[(S)-Alpha-Phenyl-2-Pyridine-Ethanamine Dihydrochloride], A Low Affinity Uncompetitive N-Methyl-D-Aspartic Acid Antagonist, Is Effective in Rodent Models of Global and Focal Ischemia¹

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ABSTRACT

[(S)-alpha-phenyl-2-pyridine-ethanamine dihydrochloride] (ARL 15896AR) is a low affinity uncompetitive N-methyl-D-aspartic acid receptor antagonist that was tested in animal models of anoxia and ischemia. Pretreatment of rodents with ARL 15896AR extended survival time during exposure to hypoxia. With the rat four-vessel occlusion model of global ischemia (20 min), oral dosing commencing at reflow, resulted in significant protection of the CA1 hippocampal neurons. ARL 15896AR was, however, ineffective in the rat two-vessel occlusion model and in the gerbil models of forebrain ischemia, the latter due to an inability to attain suitable plasma levels. In the spontaneously hypertensive rat model of middle cerebral artery occlusion (MCAO) (2 hr plus 22 hr reflow), acute dosing with ARL 15896AR (i.p.) beginning from 30 min before or up to 1 hr post-MCAO significantly reduced cortical infarct volume. The ability of ARL 15896AR to influence infarct size, as well as functional correlates was examined in SHR after 90 min of MCAO. T₂ weighted magnetic resonance images taken at 2 and 6 days post-MCAO revealed significantly smaller lesion sizes in the group receiving injections with ARL 15896AR beginning 30 min after occlusion. Spontaneously hypertensive rats were subsequently tested (30–42 days post-MCAO) and found to be deficient in skilled use of the forepaws (staircase test). The contralateral forepaw was most severely impaired, however, ARL 15896AR treatment prevented motor impairment in only the ipsilateral forepaw. Histopathological examination of cortical infarct size was unremarkable between treated and control rats. The findings indicate that ARL 15896AR exhibits neuroprotection in global and focal models of ischemia.

The discovery that a major underlying mechanism for neuronal damage after anoxia/ischemia is related to the massive release and postsynaptic action of excitatory amino acid transmitters (Olney, 1978) has led to a host of pharmacological investigations that have demonstrated a therapeutic role for the modulation of the NMDA subtype of the glutamate receptor (Pulsinelli and Buchan, 1990; Gee, 1994; Harris, 1995). In focal models of cerebral ischemia, NMDA receptor antagonists are effective in reducing cortical infarct volume, but findings regarding beneficial actions in animal models of global ischemia remain controversial (Buchan, 1990). Moreover, MK801 and the other high affinity uncompetitive NMDA receptor antagonists cause numerous side effects including motor hyperactivity and ataxia, deficits in learning and the potential for phencyclidine-like abuse. As a result, low therapeutic indices are characteristic for this drug group (Rogawski, 1992). With the discovery of low affinity uncompetitive NMDA receptor antagonists such as remacemide

ABBREVIATIONS: ANOVA, analysis of variance; ARL 15896AR, [(S)-alpha-phenyl-2-pyridine-ethanamine dihydrochloride]; AUC, area under the curve; b.i.d., twice a day dosing; CBF, cerebral blood flow; Cmax, maximal plasma concentration; H & E, hematoxylin and eosin; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MRI, magnetic resonance imaging; NMDA, N-methyl-D-aspartic acid; SHR, spontaneously hypertensive rat; t1/2, plasma half life; Tmax, time to peak plasma concentration; V/f, volume of distribution/fraction absorbed; 2-VO, 2-vessel occlusion; 4-VO, 4-vessel occlusion.
hydrochloride (Palmer et al., 1995a), ARL 13950AA (Palmer et al., 1995b), ADCI (Rogawski et al., 1991) and HU-211 (Biegan and Joseph, 1995) many of these unfavorable attributes of the high affinity antagonists are surmounted (Rogawski, 1992).

In our laboratories a rational chemical synthesis and screening approach to discover useful, safe, single enantiomeric forms of low affinity uncompetitive NMRA receptor antagonists identified ARL 15896AR [(S)-alpha-phenyl-2-pyridine-ethanamine dihydrochloride, formerly designated FPL 15896AR] (fig. 1). In addition to its neuroprotective activity in rodent models of global and focal ischemia, ARL 15896AR is nesedative. It also lacks phencyclidine-like stereotyped behavior (open field observations) nor, unlike phencyclidine, will rats self administration ARL 15896AR. Furthermore, ARL 15896AR does not influence either motor activity or learning (Hudzik and Palmer, 1995; Hudzik et al., 1996; Palmer et al., 1996). In addition, ARL 15896AR enters the brain rapidly and exhibits a favorable pharmacokinetic profile (Mahmood et al., 1994). With the successful completion of 30-day toxicology evaluation and phase I of clinical trials, phase IIa of clinical testing in stroke patients is in progress. Our investigation using rodent models of anoxia/ischemia details the preclinical work to date which provided the rational for ARL 15896AR to be tested in patients.

Methods

Experimental Subjects

Experimental protocols as well as housing and care of animals were in accordance with stringent requirements established by the Institutional Animal Care and Use Committees from the centers involved in the various studies. All drug doses are calculated to the free base form of ARL 15896AR.

Mice. For hypoxia studies male CF1 mice weighing 18–30 g were purchased from Harlan Laboratories (total used = 170).

Rats. Young adult male Sprague Dawley rats weighing approximately 140 to 200 g used for the hypoxia investigation were obtained from Harlan Laboratories. The weight range of the male Sprague Dawley rats (Harlan Laboratories) used for the 4-vessel occlusion studies varied from 275 to 350 g. For the 2-vessel occlusion models, adult male Sprague Dawley rats weighing from 250 to 350 g, were obtained from either Harlan Labs (Astra Arcus USA study) or Charles River, Montreal (University of Manitoba studies). For the anoxia/global ischemia investigations rats were maintained on low vitamin E content Rodent Lab Chow (Teklad no. 5001, Harlan) (total Sprague Dawley rats used = 133). SHR weighing from 230 to 280 g (Charles River, Montreal, Canada) were used for the focal ischemia experiments (total used = 90). Rats used in the various ischemia investigations were fasted overnight before surgery.

Gerbils. Female Mongolian gerbils weighing 50 to 70 g were obtained from High Oak Ranch, Goodwood, Ontario, Canada (total used = 46).

Global Ischemia Models

Hypoxia studies: mice. Testing for efficacy of compounds to extend survival time of mice exposed to an hypoxic environment has been described (Palmer et al., 1995b). Separate groups of mice received different doses of ARL 15896AR (10 per dose, 4–6 doses). Five min after i.v. dosing (5, 10, 20 and 25 mg/kg) and 20 min after oral dosing (5, 15, 25, 50, 62.5 and 75 mg/kg) the animals were placed in a plexiglass chamber consisting of five interconnected compartments. This apparatus was in turn placed into a QUEUE environmental chamber maintained at 35 ± 0.2°C. The mice remained in the QUEUE chamber for 10 min before induction and throughout the period of hypoxia. Hypoxia consisted of allowing a gas mixture (96% N₂/4% O₂) to flow into the individual compartments at a pressure of 1.79 kg/cm² (10 pounds per square inch) and at a flow rate of 0.01 m³/min. The time to last gasp was monitored with an electric timer controlled by an observer viewing the animal through a glass door. The environmental chamber eliminates drug-induced hypothermia which produces a "false positive" extension in survival time (Milde, 1988). Care was also taken to reduce AM/PM bias by running the experiments between 09:00–11:00 A.M. Groups of control mice were run concurrently with each dose of ARL 15896AR. The doses at which survival times increased by 50 and 100% were determined from linear regression analysis using least squares (Regres Program, ISS).

Hypoxia studies: rats. Efficacy for ARL 15896AR in extending the times to loss of the righting reflex and mortality was evaluated in rats maintained at ambient temperature for two reasons; 1) to determine proper dose ranges for the more labor intensive global ischemia studies and 2) to ascertain any effects of drug doses on body temperature. Starting oral doses were determined from previous information using ED₅₀ values obtained from antisiezure tests using maximal electroshock (Knowles et al., 1995, Palmer et al., 1996). Rats were dosed (p.o., n = 9–15/dose) with ARL 15896AR, placed in turn into individual clear plastic sealed chambers and 30 min later exposed to the gas mixture (97.5% N₂/2.5% O₂). An observer with a stop watch recorded the times to loss of the righting reflex and death (Palmer et al., 1995b).

Rat 4-VO model of global ischemia. An oral dose of 24 mg/kg of ARL 15896AR (active in the rat hypoxia studies) was selected for further evaluation in the rat 4-VO model of global ischemia.

The method of 4-VO global ischemia is an adaptation (Pulsinelli and Buchan, 1992) from the original procedure described by Pulsinelli and Brierly, 1979) and previously used by us (Ordy et al., 1992; Palmer et al., 1995b). In brief, rats were anesthetized with halothane and secured in a stereotaxic apparatus. Anesthesia was induced by 4% halothane and maintained using a mixture of nitrous oxide (70%)/oxygen (30%) plus halothane (2%). A midline dorsal neck incision was made, the alar foramina exposed via blunt dissection and an intermittent application of current via a small electro-cautery was used to cauterize the vertebral arteries. Direct visualization of the perimeter of the bony tunnel was performed to insure complete occlusion of the arteries. A suture was placed through the cervical region such that it lies posterior to the trachea, esophagus, external jugular veins and common carotid arteries, but anterior to the cervical and paravertebral muscles. The rat was then rotated and a ventral midline incision made in the throat region, the common carotid arteries located, looped with a suture and the wound secured with surgical clips. After a 24-hr recovery, the animals were lightly anesthetized with halothane, the clips removed, the carotid arteries quickly located, clamped for 20 min with aneurysm clamps and the cervical suture tightened. Rats failing to lose the righting reflex were

Fig. 1. Structure of ARL 15896AR
discarded from the study, because such animals do not develop complete bilateral CA1 neuronal damage. Also animals exhibiting seizures were excluded. Ischemic rats were placed on a heated pad maintained at 37°C throughout the period of ischemia and during 4 hr of recovery. Rectal temperature was maintained at 37°C by use of thermostatically regulated heat lamps and at 15-min intervals the head temperature checked with a tympanic probe. At the 20-min time point when the aneurysm clips were removed from the carotid arteries, the rats received an oral dose of 24 mg/kg of ARL 15896AR (2 ml/kg body weight) or equivalent volume of saline followed by a subsequent dose 6 hr later and b.i.d. for 6 additional days (total dosing regimen = 7 days). The delayed cell death produced by the 4-VO ischemia fully matures in about 7 days.

**2-VO/hypotension model of global ischemia.** The protocols for the surgical procedures between the two laboratories (Astra Arcus USA and the University of Manitoba) were standardized. The procedure is adapted from the original by Smith et al. (1984). On the day of the experiment, anesthesia was induced with either: 1) atropine (0.5 mg/kg i.p.) to decrease esophageal secretions followed by pentobarbital (65 mg/kg, i.p., dose volume 0.1 ml/100 g) or 2) nitrous oxide/oxygen (70%/30%) and 2% halothane administered via a cup fitted over the nose and mouth. For the pentobarbital experiments the rats were intubated and ventilated on a respirator. Two incisions were made (ventral neck and tail). Throughout the procedure and recovery, brain temperature was monitored by a thermocouple placed through the tympanic membrane. A temperature of 37.5°C was maintained by means of thermostatically controlled heat lamps and water blankets. Blood pressure was monitored via a 24-gauge catheter inserted into the tail artery. Blood gas samples were taken before ischemia and the ventilation adjusted to maintain normal measurements of PO2 (80–200 mm Hg), PCO2 (35–45 mm Hg) and pH. Both common carotid arteries were exposed and isolated using loose ligatures. Normal blood pressure was recorded for 5 min followed by exsanguination of blood from the arterial line to lower pressure to 45 to 50 mm Hg. Once the <50 mm Hg pressure stabilized, ischemia was induced for 12 min by occlusion of the common carotids using temporary aneurysm clips. During the ischemic period, blood pressure was maintained from 45 to 50 mm Hg by withdrawal or infusion of blood. Ischemia was terminated by removal of the arterial clips, the exsanguinated blood reinjected into the animal over a 1-min period and the arterial line flushed with 1 ml heparinized saline. Monitoring of blood pressure and blood gases were continued over 5 additional min at which time the incisions were secured. Anesthesia was discontinued for the halothane anesthetized rats whereas the pentobarbital anesthetized animals were taken off the ventilation system and placed on a heating pad for recovery.

For the experiments conducted at the University of Manitoba, the dose of ARL 15896AR administered was either 12 mg/kg (pentobarbital anesthesia, n = 7) or 24 mg/kg (halothane/nitrous oxide anesthesia, n = 9) given s.c. in phosphate buffered-saline (pH 7.4) that served as the control group. Injection of ARL 15896AR was initiated at the time of reperfusion and was repeated every 12 hr for 7 days. Body weights were monitored daily until death.

For the 2-VO study completed at Astra Arcus USA, ARL 15896AR was administered i.v. in two loading doses (80 or 40 μg/min) over a 30-min period to attain steady-state plasma concentrations of 500 (n = 3) and 1000 (n = 10) ng/ml. These were followed by maintenance doses of either 9.0 or 4.5 μg/kg/min administered for 7 days by primed ALZET osmotic minipumps implanted in a subcutaneous pocket within 15 min after reperfusion. Control animals received vehicle. Blood samples were taken at intervals and the drug concentrations determined (see below).

**Gerbil model of global forebrain ischemia.** Great care was taken with gerbils to maintain brain and body temperature because of the extreme susceptibility of this species to the ameliorative actions of hypothermia (Corbett et al., 1990). One week before ischemia gerbils were implanted with a guide cannula to allow a brain temperature probe to be inserted into the striatum. Baseline brain temperatures were recorded 2 days before ischemia. Rectal and brain temperatures were maintained near 37°C during carotid occlusion and for several hours after reflow. For the ischemia procedure, animals were anesthetized under 1.5 to 2.0% halothane anesthesia, the common carotid arteries rapidly isolated and clamped for 3 min. Treatment with ARL 15896AR (24 mg/kg s.c., b.i.d./4 days) or vehicle (phosphate buffered saline, pH 7.4) commenced at the time of reflow. In a separate group of gerbils elimination kinetics of ARL 15896AR were determined after an s.c. dose of the free base at 24 mg/kg with plasma samples taken at 0.083, 0.5, 0.833, 1.5, 2, 4, 6, 8 and 24 hr after dosing (three animals per time point). The brains from the 1.5-hr group were removed, frozen at −70°C and stored for subsequent analysis (see below).

**Focal Ischemia Models—MCAO Surgical Procedure**

The procedure for focal ischemia consisted of a unilateral carotid artery ligation in tandem with 120 or 90 min of transient MCAO in the SHR as described in detail by Buchan et al., (1992). Anesthesia for the SHR was with 3% halothane/70% N2/30% O2 with subsequent maintenance on 1 to 2% halothane. The anesthesia was adjusted to sustain mean arterial blood pressure above 90 mm Hg during the surgical procedure. The tail artery was cannulated for physiological monitoring. Mean arterial pressure, blood gases (pH, PCO2, PO2), CBF, hematocrit and serum glucose were measured before the onset of ischemia, during ischemia and at the time of reperfusion. Body temperature was rigorously maintained at 37.5 ± 0.5°C during and after surgery with a rectal thermometer coupled to a heating lamp. For surgery the ipsilateral common carotid artery was isolated. A 1-cm incision perpendicular to and bisecting a line between the lateral canthus of the right eye and the external auditory canal was made, the underlying temporals muscle excised and under direct visualization, the right MCA was exposed through a 3-mm burr hole drilled 2 to 3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. The MCA was visualized where it crosses the inferior vein, which lies within the rhinal fissure. This site is proximal to the MCA bifurcation, but distal to the origin of the lenticulostriate arteries. After obtaining basal physiological and CBF measurements, the ipsilateral (right) common carotid was occluded, the dura overlying the MCA, cut and retracted, a no. 1 microclip (Codman, Boston, MA), placed on the MCA and flow interruption observed. The incisions were secured, anesthesia discontinued and the animals returned to their home cages.

In selected studies, to establish baseline information, regional CBF was monitored in the “core” ischemic region using laser Doppler flow measurements (TSI, St. Paul, MN) at a single location, which corresponds to the region that histologically becomes the center of the infarction. A 1- to 2-mm diameter burr hole was drilled 3 mm dorsal to the site of MCAO and 3 mm caudal to the bregma. The dura at the laser Doppler flow probe site was left intact and the probe (0.8 mm diameter) advanced with a micromanipulator (Narishige Instruments, Tokyo, Japan) under microscopic guidance to a site free of large pial vessels. The probe was positioned to rest on the surface of the dura without indenting the cortex. Cerebral blood flow was measured prior to the MCAO (determine baseline flow) and immediately after occlusion. Blood flow was recorded as the mean of the maximum and minimum recorded flows over a period of 5 min and expressed as a percentage of pres ischemic baseline values.

**MCAO therapeutic window: SHR.** The study was carried out at the Neuroscience Unit of the Ottawa Civic Hospital, Ottawa, Canada. The period of focal MCAO was 2 hr followed by 22 hr reflow. Rats were given i.p. injections of saline (1 ml x 3 doses) or ARL 15896AR (12 mg/kg x 3 doses). Initial doses were administered 30 min pres ischemia, at the onset of ischemia, or at 30, 60 or 120 min post-MCAO. The two subsequent doses were injected at 4 and 12 hr from the time of the initial doses.
Magnetic Resonance Imaging

Magnetic resonance imaging was performed using a Bruker MSL-X BoSpec 7/21 Spectrometer. For each imaging study taken on day 2 and day 6 post-MCAO, the rat was anesthetized with pentobarbital (50 mg/kg, i.p.) and placed in a holder with the head positioned in a 3-mm diameter saddle coil using an incisor bar. Spin-echo scout images were acquired first in the coronal and then in the sagittal planes to select reproducible coronal slice positions. Sixteen contiguous coronal slices, centered 1.5 mm posterior to bregma, were imaged in two interleaved sets to minimize interslice excitation. For all coronal images, the matrix size was 256 × 256. Four averages were accumulated using a field of view of 3.5 × 3.5 cm² and a slice thickness of 1 mm. A field of view extension factor of 2 was used in the read direction, and 256 phase-encoding steps were acquired. Multislice multi-echo T₂-weighted spin-echo images were acquired with echo times of 20, 40 and 60 msec and TR of 1500 msec. Total acquisition time was 52 min.

Histological Procedures

For all procedures involving processing of the brain the animals were anesthetized with sodium pentobarbital (64 mg/kg, i.p.) and perfused transcardially first with heparinized saline and then with 10% formalin fixative solution. The head was removed, wrapped in plastic or left in fixative and placed overnight in a refrigerator to allow postfixation in situ. The next day the brain was removed and placed in perfusion solution for another 24 to 48 hr.

4-VO and 2-VO experiments conducted at Astra Arcus USA.

One coronal brain block from the fixed rat brain that includes the entire hippocampus was prepared using a brain dissection guide (Harvard Instruments Large Rat Brain Matrix—Coronal, no. 52–4512c). The block is defined by the blade-guide grooves in the dissection guide that are the 9th and 15th from the rostral pole of the brain. Individual blocks were imbedded in paraffin and processed to obtain 6-μ thick coronal sections. Staining was by H & E, selected because it shows overall damage (including microglia infiltration, neovascularization) to the brain, as well as assessment of neuronal loss. One section was collected every 100 μ through the entire depth of the block. For histological ratings, six consecutive sections from each animal were evaluated beginning with the section that most closely matches a coronal section approximately 3.2 mm caudal to the bregma according to the Paxinos and Watson (1982) atlas. The extent of ischemic damage was evaluated bilaterally septal to mid-septal hippocampus in six separate hippocampal subregions. Two raters blinded to the treatment condition classified the slides. Gross ischemic damage (evidenced by loss of the CA1 pyramidal cells, the presence of acidophilic cell “ghosts,” extent of neovascularization and microglial infiltration) was scored using a 4-point scale: 0 = no damage, 1 = less than 33% cell loss, 2 = 34 to 66% damage and 3 = 67 to 100% damage. Individual neuronal counts of left and right sides of the entire CA1 region were made in alternate sections (2, 4 and 6) using 20 × magnification. The beginning of the CA1 zone was designated from the CA2/CA1 transition zone. The end of CA1 was determined by an imaginary line drawn from the apex of the dentate gyrus, perpendicular to the CA1 cell layer.

Statistical analyses were performed using the BMDP program (1981) on an in-house VAX system. The Newman Keuls range test was used to compare between groups (ISS program). An overall ANOVA compared the data between raters, left vs. right sides, as well as comparisons between treatment groups followed by the Newman Keuls range test for determination of statistical differences between individual data points (Tallarida and Murray, 1987).

2-VO conducted at Winnipeg. Perfusion-fixed rat brains were coded and processed for histology (8-μm sections stained with H&E). Striaital damage was quantified by examining two 630 μm diameter microscopic fields in the dorsolateral caudate at the coronal level of septal nuclei (±0.2 mm from Bregma) and involved direct counting of acidophilic neurons. The hippocampus was examined at 6 levels along its septotemporal axis. Damage at each level was expressed as a percentage of the total CA1 sector. If only a few necrotic neurons were present, these were counted and divided by the total number of known neurons. If the majority of neurons were necrotic, then the remaining viable neurons were counted and necrosis obtained by subtracting this value from the total neuronal counts. Statistical analyses were performed using non-parametric tests.

Forebrain ischemia gerbils. On day 10 of the experiment the brains were perfusion-fixed as described above followed by sectioning (10 μm) and Cresyl violet staining. The degree of hippocampal CA1 injury was quantified according to the following rating (Hewitt and Corbett, 1992): 4 = 90–100% normal appearing neurons; 3 = 60 to 89%; 2 = 30 to 59%; 1 = 6 to 29% and 0 = less than 5% appearing normal. The rating scale was applied to each of three sectors of the dorsal CA1 region (medial, middle and lateral) from both hemispheres in the rostral hippocampus (~1.7 mm posterior to Bregma) in addition the ventral hippocampus from the middle and lateral sectors was rated separately. Thus an animal with little or no CA1 loss would be rated as a 23 or 24 whereas an animal with severe loss would be rated close to 0. Previous work demonstrated the rating method correlated well (r = 0.97, P < 0.001, n = 56) with actual cell counting techniques. All rating techniques were performed blind with respect to the treatment condition.

MCAO rats. Perfusion-fixed brains from SHR were processed for histology at 22 hr post- MCAO in the acute “therapeutic window” investigation. In the collaborative study the histology was performed at 42 days after MCAO. Brains were cut into 2-mm thick coronal slices, dehydrated in graded ethanol, followed by clearing in xylol and embedding in paraffin. Coronal sections were taken every 500 μm, yielding 25 coronal planes encompassing the infarct. Under a light microscope the necrotic areas of the cerebral cortex and the striatum were traced on images of the slices, controlling for the infarct borderline. Areas of the ipsilateral and contralateral hemispheres were also measured. This allowed for calculation of damage as percent of the hemisphere, thus controlling for variations in atrophy or brain size from one rat to another. Data were compared using one-way ANOVA (Zhu and Auer, 1995).

Pharmacokinetics and Plasma Analysis

Plasma levels. For sample analysis, 50 μl of internal standard containing 500 ng of ARL 15759XX (1-methyl-1-phenyl-2-pyridine ethanamine free base) were added to each tube containing 250 μl of plasma followed by the addition of 40 μl of 1 M NaOH. Mixing was followed by addition of 2 ml of 10% n-butanol/hexane, mixing for 10 min with low speed centrifugation (822 × g for 10 min). The organic phase was removed and added to tubes containing 125 μl of 0.1 M HCl followed by mixing, centrifugation (822 × g) and removal of the organic layer. Fifteen μl of 1.0 M NaOH were added to the tubes, followed by brief mixing and transfer to an HPLC autosampler. Standards were prepared in plasma from naive rats at concentrations between 5 and 10,000 ng/ml. The method is specific and the peak height ratios versus concentration are linear over the measured range of standards. Samples were analyzed by HPLC using reverse phase chromatography with a mobile phase of 13.8% acetonitrile, 86% 0.05 M KH₂PO₄, pH 3.75, and 0.2% triethylamine. Using a variable wavelength detector at 260 nm with a 250 × 4 mm LiChropur 60 RP with 5 μm particle size, the detection limit for ARL 15896AR was 5.0 ng/ml in plasma. Levels of ARL 15896AR were reported as the free base.

Pharmacokinetics: rat. Determination of pharmacokinetic parameters of ARL 15896AR was made in Sprague-Dawley rats after an acute dose of 100 mg/kg, s.c. Blood samples were taken at 0.083, 0.5, 0.833, 1.5, 2, 4, 8 and 24 hr from six rats with jugular vein cannulas. The data were fit to a one-compartment model using the computer program PCNONLIN V4 (SCI Software, Statistical Consultants, Inc., Lexington, KY). For comparison with profiles from other studies, the plasma concentration versus time profile from our
study was normalized to doses used in other studies (Mahmood et al., 1994; Palmer et al., 1996), as well with data from the present investigations namely, 4-VO, 2-VO, gerbil ischemia study and the collaborative investigation with the MCAO.

For the collaborative MCAO study the measured concentrations were compared to the expected concentration profile modeled for this dosing regimen, i.e., two s.c. doses of 12 mg/kg at 0 and 4 hr.

Pharmacokinetics: gerbils. Elimination kinetics of ARL 15896AR were determined in 30 female Mongolian gerbils after a single s.c. dose of 24 mg/kg, a dose previously shown to be effective in the rat 4-VO study. The study compared parameters of ARL 15896AR between gerbils and rats. Blood was taken from gerbils (CO2-induced mortality) at 0.083, 0.5, 0.833, 1.5, 2, 4, 6, 8, 12, and 24 hr after dosing (three animals per time point). Vena cava samples were transferred to heparinized Vacutainer tubes on ice, centrifuged and the plasma removed and stored at −70°C until analyzed.

Testing for Motor Behavior

Crude motor behavior. The tests conducted on SHR included the following.

1) Ipsilateral circling—graded from 0 (no circling) to 4 (continuous circling) (Ungerstedt and Arbuthnott, 1970).
2) Contralateral hindlimb retraction—measuring the ability of the animal to replace the hindlimb after it was displaced 2 to 3 cm laterally (graded 0 for immediate replacement to 4 for very slow or no replacement) (Feeney et al., 1981).
3) Beam walking—graded from 0 for an animal who could not traverse a 2.4-cm wide beam to 4 for a rat unable to accomplish the task (Feeney et al., 1981).
4) Bilateral grasp—graded from 0 for an animal with normal forepaw grasping behavior to 4 for one unable to grasp with the forepaws (Bederson et al., 1986).

The total neurological deficit score was taken as the sum of the scores from all four tests (maximum deficit score = 16).

Skilled forepaw use. The “Staircase Testing Paradigm” for evaluation of complex motor behavior was conducted on SHR which were food deprived for 24 hr before testing. The apparatus consists of an elevated platform on which the rat rests with staircases on either side of the animal. A staircase contains seven steps with a food well in each step. Each day the rat was placed in the apparatus with two Noyes pellets on the platform and each food well baited with 3 Noyes pellets. The animals were left in the apparatus for 20 min and the number of pellets consumed noted. After the first test session each rat was given 6 Noyes pellets and returned to the home cage which was devoid of food. The next day the rats were returned to the staircase apparatus, again with 2 Noyes pellets present on the platform and the food wells baited. At the end of the second test session and after subsequent test sessions all rats were given 10 g of rat food in their home cages. This amount of food plus whatever food was obtained in the staircase test maintained the animals at approximately 85% of their free-feeding weight. A total of nine test sessions were conducted per animal in the staircase apparatus. The data obtained were based on the average number of pellets consumed over the last three test sessions. The animals were unable to retrieve any spilled pellets. Thus the amount actually consumed was determined from the number of pellets present at the start of the test session minus the number left in food wells or spilled to the chamber floor. Because the rats could reach the top two steps of the staircase and obtain pellets by using their tongues or the “good limb” (i.e., limb ipsilateral to the occlusion) the total number of pellets consumed per test session did not include pellets eaten from the top two steps nor the two “free” pellets placed on the platform. In other words, data were based on food eaten/spilled/uneaten from the bottom five steps of each staircase (Montoya et al., 1991; Grabowski et al., 1993).

Experimental Plan for MCAO Collaborative Study: SHR

Surgery for the 90 min MCAO, dosing, sampling of plasma, T2 weighted MRI and crude motor assessments were carried out at the University of Manitoba. Analyses of plasma samples were conducted at Astra Arcus USA, staircase testing was performed at Memorial University and resultant histopathological examination of the brains at Foothills Hospital. All findings were coded until final completion of the study.

Four groups of animals were prepared: group I—sham-operated control (n = 6), group II—post MCAO treatment (30 min) with phosphate buffered saline vehicle (pH = 7.4, n = 6), group III—post MCAO treatment (30 min) with ARL 15896AR (n = 6) and group IV—pre-MCAO treatment (15 min) with ARL 15896AR (n = 4). Dosing with ARL 15896AR was 10 mg/kg, s.c. The second and third doses were administered at 4.5 and 12 hr post-MCAO. A plasma sample for determination of ARL 15896AR levels was removed 5 hr after MCAO from groups II and III. Physiological measurements were made before and after ischemia from six rats selected at random from the different experimental MCAO groups. Now that the reproducibility of the model has been established these served as spot checks only. Multi-slice T2 weighted MRI were taken at 2 and 6 days post-MCAO. After MCAO the rats were tested for crude motor performance at 4 hr and 1, 2, 4, 6, 8 and 10 days post-MCAO. At 12 days after surgery the rats were flown to Memorial University and tested from days 30 to 42 for complex motor behavioral assessments involving the staircase apparatus. Histopathological evaluation included comparisons of both the volumes of infarction (mm3), as well as percent change from the contralateral undamaged hemisphere. Cortical infarction contains dead or dying tissue, although cortical atrophy represents loss of tissue. Atrophy is determined as the difference in volume between the control and damaged hemispheres.

Results

Global Ischemia Models

Hypoxia: mice. Pretreatment with ARL 15896AR extended the time to mortality after exposure of mice to an hypoxic environment. The i.v. dose to extend survival time by 50% was 21.0 mg/kg and by 100% it was 40.4 mg/kg (slope ± S.E. for the i.v. dose response curve = 2.6 ± 0.70). The mean ± S.E. time to mortality in the 30 control animals run with this experiment was 2.4 ± 0.1 min.

Similarly, oral pretreatment of mice with ARL 15896AR extended survival time by 50 and 100% at doses of 36.7 and 78.7 mg/kg, respectively (slope ± S.E. = 1.19 ± 0.24). The mean ± S.E. survival time of the 40 controls run in conjunction with this experiment was 2.49 ± 0.06 min.

Hypoxia: rats. Because hypothermia may influence therapeutic outcome in hypoxia/ischemia investigations (Corbett et al., 1990; Hewitt and Corbett, 1992), we first looked for possible influence of oral doses (12 and 24 mg/kg) of ARL 15896AR on rectal temperature over a 2-hr time course using naive control rats. The stress of measuring rectal temperature in both control and ARL 15896AR-treated rats (n = 10/treatment group) indicated a tendency (nonsignificant) for a slight elevation when measured 30 min later. However, neither dose of ARL 15896AR affected body temperature when assessed at 30-min intervals for up to 2 hr after administration of the compound. The 3 × 5 ANOVA indicated the following: TREATMENT: P = .1081; INTERVAL: P = .0273; TREATMENT × INTERVAL: P = .2271.

The hypoxic study used the higher dose of ARL 15896AR (24 mg/kg) which was administered orally 1 hr before induction. ARL 15896AR treatment (n = 13) lengthened the mean (± S.E.) time to loss of the righting reflex in the controls (n = 11) from 131 ± 7 to 154 ± 8 sec (P < .04, Student’s two-tailed
t test). Similarly, the mean ± S.E. time to mortality was extended from 405 ± 32 to 643 ± 65 sec (P < .003).

4-VO: rats. Monitoring of rectal temperature at 5-min intervals during and after 20 min of 4-VO global ischemia did not result in any significant change in body temperature among the sham-operated controls, the ischemic controls or the ischemic rats receiving 24 mg/kg, p.o. of ARL 15896AR (overall ANOVA: TREATMENT - P = .15; TIME - P = .14; TREATMENT x TIME - P = .99). Moreover, the mean weights of each group as monitored throughout the 7-day treatment period were unremarkable (ANOVA: TREATMENT: P = .98; DAYS - P = .001; TREATMENT x DAYS - P = .5).

At 8 days after 20 min of 4-VO ischemia, hippocampal CA1 neuronal damage was consistent throughout the vehicle-treated group. Six sections from this vehicle-treated group were evaluated for gross histological damage. The overall mean gross histological score was 2.73 (3 = total damage). The mean overall recorded gross damage score for the group treated for 7 days with ARL 15896AR was 1.33, whereas the sham-operated group was 0. Figure 2 depicts the gross histological scores taken from the six individual sections of the left and right hippocampi. In the vehicle group no differences were observed between the left and right hippocampi, however, in the ARL 15896AR treatment group there was significantly (Newman Keuls: P < .01) more damage to the CA1 region in the left than in the right hippocampus. The damage to the left and right hippocampi were, however, significantly less than the corresponding vehicle-treated controls (Newman Keuls: P < .001).

The results of counting the individual CA1 neurons from hippocampal regions 2, 4 and 6 are presented in figure 3. The overall combined mean counts ± S.E. for the number of viable neurons from the 3 regions were: sham-operated control = 441 ± 8; ARL 15896AR-treatment ischemic group = 212 ± 26; and the vehicle treatment ischemic group = 68 ± 17. When the individual sections were examined there was again a significant (Newman Keuls: P < .01) laterality difference in the ARL 15896AR treatment group; the left side had greater damage than the right. Regardless of this observation, there were consistently more viable CA1 neurons in all three hippocampal regions analyzed from the ARL 15896AR-treated group than in the ischemic group receiving saline (Newman Keuls: P < .01). Generally, as analyses were made of individual cell counts along the septo-middle-temporal axis of the hippocampus the cell counts became larger in number, i.e., more CA1 neurons in region 6 than in region 2, a finding noted previously (Palmer et al., 1995b).

2-VO: rats. Regardless of the mode of anesthesia, no 7-day treatment regimen with ARL 15896AR resulted in any significant degree of neuronal protection to the CA1 hippocampal region or the striatum (see table 1).

Forebrain ischemia: gerbils. The ARL 15896AR dosing schedule did not significantly influence brain or body temperature in either sham-operated or ischemic gerbils. Moreover, this regimen did not afford protection to the CA1 neurons from the dorsal hippocampus. The mean ± S.E. histological scores for the experimental groups were: 1) sham-operated, ARL 15896AR-treated controls, score = 24 ± 0 (n = 5); 2) ischemia, ARL 15896AR treatment, score = 2.5 ± 3.9 (n = 7); 3) ischemia, vehicle treatment, score = 5.3 ± 5.4 (n = 7).

The gerbil plasma concentrations from 0 time to 6 hr were fitted to a one-compartment first order input and output model and compared to previous information obtained with the rat (see fig. 4). The pharmacokinetic parameters are listed in table 2. After s.c. administration to gerbils, ARL 15896AR was absorbed more rapidly and Cmax were attained quicker than in the rat. ARL 15896AR was eliminated more than 10 times faster in the gerbil. This faster elimination rate in gerbils explained the lower total exposure as mea-
sured as AUC. The calculated AUC for rats receiving 24 mg/kg s.c. (57.8 μg/hr/ml) was eight times the exposure achieved by gerbils (7.1 μg/hr/ml) at a similar dose. In both species ARL 15896AR readily crossed the blood-brain barrier. The mean ± S.D. concentration at 1.5 hr after dosing in the gerbil was 6490 ± 523 ng/g brain tissue. Previously published data for the rat (Mahmood et al., 1994; Palmer et al., 1996) revealed that within 15 min after i.v administration brain levels of ARL 15896 exceeded that of plasma by a factor of 5-fold or greater and remained so elevated over 6 hr. Thus in summary ARL 15896AR is both absorbed and eliminated much faster in gerbils than rats after s.c. dosing.

**Focal Ischemia Studies**

**MCAO therapeutic window: SHR.** After 2 hr of MCAO, the one-way ANOVA analyses of the data indicated highly significant (P < .001) reductions in the volume of cortical infarction when the initial dosing occurred at 30 min preischemia, at the time of MCAO or 30 min post-MCAO. Protection remained significant, albeit less (P < .05), when the initial dosing regimen began at 60 min post-MCAO. No protection was observed in the SHR if the initial dosing with ARL 15896AR was delayed to 2 hr post-MCAO (fig. 5).

**MCAO Collaborative Study: SHR**

For the physiological measurements (plasma glucose, hematocrit, PO₂, PCO₂, plasma pH) made from the six selected rats, the mean pre-ischemia values did not differ significantly from the mean post-ischemia values (see table 3).

**Magnetic resonance imaging.** A representative multislice T₂ weighted MRI taken at 2 days post-MCAO from an

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**TABLE 1**

<table>
<thead>
<tr>
<th>Experimental</th>
<th>Hippocampal CA1 Neurons</th>
<th>Striatal Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>ARL 15896AR</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anesthesia—I</td>
<td></td>
<td></td>
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<tr>
<td>ARL 15896AR</td>
<td>2.7 ± 0.91</td>
<td>3.0 ± 0</td>
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<tr>
<td>(500 ng/ml)</td>
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<td></td>
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<tr>
<td>ARL 15896AR</td>
<td>2.5 ± 0.95</td>
<td>(n = 12)</td>
</tr>
<tr>
<td>(1000 ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentobarbital</td>
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<tr>
<td>Anesthesia—I</td>
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<tr>
<td>ARL 15896AR</td>
<td>61.1% ± 10</td>
<td>69.5% ± 8</td>
</tr>
<tr>
<td>(12 mg/kg, b.i.d./ 7 days, s.c.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrous oxide/halothane anesthesia</td>
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<tr>
<td>ARL 15896AR</td>
<td>53.7% ± 18</td>
<td>45% ± 11</td>
</tr>
<tr>
<td>(24 mg/kg, b.i.d./ 7 days, p.o.)</td>
<td>(n = 5)</td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

Values are the combined means ± S.E. of all levels of sections including left and right sides of the brain.

* In the pentobarbital anesthesia–I study hippocampal CA1 neuronal damage is quantified using a gross scoring technique rating bilaterally 6 sections taken along the mid-septotemporal hippocampus: 3 = 67–100%, 2 = 34–66%, 1 = <33% and 0 = no damage.

* In the pentobarbital anesthesia–II study and with the nitrous oxide/halothane anesthesia study, the hippocampus is examined at six levels along the septotemporal axis. Damage at each level is expressed as a percentage of the total CA1 setter (see “Methods”).

* Striatal damage is quantified by examination of two 630-μm diameter microscopic fields in the dorsolateral caudate at the coronal level of septal nuclei (+0.2 mm from Bregma). Values represent total number of acidophilic neurons in the two fields.

---

**TABLE 2**

<table>
<thead>
<tr>
<th>PK Parameters</th>
<th>Gerbil (s.c.)</th>
<th>Rat (s.c.)</th>
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</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>24</td>
<td>100</td>
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<tr>
<td>t½ (hr)</td>
<td>0.43</td>
<td>4.7</td>
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<tr>
<td>Elimination rate constant, k₁₀ (hr)</td>
<td>1.61</td>
<td>0.15</td>
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<tr>
<td>Absorption rate constant (hr)</td>
<td>21.4</td>
<td>0.42</td>
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<tr>
<td>V/F (liters/kg⁻¹)</td>
<td>2.1</td>
<td>2.7</td>
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<tr>
<td>Tₚ (min)</td>
<td>7.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Cₚ (μg/ml)</td>
<td>9.3</td>
<td>20.2</td>
</tr>
<tr>
<td>AUC (μg/hr/ml)</td>
<td>7.1</td>
<td>241</td>
</tr>
</tbody>
</table>

* V/F; V = volume of distribution; f = fraction absorbed (assumed to be unity).
The volume of apparent damage for the post MCAO ARL 15896AR treatment group III was significantly less (see above) than the ischemic control group II.

**Plasma levels.** Blood samples were taken from post-treatment group III and the control group II at 5.5 hr post-MCAO. The mean ± S.D. levels of free base ARL 15896 were 3527 ± 930 ng/ml plasma. With the exception of one outlier, the plasma levels were within the predicted range (present experiments and Mahmood et al., 1994; Palmer et al., 1996) expected after a second s.c. dose of 10 mg/kg (fig. 9).

**Crude motor behavior.** After MCAO, SHR were tested for crude motor performance. The findings for the neurological deficit scores of all rats receiving MCAO or sham operations are given in figure 10. Out of a total possible deficit score of 16 from the 4 motor/neurological impairment tests the maximal scores attained ranged from 6.5 to 8, an observation occurring immediately after the MCAO (4 hr). However, at this time the animals had not fully recovered from the effects of the anesthesia/stroke operation. By 24 to 48 hr after MCAO, significant impairment was again noted in all groups, however, the scores dropped from 5 to 6 at 24 hr to ~4 by 48 hr. From day 4 through day 10 after MCAO the crude motor impairment scores were in the range of the sham-operated control group. Neither treatment regimen with ARL 15896AR was associated with any significant improvement when compared to the control SHR subjected to MCAO.

**Complex motor behavioral assessments.** In the staircase test there were definite, significant (ANOVA with post hoc Newman Keuls analyses, P < .05) motor deficits with the contralateral forepaw regarding the number of “steps reached.” This observation was evident in all ischemic groups (fig. 11, top panel). As the staircase test also measures the number of food pellets actually picked up from the lower steps and eaten, it was readily apparent in the ischemic control SHR that there were severe deficits with both the contralateral (ANOVA, P = .0004) and ipsilateral forepaws (ANOVA, P = .03). Pre- and post-MCAO treatment of SHR with ARL 15896AR did not avert the subsequent deficit on the contralateral side, but did indeed significantly (P < .05, post hoc Newman Keuls analyses) prevent the motor deficit on the side ipsilateral to the lesion (fig. 11, bottom panel).

**Histopathology.** Histopathological evaluation of the serial brain slices indicated no significant difference between treatment groups (MCAO control and MCAO ARL 15896AR) regarding the extent of total brain damage, brain atrophy, as well as cortical or subcortical infarction volumes. Data analysis included comparisons of both the volumes of infarction (mm³), as well as percent change from the contralateral undamaged hemisphere. With regard to measurements of volume of infarction, the cerebral cortex experienced, as expected, the greatest degree of damage (all treatments = ~20 mm³). Subcortical damage was minimal (all treatments ranged from 0.75–1.25 mm³). The volume of cortical atrophy ranged from ~50 to 67 mm³ and the total brain damage ranged from ~63 to 90 mm³ for all treatments. The data in figure 12 are presented as the percent damage on the right (MCAO) side with respect to the contralateral left control side.

SHR is depicted in figure 6. In all animals the area of cortical infarction on day 2 was considerably greater (P < .001, overall ANOVA followed by post hoc Newman Keuls analysis) than on day 6. The data shown in figures 7 and 8 make these comparisons. Figures 7 (day 2) and 8 (day 6) represent apparent lesion areas on consecutive T2 weighted MR images for the 3 MCAO groups. The group treated with ARL 15896AR 30 min after MCAO (group III) had a significantly smaller lesion volume on both days (P < .05) when compared to the control MCAO group (group II). Possibly due to the smaller n value, the values for the group pretreated with ARL 15896AR (group IV) were not significantly different than the controls (group II). The composite mean volumes (mm³) ± S.D. of all the serial T2 weighted images taken at both 2 or 6 days from the three ischemic groups were: 1) group II - 2 days = 248 ± 34, 6 days = 123 ± 18; 2) group III - 2 days = 185 ± 39, 6 days = 99 ± 20; 3) group IV - 2 days = 204 ± 42, 6 days = 109 ± 25.

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Preischemia</th>
<th>Postischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mmol)</td>
<td>3.90 ± 0.66</td>
<td>3.60 ± 0.71</td>
</tr>
<tr>
<td>Hematocrit (percent)</td>
<td>53.00 ± 3.28</td>
<td>52.00 ± 0.71</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>95.90 ± 13.90</td>
<td>134.40 ± 66</td>
</tr>
<tr>
<td>PCO2 (mm Hg)</td>
<td>45.00 ± 3.31</td>
<td>43.10 ± 2.22</td>
</tr>
<tr>
<td>Plasma pH</td>
<td>7.35 ± 0.15</td>
<td>7.32 ± 0.07</td>
</tr>
</tbody>
</table>

Because the reproducibility of the model has been established, mean ± S.D. values are taken at random from the different experimental MCAO groups. The data represent six animals.
Discussion

General

ARL 15896AR is currently under development primarily as a neuroprotective compound with treatment of acute focal cerebral ischemia as the ultimate clinical target. In addition, ARL 15896AR possesses good antiepileptic potency in rodents. Prevention of seizures elicited by the excitatory amino acids, NMDA and kainate, as well as, tonic hind limb extension as a consequence of maximal electroshock, 4-aminopyridine or bicuculline have been observed (Knowles et al., 1995; Palmer et al., 1996). Unlike the more potent uncompetitive NMDA receptor antagonists, ARL 15896AR does not produce abuse liability (Hudzik et al., 1996), affect learning (Hudzik and Palmer, 1995) nor elicit motor deficits (Palmer et al., 1996).

In vitro experiments indicate that ARL 15896AR possesses a moderate degree of receptor affinity as an uncompetitive NMDA receptor antagonist. The IC50 for displacement of MK801 binding in rat brain synaptosomal preparations in vitro is 1.6 μM. When added to neuronal cultures from rat cerebral cortex, ARL 15896AR prevents excitatory amino acid-elicited cell death, translocation of protein kinase C, plus the associated Ca++ surge into the cells (Black et al., 1995). NMDA-induced depolarization in rat hippocampal slices is also prevented by ARL 15896AR (Cregan et al., 1995).

Role in Ischemia

Rationale. The ability of ARL 15896AR to influence both NMDA and possibly sigma-1 receptors (Palmer et al., 1996) indicates potential to lessen the degree of neuronal damage as a result of ischemic/anoxic insult to the brain. Such compounds have been shown repeatedly in the past to afford neuroprotection in animals subjected to various experimental paradigms of global and focal ischemia (Chapman et al., 1990; Meldrum, 1992; McCulloch, 1992; Jensen and Auer, 1991; Buchan, 1990). Notably, eliprodil, a mixed NMDA/sigma receptor antagonist, was effective in a mouse focal ischemia model (Poignet et al., 1992).

The initial in vivo experiments describing a neuroprotective effect of ARL 15896AR were published by Greene and coworkers (1996). Intrastratial injections of malonate in rats produces a toxic lesion, an event known to be mediated by the NMDA receptor. When ARL 15896AR was co-injected into the striatum (200 nM) or injected s.c. (9 mg/kg) either before or shortly after the malonate injection, the size of the striatal lesions assessed at 72 hr was reduced by more than 80%. Protection was equivalent to that observed with MK801. However, at the dose used, the latter compound caused behavioral side effects.

Our investigations focused upon the ability of ARL 15896AR to afford protection in animal models of global ischemia/anoxia and the model of focal ischemia in SHR achieved by clamping the middle cerebral artery.

Anoxia. ARL 15896AR lengthened the time of survival in mice and rats plus increased the duration to the loss of the righting reflex in rats exposed to anoxic environment. Anoxic conditions do indeed evoke a massive release of excitatory amino acid transmitters in the central nervous system and such conditions are amenable in part to treatment by both NMDA and sigma receptor antagonists (Chapman et al., 1990; Meldrum, 1992; Jensen and Auer, 1991; Poignet et al., 1992). If a chemical series is related in structure and individual compounds are active regarding lengthening survival time, the method is then useful for general rapid drug screening and for selection of initial doses for the more labor intensive investigations involving ischemia (Palmer et al., 1995a, b). Unless body temperature was controlled (e.g., mice in our study) anoxic conditions may, however, lead to identification of false positives (Milde, 1988). Nevertheless, the model has
been shown to have applicability, even to the clinical situation (Collins et al., 1989).

**Global ischemia.** In the 4-VO model of global ischemia, oral ARL 15896AR treatment beginning at reflow and continuing for 7 days resulted in protection of the highly vulnerable hippocampal CA1 neurons. We did, however, observe greater damage to the CA1 neurons on the left side. A plausible explanation for this phenomenon was the possibility of incomplete cauterization of the right vertebral artery in some of the rats. Two structurally related compounds, namely remacemide hydrochloride (Palmer et al., 1995a; Ordy et al., 1992) and ARL 13950AA (Palmer et al., 1995b), were also highly effective in the 4-VO model and we did not observe the laterality effect in these studies.

Of further interest was the lack of effect when ARL 15896AR was tested in the 2-VO/hypotension model. It is possible that the deep anesthesia required in the latter paradigm, unlike the 4-VO, interferes with the action of ARL 15896AR (Ginsberg and Busto, 1989). It was suspected that this might be the case for pentobarbital anesthesia, because of the prolonged recovery period from the anesthesia and also in view of the controversial history for barbiturates in the treatment of stroke (Steer, 1982). Nevertheless, other investigators (Meldrum, 1992; Auer et al., 1991; Chapman, 1990, for reviews) have used the 2-VO/hypotension model in the past and have reported efficacy for both competitive and uncompetitive NMDA receptor antagonists.

The ineffectiveness of ARL 15896AR in the gerbil model of global forebrain ischemia was explained due to an inability of this species to sustain an adequate, plasma level of the compound. The dose level to achieve neuroprotection was determined by pharmacokinetic modeling from the present and previous studies (Mahmood et al., 1994; Palmer et al., 1996).

We observed a similar lack of effect in the gerbil with remacemide hydrochloride (Palmer et al., 1995a).
The efficacy of NMDA receptor antagonists to prevent delayed neuronal damage to the hippocampal CA1 region after global ischemia is subject to considerable controversy and requires further study. The first issue is the debate concerning whether or not the high affinity uncompetitive and competitive NMDA receptor antagonists, notably CNS 1102 (Aptiganel or Cerestat), CGS 19755 (Selfotel) and MK801 (Dizocilpine) exhibit efficacy in the global models (Shuaib et al., 1993; Auer et al., 1991; Chapman, 1990; Meldrum, 1992).

MK801 was shown to be ineffective in both the rat 4-VO and gerbil models of forebrain ischemia when experimental conditions were such that brain and body temperatures were rigorously maintained (Buchan, 1990; Busto et al., 1987; Corbett et al., 1990). In contrast, are the claims for neuronal protection to the CA1 neurons after treatment by the low affinity uncompetitive NMDA receptor antagonists, such as HU-211, remacemide hydrochloride, ARL 13950AA (Biegnon and Joseph, 1995; Ordy et al., 1992; Palmer et al., 1995ab) and ARL 15896AR (our study) under experimental conditions controlling for hypothermia.

**Focal ischemia.** NMDA receptor antagonists are especially effective as neuroprotective agents in focal models of ischemia (for reviews see McCulloch, 1992; Meldrum, 1992; Chapman, 1990; Buchan, 1990). Thus ARL 15896AR was similarly shown to be protective in the rat model of malonate-induced striatal lesions (Greene et al., 1996). In our study using the 2-hr MCAO model in the SHR, ARL 15896AR was effective in reducing resultant cortical damage when initial doses were administered up to 1 hr after MCAO. This encouraging result led to the design of the collaborative investigation that is discussed in the following paragraphs.

In the collaborative study, the SHR received 90 min MCAO in conjunction with an acute ARL 15896AR dosing regimen. Drug administration commenced at either 15 min before (pretreatment group) or 30 min after (posttreatment group) MCAO. The second and third doses were given at 4.5 and 12.5 hr after MCAO. Plasma levels were monitored in the posttreatment group at 5.5 hr after MCAO. The 10 mg/kg, s.c. doses were calculated to yield a mean plasma level of 3000 ng/ml which has been shown using pharmacokinetic modeling studies from these and other experiments (Mahmood et al., 1994; Palmer et al., 1996) to predict neuroprotection. Maintenance of adequate plasma levels of NMDA antagonists, such as dextrorphan, is a critical factor for neuroprotection during experiments involving transient focal ischemia (Steinberg et al., 1991).

In the post-MCAO treatment group receiving ARL 15896AR, we were able to demonstrate a significant reduction in the volume of tissue appearing hyperintense on T2-weighted magnetic resonance images at days 2 and 6 after ischemia. Unlike diffusion-weighted magnetic resonance images, T2-weighted images taken immediately after stroke do
not accurately demonstrate the volume of tissue that will progress to infarction (Minematsu et al., 1993; Knight et al., 1994). However, by 2 days after ischemia, T2 weighted images accurately demonstrate infarcted tissue (van Bruggen et al., 1992; Herz et al., 1996), at which time there is excellent correlation with histologically assessed infarct volume (Allegrini and Sauer, 1992; Rudin and Sauer, 1992). The decrease in apparent volume of infarction between 2 and 6 days probably reflects some degree of atrophy in the infarcted hemisphere and a decrease in volume due to decreased brain swelling, a finding supported by the work of Knight and co-workers (1994).

Uncompetitive NMDA receptor antagonists have been demonstrated to be effective in reducing lesions appearing early on T2-weighted images after induction of focal ischemia in rats and rabbits (Minematsu et al., 1993; Steinberg et al., 1991). The T2-weighted MRIs taken herein after a relatively short period of MCAO do indicate the sensitivity of the method to identify cerebral damage in the absence of histopathological correlations.

In both control and ARL 15896AR-treated SHR, motor deficits were most severe shortly after MCAO and began to subside from 1 to 2 days, eventually reaching values analogous to the sham-operated controls from 4 to 6 days. In contrast, using a different model, Belaey and coworkers (1995) recently showed that administration of HU-211 during MCAO to Wistar rats was associated with improvement of crude motor behavior, as well as, reduction in cortical infarct volume.

After more than a month after MCAO and treatment thereof with ARL 15896AR in the present experiments, there were deficiencies in forepaw dexterity when the SHR were tested on the staircase apparatus. The rats were impaired in reaching, retrieving and eating food pellets from the lower steps of the apparatus when using their contralateral forepaw. Interestingly, the ipsilateral forepaw was also deficient and motor acts consisting of retrieving and eating the food pellets were most amenable to improvement after the acute dosing schedules with ARL 15896AR.

Histopathological analyses of the brains at the completion of the study did not reveal significant differences in the volume of infarction between ARL 15896AR-treated or control SHR experiencing MCAO. Two factors may account for this observation: 1) the resultant degree of reduction in the lesion size on T2 weighted MRI in the ARL 15896AR post-treatment group was not sufficiently robust to be correlated with histopathology; and 2) maturation and shrinkage of the lesion after the insult due to reduction in brain edema, phagocytosis, removal of necrotic tissue by microglia and finally transsynaptic atrophy. Because of the long survival time of 6 wk, the worst type of damage was atrophy (tissue loss from the ischemic hemisphere).

In summary, after an episode of focal ischemia in the SHR, the early lesion detected by T2 weighted MRI correlates better in time with behavioral outcome than does the late lesion size as determined by histopathology. At the end of 7 wk the atrophy of the brain is presumably such that subtle differences accounting for behavior changes are no longer apparent. The investigation, albeit preliminary in nature, does reveal protection of these precise cortical motor mechanisms after an acute dosing regimen with ARL 15896AR. The study could be used as a model for future detailed examination of drug efficacy in focal ischemic conditions.

Conclusion

The preclinical information reported herein and elsewhere (Black et al., 1995; Greene et al., 1996) which shows efficacy of ARL 15896AR in animal and cellular models of ischemia/excitotoxicity support the ongoing phase II clinical trials of the compound for use in patients experiencing acute focal ischemia. Moreover, the rapid attainment of plasma and brain levels after i.v. administration of ARL 15896AR (Mahmood et al., 1994), as well as its advantageous safety profile (Hudzik and Palmer, 1995; Hudzik et al., 1996), would further argue favorably for its use in acute ischemic conditions. In fact, single or multiple doses of ARL 15896AR up to 160 mg, i.v. have been well tolerated in human volunteers and the pharmacokinetic exposure as determined as AUC is well within or exceeds the range required for neuroprotection based on animal models (Palmer et al., 1996).

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References


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