain body temperature normothermic throughout the experiment. [The facilities in which the animals were maintained are fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.]

For EEG experiments, 55 additional drug-naive male Sprague-Dawley rats (250–300 g) were anesthetized with ketamine HCl and xylazine (70 mg/kg and 6 mg/kg i.m., respectively) and surgically prepared with a chronic indwelling jugular vein catheter and epidural stainless steel screw electrodes (0-80 × 1/8 inches; Small Parts Inc., Miami Lakes, FL) chronically implanted and affixed to the skull with the use of dental acrylic cement (Tortella et al., 1997c). The four recording electrodes were implanted bilaterally over the right and left frontal (3 mm anterior, 2 mm lateral to bregma) and parietal (3 mm posterior, 2 mm lateral to bregma) cortices. A fifth reference electrode was implanted over the occipital cortex. After surgery, all animals were allowed 3 to 5 days for recovery before drug testing. During this time, they were provided food and water ad libitum and allowed to acclimate to the test environment by being individually housed in clear Plexiglas recording chambers (30 × 30 × 49 cm) under a 12-h light/dark cycle (lights on at 6:00 PM).

MCAo Experiments. Before MCAo surgery, the animals were randomly divided into three treatment groups: vehicle ($n = 14$, distilled deionized water), AHN649 (0.156–20 mg/kg, $n = 7$/dose), or DM (0.156–10 mg/kg, $n = 7$/dose). Beginning at 30 min after occlusion and again at 1, 2, 4, 6, 24, and 48 h after injury, each rat received an i.v. injection of the appropriate treatment. Before each injection, the body temperature was recorded, and a neurological examination was performed (see below). Importantly, so as not to compromise the results of the functional neurological examination at 2 h, it was performed immediately before reperfusion and the 2-h injection. As such, the actual 2-h injection was administered immediately after recovery from the reperfusion surgical procedure (approximately 125–130 min after injury). Food and water were provided ad libitum, and the animals were individually housed in clear Plexiglas chambers (30 × 30 × 49 cm) under a 12-h light/dark cycle (lights on at 6 PM). After 70 h of reperfusion/recovery, the rats were euthanized by decapitation, and their brains were removed for quantification of infarction.

Infarct Analysis. From each rat brain, seven coronal sections (2 mm thick) were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction. Analysis of ischemic cerebral damage, including total core infarct...
volume and hemispheric infarct size, was achieved using the 2,3,5-triphenyltetrazolium chloride (TTC) staining method and computer-assisted image analysis as described in detail elsewhere (Britton et al., 1997). Briefly, the posterior surface of each TTC-stained forebrain section (and the anterior surface of the first section) was digitally imaged (Loats Associates, Westminster, MD). Infarction and hemispheric areas (mm²) were quantified to provide subsequent determinations of the various infarct characteristics, including: 1) total volume (mm³) of core injury, 2) the percentage of volume of cerebral tissue representing infarction (percentage of hemispheric infarction), and 3) hemispheric swelling. Total core infarct volume, where core injury was defined as brain tissue completely lacking TTC staining, was calculated by sequential integration of the respective surface areas. Similarly, ipsilateral and contralateral hemispheric volumes were calculated to account for the contributing effect of brain swelling to infarct volume (Barone et al., 1993) where hemispheric swelling representing tissue edema was expressed as the percentage of increase in the size of the ipsilateral (occluded) hemisphere compared with the contralateral (uninjured) hemisphere (Britton et al., 1997).

**Neurological Examination.** A neurological examination was performed on each rat immediately before MCAo surgery and again before each injection using a modification of the procedure described by Bederson et al. (1986). Briefly, neurological scores (NS) were derived using a 10-point sliding scale. Each animal was examined for reduced resistance to lateral push (score, 4), open field circling (score, 3), and shoulder adduction (score, 2) or contralateral forelimb flexion (score, 1) when held by the tail and suspended approximately 0.5 m above the floor. Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as an NS of 10. In the present study, all vehicle-treated rats subjected to MCAo exhibited a neurological score of 10 when examined 2 h after ischemia or immediately before reperfusion (see Fig. 6).

**EEG Experiments.** The recording chambers described above were equipped with custom-designed multichannel mercury swivel commutators (Dragonfly Inc., Silver Spring, MD). On the morning of the EEG experiment, the rats were connected to the swivel system by flexible shielded cables providing a noise-free connection from the unrestrained rat to a Grass model 7D polygraph while permitting freedom of movement by the animals during all phases of the experiment. Control EEG recordings were collected such that drug testing was initiated only after each subject exhibited normal behavioral and EEG slow-wave sleep (SWS) patterns. All EEG experiments routinely began between 9:00 and 11:00 AM and were considered complete on the reemergence of SWS. Using this protocol, each animal served as its own control. Throughout these experiments, all rats were drug naïve and used only once. After the onset of normal SWS rhythms and behavior, the experiment was initiated by administering an i.v. injection of the control vehicle (1 ml/kg distilled deionized water). With the reemergence of EEG SWS after the control injections, the animals (n = 5/dose) were injected i.v. with either AHN649 (10–80 mg/kg) or DM (12.5–31.25 mg/kg). For the duration of the EEG experiments, each animal was continuously observed by two of the authors for signs of abnormal behavioral activity, including sedation; ataxia; enhanced locomotion; stereotypic activity including head-weaving, grooming, preening, and scratching; and signs of clonic (convulsant) muscle activity. The behavioral state of each animal was defined subjectively by the clinical appearance, at any time before the onset to SWS, of the following behaviors: 1) sedation, the absence of overt behaviors in an otherwise awake state and distinguished from normal quiet awake behavior by the appearance of drug-induced, synchronized EEG slowing; 2) ataxia, unsteady or impaired gate during walking/locomotion; 3) enhanced locomotion, running or increased sniffing and searching behavior; 4) clonus, a rapid, repetitive movement or twitching of a muscle or muscle group; or 5) sleep, normal sleep posture, eyes closed, and EEG SWS patterns. The behavioral responses for each animal were noted and recorded on the EEG polygraph records as a correlate to their respective changes in EEG activity.

**Data Analysis.** Data are presented as the mean ± S.E. For the MCAo infarct volume and neuroprotection results, statistical analysis were made using an ANOVA and post hoc Dunnett's test for multiple comparisons. Regression analysis and potency comparisons were performed on the percentage of neuroprotection dose-response data. For EEG results, the Student's t test for paired data was used where appropriate. ED₅₀ (the dose required to produce a response in 50% of the animals tested per group) and LD₅₀ (the dose required to produce death in 50% of the animals tested per group) values were derived from quantal dose-response data. For the neuroprotection ED₅₀, the criteria for identifying a positive responder were defined empirically as a drug-treated MCAo animal whose measured infarct volume was less than 140 mm³ or the mean infarct volume minus 1 S.D. for the vehicle control group (or 232 mm³ – 92 mm³, respectively, in the present study). For the seizure ED₅₀, an animal was considered a responder if its postdrug EEG contained ictal activity, regardless of the pattern or severity. All statistical tests were performed using the Pharmacological Calculations Computer Programs described by Tallarida and Murray (1986).

**Compounds.** AHN649 was synthesized at the Walter Reed Army Institute of Research (Division of Experimental Therapeutics) as previously described (Newman et al., 1992), and DM was purchased from Research Biochemicals Inc. (Natick, MA). All compounds were dissolved in distilled deionized water immediately before testing and administered as a 1 ml/kg volume. All injections were administered as a 1-min i.v. infusion without handling or disturbing normal animal behavior.

**Results.** In control vehicle-treated rats, 2 h of MCAo followed by 70 h of reperfusion resulted in core infarction of the ipsilateral temporal/parietal cortex and underlying striatal tissue (Fig. 1). Significant forebrain infarction was measured from approximately 3 to 13 mm from the frontal pole (Fig. 2). Total infarct volume in control rats was 232 ± 25 mm³ (Fig. 3), representing approximately 25% contralateral hemispheric infarction (Fig. 5B). MCAo resulted in significant hemispheric edema representing an approximately 8% increase in cerebral volume compared with the contralateral, uninjured hemisphere (Fig. 5A). All vehicle-treated rats subjected to MCAo exhibited a neurological score of 10 when examined 2 h after ischemia (see Fig. 6). Although neurological function was severely impaired (NS = 10 ± 0), by 72 h postinjury, a significant degree of spontaneous recovery was measured in the control animals (NS = 3.8 ± 0.4). However, none of the injured, vehicle-treated animals completely recovered neurological function (NS = 0), with at least contralateral forelimb flexion and shoulder adduction (NS = 3) still evident in all rats examined at 72 h after injury.

Regardless of the treatment group, all MCAo animals lost approximately 15 to 20% b.w. over the 72-h recovery period, and there were no significant differences in body weight loss between groups. Similar to our earlier findings (Britton and Tortella, 1997), in the vehicle-control animals MCAo caused a transient, mild hyperthermia (38.3 ± 0.2 and 37.6 ± 0.3°C at 2 and 4 h postocclusion, respectively) that returned to normal (36.9 ± 0.4°C precooclusion) by 4 to 6 h postocclusion (36.9 ± 0.3°C). Interestingly, at 48 to 72 h after injury, control animals exhibited a mild and unexpected hypothermia (35.9 ± 0.2 and 35.4 ± 0.5°C, respectively). Only the highest doses of AHN649 (10 and 20 mg/kg) significantly blocked the MCAo-induced hyperthermia. At all other doses
and timepoints, temperature measurements from AHN649- or DM-treated animals were not significantly different from the corresponding control, vehicle-treated animals.

AHN649 or DM post-treatment significantly reduced ischemic infarction (Figs. 1 and 2). Both drugs were equipotent (potency ratio = 0.643, P = NS; Table 1). However, AHN649 was more efficacious than DM, decreasing infarct volume to 100 ± 33 mm³ (Fig. 3) and resulting in a maximal neuroprotection of 57 ± 14% at the highest dose tested (Fig. 4). At both the intermediate (0.625 mg/kg) and high (10 mg/kg) doses of DM, the maximal decrease in infarct volume measured was 137 ± 31 mm³ (Fig. 3), resulting in a maximal neuroprotection of only 41 ± 13% (Fig. 4). Importantly, only the neuroprotective effects of AHN649 were positively correlated with dose (r = 0.90, P < .026; Fig. 3). By comparison, the neuroprotection dose-response effect of DM was significantly nonlinear (r = 0.55, P < .23; Fig. 3). Although both compounds produced a trend toward reducing the amount of injury-induced cerebral edema, these effects were not significant (Fig. 5A). As expected based on the infarct volume data, both AHN649 and DM significantly reduced the overall percentage of hemispheric infarction (Fig. 5B). However, again, the effect of DM was significant at lower doses and nonlinear.

The results shown in Table 1 summarize the neuroprotection and lethality data obtained for each compound. Although the potency (i.e., ED₅₀ value) is similar for both drugs, the large CLs associated with DM reflect the nonlinearity of its effect to decrease cerebral infarction. A comparison of the respective LD₅₀ values derived for AHN649 and DM from normal, uninjured animals reveals a 2.9-fold difference between the two drug groups (79 versus 27 mg/kg, respectively). Critically, the calculated therapeutic indexes (TI) for both drugs indicate a wide margin of safety, and comparisons of their respective TIs indicate that AHN649 is approximately 4.5 times safer than DM.

Compared with vehicle-treated controls, neurological function significantly improved after AHN649 (Fig. 6A) or DM (Fig. 6B) treatment. Improvements in neurological function...
were time and dose dependent for both drugs. When evaluated at comparable times after MCAo, drug-induced neurological improvements were first seen beginning 24 h after injury with continued improvement measured at 48 h (not shown) and 72 h. At the highest doses tested, the NS for AHN649 (20 mg/kg) at 72 h was 0.7 ± 0.4 compared with an NS of 3.8 ± 0.4 for the corresponding controls. In contrast, an optimal improvement in DM-induced NS (2.0 ± 0.6) was measured after the intermediate dose of DM (0.625 mg/kg), with a slight worsening in the NS at higher doses. Of the AHN649-treated rats, 20% completely recovered neurological function (NS = 0), whereas an additional 57% recovered to an NS of 1. In contrast, although neurological function recovered to an NS of 1 in 52% of all DM-treated rats, no rats in this treatment group recovered complete neurological function (similar to nontreated injured rats).

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Neuroprotection ED$_{50}$ (95% CLs)</th>
<th>Lethality LD$_{50}$ (95% CLs)</th>
<th>TI LD$<em>{50}$/ED$</em>{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN649</td>
<td>0.80 (0.15–4.07)</td>
<td>79 (62–100)</td>
<td>99</td>
</tr>
<tr>
<td>DM</td>
<td>1.25 (0.11–13.49)</td>
<td>27 (24–31)</td>
<td>22</td>
</tr>
</tbody>
</table>

*a* Dose producing neuroprotection (as defined in the text) in 50% of the rats subjected to MCAo.

*b* Dose producing lethality in 50% of normal, drug-naive uninjured rats (derived from the EEG experiments).
Results of the EEG and behavior studies are summarized in Table 2 and Figs. 7 to 9. AHN649 (Fig. 7A) produced moderate EEG slowing and sedative behavior but no evidence of EEG seizure activity. DM also induced similar EEG slowing and sedation in animals not exhibiting convulsant activity (Fig. 7B). However, in contrast to AHN649, injections of DM produced mild EEG seizure activity at doses as low as 12.5 mg/kg (not shown) and intense ictal activity characterized by polyspike and spike-wave complexes consistently observed at higher doses (Fig. 8). Typically, the DM-induced EEG seizure activity emerged within minutes after injection and was accompanied by clonic movements followed by postictal depression. From these results, the calculated safety index (ratio of the neuroprotection ED$_{50}$ to the seizure ED$_{50}$) was 15.2 for DM and more than 100 for AHN649. Importantly, as shown in Fig. 9, lethality induced by either compound: 1) occurred within minutes of the injection and 2) was not necessarily associated with brain seizures.

Both AHN649 and DM dose dependently delayed the onset to behavioral and EEG SWS. Normal SWS latency after the control i.v. vehicle injections ranged from 7 to 23 min across both treatment groups. In AHN649-treated rats, a significant increase in SWS latency was not obtained until the 20 mg/kg dose (35.6 ± 6.2 min; $p < .05$, paired $t$ test) and was maximal at the 60 mg/kg dose (107 ± 21.2 min). In DM-treated rats, the latency to SWS was markedly increased (47.4 ± 9.3 min; $P < .01$, paired $t$ test) even at the lowest dose tested (12.5 mg/kg) and continued to increase to a maximum of 153 min measured in the only animal surviving the highest dose tested (31.25 mg/kg).

**Discussion**

The present results describe a comprehensive, dose-response characterization of the neuroprotective and neurotoxic profiles of the DM analog AHN649. Similar to earlier findings derived from a rat model of global forebrain injury (Tortella et al., 1997a), we demonstrate here the ability of AHN649, administered i.v. as postinjury therapy, to significantly decrease brain infarction caused by experimental, focal cerebral ischemia and reperfusion. The neuroprotective effect of AHN649 was associated with significant improvements measured in postinjury neurological function and appears to be unrelated to any adverse cardiovascular or temperature effects. Furthermore, EEG studies in normal rats failed to reveal signs of neurotoxic side effects.

An important feature of the injury model used in this study is its pharmacological sensitivity to neuroprotective agents. For example, in similar preclinical models of transient MCAo-induced cerebral injury, it has been demonstrated that some (but not all) antioxidant treatments can reduce infarction by 40% (Clemens and Panetta, 1994). Neuroprotection treatments targeting upstream mechanisms (Lu et al., 1998) or specific AMPA receptor mechanisms (Kawasaki-Yatsugi et al., 1998) of excitotoxic glutamatergic activity have also been described to reduce cerebral damage caused by transient MCAo. Not surprisingly, the high-affinity non-competitive NMDA antagonist MK801 also significantly re-
duces infarction in rats or cats by approximately 30% (Dezsi et al., 1992; Margaill et al., 1996), whereas the low-affinity noncompetitive NMDA antagonist ARL 15896AR [(S)-α-phenyl-2-pyridine-ethanamine dihydrochloride] has been shown to reduce infarct volume by approximately 23% (Cregan et al., 1997). By comparison, our results describe a neuroprotective efficacy with AHN649 approaching a 60% reduction in infarct volume at dose levels correlating well with significant improvements in neurological recovery and devoid of EEG or behavioral neurotoxic side effects.

Collectively, using both the MCAo and EEG models, experimental conditions were established permitting a direct, quantitative comparison of the neuroprotective, neurotoxic, and lethal potencies between AHN649 and DM. Although both compounds exhibited similar neuroprotection potencies, the efficacy of DM to decrease cerebral infarction (maximum of 41%) was more limited and likely compromised by neurotoxic actions resulting from brain seizures, a problem also reported with MK801 (Tortella and Hill, 1996). By comparison, the neuroprotective efficacy of AHN649 does not appear to be compromised by behavioral (Tortella et al., 1994) or seizurogenic (present study) neurotoxic side effects. Although AHN649 produced mild sedation and related EEG slowing in normal rats, there was no EEG or behavioral evidence of ataxia, marked sedation, or severe EEG slowing. Critically, there was no evidence of EEG seizures or behavioral convulsant activity, even at the highest (and lethal) doses tested (i.e., 80 mg/kg). This indicates that the protective index for AHN649, calculated as the ratio of the seizure ED50 dose (>80 mg/kg) to the neuroprotective ED50 dose (0.8 mg/kg), is greater than 100. By comparison, the protective index for DM, although still considered relatively safe, is only 15.2.

TABLE 2
Sedation ED50 and EEG seizure ED50 values and 95% CLs

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Sedation ED50 (95% CLs)</th>
<th>EEG Seizures ED50 (95% CLs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN649</td>
<td>35</td>
<td>25 (13–45)</td>
<td>No effect</td>
</tr>
<tr>
<td>DM</td>
<td>25</td>
<td>30 (13–66)</td>
<td>19 (14–28)</td>
</tr>
</tbody>
</table>

a Total number of rats tested per drug group.
b Dose producing sedation or EEG seizure activity in 50% of normal, drug-naïve uninjured rats.

duces infarction in rats or cats by approximately 30% (Dezsi et al., 1992; Margaill et al., 1996), whereas the low-affinity noncompetitive NMDA antagonist ARL 15896AR [(S)-α-phenyl-2-pyridine-ethanamine dihydrochloride] has been shown to reduce infarct volume by approximately 23% (Cregan et al., 1997). By comparison, our results describe a neuroprotective efficacy with AHN649 approaching a 60% reduction in infarct volume at dose levels correlating well with significant improvements in neurological recovery and devoid of EEG or behavioral neurotoxic side effects.

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Although we have not established the neuroprotective potency of MK801 in our MCAo model, other studies have reported similar quantitative neuroprotective effects of MK801 to reduce focal ischemic infarction at a dose of 1 mg/kg (Dezsi et al., 1992; Margaill et al., 1996). Consequently, the protective ratio for MK801 would be projected to be less than 1. Therefore, using experimental stroke and EEG neurotoxicity as outcome measures, AHN649 is approximately 7 times safer than DM and 100 times safer than MK801.

Interestingly, although DM produces seizures, seizure activity per se appeared to be unrelated to the lethal properties of this drug or of AHN649 (Fig. 10). DM was, however, determined to be approximately 3 times more lethal than AHN649 after systemic i.v. injections. Therefore, using the respective LD50 and neuroprotection ED50 data for each compound, the respective TIs (see Table 1) indicate that the 3-amino substitution of DM yields a potential therapeutic drug with a margin of safety at least 5 times greater than its parent molecule.

It is highly unlikely that the neuroprotective effect of AHN649 or DM in cerebral ischemia can be attributable to indirect effects on key physiological parameters such as blood pressure, heart rate, blood pH or gases, or brain/body temperature. In previous studies (Britton et al., 1997; Tortella et al., 1997b), it has been shown that doses of DM or AHN649 in the range of those used in the present study had no effect on these physiological variables in normal rats. Furthermore, in the present study, careful monitoring of rectal “core” body temperature, which has been reported in similar ischemia studies to closely reflect brain temperature (Xue et al., 1992; Zhang et al., 1994), failed to reveal a drug-induced hypothermia associated with the decrease in brain infarction.

It is also unlikely that the neuroprotective effects of AHN649 were due to general barbiturate-like sedative effect on brain metabolism. Profound cortical EEG slowing may reflect a functional state of depressed cerebral metabolic activity. By decreasing the metabolic demands of neuronal tissue during periods of severe brain ischemia, such as one might achieve during a drug-induced coma, preservation of neuronal tissue may be improved. However, in the case of AHN649, it was determined that even at doses several times greater than those required for neuroprotection, there was no EEG evidence of severe cortical slowing. This contrasts with EEG results described elsewhere for high-affinity noncompetitive NMDA antagonists such as MK801 or PCP in which marked cortical EEG slowing can be seen at neuroprotective doses (Marquis et al., 1989; Tortella and Hill, 1997).

Another important finding of this study was the absence of brain neurotoxicity seen with AHN649 as measured by EEG seizures. It has been reported for MK801 and other high-affinity PCP and/or σ ligands that relatively high i.v. doses in rats may be associated with EEG brain seizures and, in some cases, the emergence of proconvulsant properties leading to clinical signs of behavioral convulsant activity (Marquis et al., 1989; Echevarria et al., 1990; Tortella and Hill, 1997). At the cellular level, it is possible that the seizurogenic actions of MK801 and related compounds result from actions leading to an increase in cerebral glucose utilization or the induction of subcortical vacuolization (Nehls et al., 1990; Hargreaves et al., 1993). Importantly, concentrations of AHN649 up to 200 μM are not neurotoxic in cultured rat neurons (Tortella et al., 1995), and light microscopic assessments of cortical tissue derived from normal or ischemic rat forebrain have not shown evidence of AHN649 neuropathology (Tortella et al., 1997a).

Although the neuroprotection mechanism of action of AHN649 remains unresolved, it likely involves antagonism of the NMDA/calcium channel ionophore and blockade of excitotoxic intracellular calcium signaling (Tortella et al., 1995). Also, as reviewed above and in earlier reports (Newman, 1996; Newman et al., 1996) AHN649 and related compounds are relatively selective ligands at low-affinity NMDA-binding sites and the pharmacological profile of AHN649 is consistent with that of an NMDA antagonist (Tortella et al., 1994,
1995). Furthermore, its lack of serious neurobehavioral side effects as determined by EEG analysis (present study), rotorod performance, and overt behavior (Tortella et al., 1994) and morphology (Tortella et al., 1995, 1997a) is predictive of a low-affinity ligand with rapid on-off kinetics (Rogawski, 1993). Other possible mechanisms, including interactions with $\sigma_1$ receptors or blockade of neuronal sodium channels, also warrant consideration. AHN649 is selective for $\sigma_1$ ($K_i = 0.98 \mu M$) versus $\sigma_2$ (>10 $\mu M$) binding sites (Newman et al., 1996), and we have recently determined using NovaScreen receptor competition analysis that AHN649 (1 $\mu M$) produces 78% inhibition at neuronal sodium channels (F.C.T. and A.H.N., unpublished data). Consistent with these mechanisms, $\sigma$ ligands can modulate NMDA channel function and produce neuroprotection (DeCoster et al., 1995; Klette et al., 1995; Couture and Debonnel, 1998), and several sodium channel antagonists are neuroprotective (Taylor and Mel- drum, 1995).

The possible induction of PCP-like psychotomimetic reactions continues to raise serious concerns about the potential clinical value of first-generation high-affinity NMDA antagonists such as MK801. Because DM is rapidly metabolized to the potent PCP-like drug dextroxtorphan, the psychotomimetic risk potential could be considerable. As such, our singular objective has been to synthesize novel analogs of DM that would not metabolize to dextroxtorphan or would do so at a slower rate, and thereby exhibit a neuropharmacology distinct from PCP-like drugs. Experimentally, AHN649 appears to closely fit this profile because it is devoid of PCP-like behavior, EEG, or receptor-binding activity, yet remains many of the clinical actions of DM. Other analogs of DM have been synthesized that also exhibit a similar pharmacological profile and also have been shown to be neuroprotective, at least in in vitro models of neuronal injury (Tortella et al., 1995). These novel analogs of DM remain to be studied in experimental and in vivo brain injury models.

In conclusion, comprehensive dose-response studies have confirmed potent neuroprotective actions of AHN649 in a clinically relevant experimental model of focal cerebral ischemia or stroke. In addition, the results obtained from our EEG studies indicate that AHN649 is safer and relatively devoid of the neurotoxicity normally associated with other NMDA antagonists like DM or MK801. The improved pharmacological profile has been proposed to confer added safety directly attributable to lower-affinity binding at the NMDA receptor complex while retaining neuroprotective efficacy, further supporting the feasibility of targeting low-affinity NMDA site ligands as a postinjury therapy for injuries to the brain. Critical studies aimed at determining the postinjury therapeutic window for AHN649, which has been reported to be relatively short (i.e., less than 30 min) for MK801 (Margail et al., 1996), and therapeutic efficacy in longer recovery models of MCAo injury are under way.

References
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