[S]-AR-R 15896AR—A Novel Anticonvulsant: Acute Safety, Pharmacokinetic and Pharmacodynamic Properties

GENE C. PALMER, ROBERT J. MURRAY, CARRIE L. CRAMER, MARY L. STAGNITTO, MARILYN K. KNOWLES, LOU R. FREEDMAN, MARK S. EISMANN, NIK MAHMOOD, MIKE BALESTRA, ALFONSO R. BORRELLI, THOMAS J. HUDZIK and DENNIS J. McCARTHY

Astra Arcus USA, Rochester, New York

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ABSTRACT

A rational, chemical, synthetic effort to identify promising low-affinity uncompetitive *N*-methyl-D-aspartic acid receptor antagonists for use as antiepileptic drugs led to the discovery of AR-R 15035AR, or [RS]-α-phenyl-2-pyridine-ethanamine·2HCl. Chiral separation followed by intensive in vivo screening resulted in the selection of the [S] enantiomer, AR-R 15896AR, as the best compound for further preclinical development. AR-R 15896AR prevented tonic seizures in rodents for up to 6 to 8 h in response to maximal electroshock (MES), 4-aminopyridine, bicuculline, or strychnine, as well as characteristic seizures following injections of *N*-methyl-DL-aspartic or kainic acids. AR-R 15896AR was ineffective in two kindling models of epilepsy, did not produce tolerance to MES, and was devoid of proconvulsant and phencyclidine-like properties in mice and rats, respectively. Therapeutic indices for AR-R 15896AR were comparable to or exceeded those for standard anticonvulsants. Orally administered AR-R 15896AR rapidly entered the rat brain and was eliminated in parallel from the plasma and plasma-free compartment. A dose-response relationship between plasma and brain levels after p.o. or i.v. administration of AR-R 15896AR and protection against MES was highly correlative. The time course for loss of protection against MES mirrored the elimination of the compound from brain and plasma. The total brain concentration (25 μM) of drug at the ED_{50} value ($\sim$3 mg/kg) for protection against MES seizures was consistent with the reported affinity of AR-R 15896AR at the *N*-methyl-D-aspartic acid binding site (IC_{50} value = 1.3 μM). The present findings demonstrated the attractiveness of AR-R 15896AR as a candidate for further development to treat epilepsy.

Excessive quantities of the excitatory amino acid, glutamate, are released from neuronal synaptic endings as a consequence of anoxia, seizures, and ischemia (Bradford, 1995). Glutamate in turn activates postsynaptic receptor subtypes, one of which is selectively responsive to the glutamate analog, *N*-methyl-D-aspartic acid (NMDA) (Harris, 1995). NMDA receptor stimulation results in the opening of a receptor-operated Ca^{++} channel (Harris, 1995). Progressive accumulation of intracellular Ca^{++} leads sequentially to seizure initiation, seizure spread (Bradford, 1995), and eventually neuronal death, i.e., the "excitotoxicity hypothesis" advanced by Olney (1978). Therapeutic agents have been sought that are capable of interrupting or preventing the pathological processes associated with an action at the NMDA receptor. For example, MK801 (dizocilpine) and CNS 1102 (aptaganel) possess exceptionally high affinities for the ionic channel subsite, the "phencyclidine or PCP-site", associated with the NMDA receptor. These compounds are effective broad-spectrum anticonvulsants and likewise prevent the pathological sequelae of cerebral ischemia (Chapman et al., 1990; Albers et al., 1992; Meldrum, 1992). However, preclinical and clinical testing of the two agents, especially MK801, revealed narrow separations between effective treatment doses and doses producing severe side effects, namely, impairments in motor function, learning, and memory, as well as abuse liability (Willetts et al., 1990; Rogawski, 1992; Hudzik and Palmer 1995; Grant et al., 1996). On the other hand, low-affinity NMDA receptor antagonists have shown promise for the treatment of stroke (Cregan et al., 1997; Palmer and Hutchison, 1997), Alzheimer's disease (Muller et al., 1995), and epilepsy (Rogawski et al., 1991; Rogawski, 1992; Palmer et al., 1995; Palmer and Hutchison, 1997). Low-affinity compounds exhibited more favorable therapeutic indices (Rogawski, 1992; Hudzik and Palmer 1995; Muller et al., 1995; Grant et al., 1996; Hudzik et al., 1996). Other mechanistic differences between the two classes of uncompetitive NMDA

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartic acid; ANOVA, analysis of variance; AUC, area under the plasma concentration versus time curve; C_{max}, maximal plasma concentration; t_{max}, time to peak plasma concentration; $t_{1/2}$, plasma half-life; IA, inactive; s.i.d., dosing one time per day; MELD, median estimated lethal dose; MES, maximal electroshock; NMDLA, *N*-methyl-DL-aspartic; PCP, phencyclidine; PTZ, pentylenetetrazol or metrazol; TI, therapeutic index; v/f, volume of distribution/fraction absorbed.

antagonists are that the low-affinity antagonists: 1) exhibited faster off-rate kinetics at the ion channel (Black et al., 1995, 1996; Muller et al., 1995; Subramaniam et al., 1996); 2) acted rapidly to retard the entry of NMDA-triggered $[Ca^{++}]_{in}$ into cultured neurons, whereas high-affinity antagonists exerted a slow but complete block of the NMDA-triggered $[Ca^{++}]_{in}$ response (Black et al., 1995, 1996); 3) possessed differences in regional binding affinity to NMDA receptors (Greene et al., 1996); and 4) displayed less specificity for NMDA receptor subtypes (Monaghan and Larsen, 1997).

The goals of the investigation were to develop a safe effective low-affinity NMDA receptor antagonist for possible clinical use in epilepsy and/or stroke. Desirable characteristics of the selected compound would include: chiral separation into the most favorable isomer, efficacy against animal seizure models, safety, and a predictable, uncomplicated pharmacokinetic/pharmacodynamic profile.

Based on their affinity to displace MK801 from rat synaptosomal membranes, more than 140 active compounds were tested in mice for effectiveness in the maximal electroshock (MES) test. Compounds were sought with favorable efficacy with selections limited to those with the best therapeutic indices, i.e., efficacy versus neurotoxicity ratios. [RS]-AR-R 15035AR ([RS]-α-phenyl-2-pyridine-ethanamine·2HCl) was identified from the screening paradigm as the most promising lead. Following chiral separation of the racemate and further testing of the enantiomers, the [S]-isomer, AR-R 15896AR (Fig. 1), was found to be superior to the [R]-isomer, AR-R 15895AR, in all of the models tested. AR-R 15896AR was further shown to be active in rodent models of focal and global ischemia, findings that support the ongoing Phase II clinical trials for patients with acute stroke (Palmer et al., 1996; Cregan et al., 1997). The preclinical anticonvulsant, acute safety, pharmacokinetic and pharmacodynamic profiles of AR-R 15896AR are reported here.

Materials and Methods

All experiments for animal testing were approved by the Institutional Animal Care and Use Committee. The handling of rodents conformed to the guidelines of the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 91-579). Astra Arcus USA is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Statistical procedures for the analysis of variance (ANOVA), the Newman Keuls range test, Fieller's theorem for comparing ratios, the Z test for comparisons of proportions of animals seizing in a population, the Mann-Whitney U test for nonparametric, nonsymmetrical data and the Student's two-tailed t test are referenced in Zar (1984). The resultant effective dose$_{50}$ (ED$_{50}$) and toxic dose$_{50}$ (TD$_{50}$) values, i.e., doses preventing seizures or neural impairment in 50% of the animals, were calculated using the AEL50 Program, which likewise gives the 95% confidence limits (Finney, 1971).
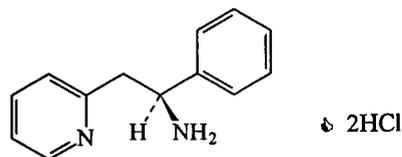
Chemical structure of [S]-AR-R 15896AR

Fig. 1. Structure of AR-R 15896AR.

Materials

Mice. Male CF1 mice weighing 20 to 30 g (Harlan Laboratories, Indianapolis, IN) were housed under a 12-h light/dark cycle at 20 to 22°C with a relative humidity of 30 to 50%. Standard food (Purina Rodent Lab Chow 5001) and water were available ad libitum until the time of drug testing. Mice were quarantined in the institutional animal facility for at least 7 days before experiments began.

Rats. Young adult Sprague-Dawley rats weighing approximately 70 g were obtained from Harlan Laboratories. Unless otherwise specified, male rats were used for the majority of experiments. Rats were quarantined for a minimum of 7 days in the animal quarters under conditions similar to those described for mice.

Compounds. [RS]-AR-R 15035AR, [S]-AR-R 15896AR, and [R]-AR-R 15895AR (the suffix "AR" designates the dihydrochloride salt form) were synthesized at Astra Arcus USA. The following were provided as gifts from the manufacturers: felbamate (Carter Wallace, Cranbury, NJ), clonazepam (Roche Laboratories, Nutley, NJ), valproate (Abbott Laboratories, North Chicago, IL) and lamotrigine (GlaxoWellcome, Research Triangle Park, NC). Phencyclidine, flunarizine, strychnine, 4-aminopyridine, pentylenetetrazol, picrotoxin, bicuculline, kainate, N-methyl-DL-aspartate, phenobarbital, carbamazepine and phenytoin were purchased from Sigma. All drug doses were expressed as mg/kg of body weight calculated to the free base form of the compound. Drugs were routinely made up on the day of dosing by dissolution in distilled water or saline. Routes of administration were i.v. , p.o., i.p., or s.c. Dose-response curves consisted of at least five different concentrations of drug, with 10 animals normally used per dose.

Seizure Models

MES in Mice. For the MES test, a drop of saline containing 1% butacaine was applied to the eyes of mice pretreated with test compound or water. The MES was produced bicorneally (50 mA, 60 Hz for 0.2 s) using a constant current shock generator (model 11A; IITC, Landing, NJ). The instrument has a built-in high internal resistance to compensate for current loss through the animal. Seizures consisted of clonic flexion of the hind limb changing into tonic extension in 0.2 to 0.5 s and then into generalized clonic convulsions followed by depression and recovery. Mice were tested at 30 min following compound administration, except for i.v. dosing, for which testing occurred at 15 min (Swinyard and Woodhead, 1982; Palmer et al., 1995).

MES in Rats. At 24 h before testing of anticonvulsant compounds, rats were prescreened for their ability to develop full tonic extension in the MES test. The selection was necessary because approximately 15% of rats do not develop full tonic extension in the MES test. On the evaluation day, the rats received a stimulus of 150 mA (0.2-s duration) (model H electroshock stimulator obtained form Wahlquist Instrument Co., Salt Lake City, UT) delivered via corneal electrodes (eyes premoistened with 1% butacaine in saline). A dose-response curve was generated following i.p., p.o., s.c., or i.v. dosing. Rats were usually tested at 1 h postdose (Palmer et al., 1995). In a separate AR-R 15896AR dose-response test, the rats were sacrificed within 5 min after MES testing and their brains and blood removed and stored frozen until later determination of AR-R 15896AR concentrations.

In both species abolition of the hind limb extensor component after drug treatment was considered the endpoint for the test. The tonic component was judged abolished if the hind limb extension did not exceed a 90° angle with the plane of the body (Swinyard and Woodhead, 1982).

Chemically Induced Seizures in Mice. Testing for efficacy against chemically induced convulsions occurred at 30 min postdose. Chemical convulsants were administered by various routes at doses that caused convulsions in 97 or 98% of the animals (ED$_{97,98}$ values). Following injections of chemical convulsants, mice were placed into individual clear plastic cages and usually observed continuously for

30 min. All anticonvulsants were administered i.p. for the 4-aminopyridine, NMDLA, kainate, pentylenetetrazol, and picrotoxin experiments and p.o. for the bicuculline and strychnine experiments.

4-Aminopyridine Tonic Seizure Test. 4-Aminopyridine (12 mg/kg, s.c.) was administered and mice were observed for the presence or absence of hind limb tonic extension (Yamaguchi and Rogawski, 1992; Cramer et al., 1994).

NMDLA Seizure and Mortality Test. NMDLA was injected (150 mg/kg, i.v.) into mice, and immediately afterward the animals were observed for 5 min for the presence or absence of the characteristic seizures (circling, myoclonic jerks, wild running, and finally severe clonic convulsions with loss of the righting reflex), and for the next 60 min for mortality as described (Czuczwar et al., 1985).

Kainate Seizure and Mortality Test. After injection of kainate (15 mg/kg, i.v.) the mice were observed for 5 min for the presence or absence of the characteristic seizures (clonic convulsions, persistent postural freezing, and/or hind limb tonic extension), which usually occur within 2 to 3 min and 30 min for protection against mortality (Braun and Freed, 1990; Cramer et al., 1994).

Bicuculline Seizure Threshold Test. Bicuculline (2.7 mg/kg, s.c.) was administered. Mice were observed for the presence or absence of the following: 1) clonic seizures, 2) tonic seizures, and 3) mortality (Swinyard and Woodhead, 1982).

Strychnine Seizure Pattern Test. Strychnine (1.2 mg/kg, s.c.) was administered, and mice were observed for the presence or absence of the hind limb extensor component of the characteristic spinal seizures. Measurements were also made regarding the latencies to seizures and mortality (Swinyard and Woodhead, 1982).

Pentylenetetrazol (PTZ) Seizure Threshold Test. PTZ (85 mg/kg, s.c.) was administered, and mice were observed for the presence or absence of clonic seizures. A threshold convulsion was described as an episode of clonic spasms lasting at least 5 s. Absence of this threshold convulsion for 30 min indicated that the test compound possessed the ability to raise the PTZ seizure threshold (Swinyard and Woodhead, 1982).

Picrotoxin Seizure Threshold Test. Picrotoxin (3.15 mg/kg, s.c.) was injected, and the animals were placed in isolated clear plastic cages and observed over the ensuing 45 min for the presence or absence of convulsions (Swinyard and Woodhead, 1982).

Kindled Seizures in Rats

Established Bicorneal Kindled Seizures. Rats were "kindled" by application of subthreshold electrical stimulation (8 mA, 60 Hz for 2 s, dosing two times per day, 5 h apart) applied via corneal electrodes until all animals exhibited grade IV to V seizure activity. All rats were kindled an equal number of times (11 total stimulations) although some demonstrated grade IV convulsions after fewer stimulations (~5 days). Seizures consisting of progressive development of symptoms are graded according to the following criteria established by Racine (1972) and previously used in our laboratory (Palmer et al., 1992, 1995):

• Grade I—facial movements;
• Grade II—facial movements with prominent head nodding;
• Grade III—symptoms as in II plus raising to the sitting position with mild forelimb clonus;
• Grade IV—marked rearing to a vertical position moving the head from side to side with prominent oral movements, and forelimb clonus; and
• Grade V—symptoms as in IV progressing to falling and clonic seizures.

In these established kindled seizures, the test for drug efficacy was performed after the last kindle and 1 h after dosing with AR-R 15896AR, phenobarbital, or valproate. Protection was indicated by a significantly lower seizure score by the group of drug-treated animals as compared with controls. Because the distribution of the data

populations were nonsymmetrical, the nonparametric Mann-Whitney U test was used for statistical comparisons (Zar, 1984).

Development of bicorneal kindled seizures. Another study was designed to determine whether AR-R 15896AR would retard or prevent the kindling process. Animals were dosed p.o. [once per day (s.i.d.) for 5 days] with test compounds or saline and 1 h later received the first subthreshold stimulus; the second subthreshold stimulus was given 5 h after dosing. Seizure scoring was as described above. After 5 days the animals were granted a 2-day weekend washout (drug free period) followed by a final single stimulation on the following Monday (day 8). The purpose of the washout was to determine (assuming the compound was active) whether the presence of the drug was required to inhibit the seizures or whether the seizure process itself had been prevented (Palmer et al., 1992).

Known standards that have been previously shown to be effective versus kindling seizures were evaluated as positive controls, namely, phenobarbital (for established seizures, Garske et al., 1991) and valproate (for development of seizures, Applegate et al., 1997). Seizure scoring was performed by two raters, one of whom was unaware as to the mode of treatment. Comparisons were performed using an analysis of variance (ANOVA) followed by the Newman Keuls range test (Zar, 1984) to determine differences between individual data points.

Febrile-Kindled Seizures in Weanling Rats. Rats were dosed p.o. with test agents or appropriate vehicle 1 h before placement in a water bath maintained at 45 ± 0.1°C for 4 min (Palmer et al., 1998). An adjustable perforated base was placed in the bath as a means of hind limb support to prevent forced swimming. The sides of the bath were of sufficient height to avert escape. Animals were kept under constant observation for the 4 min in the water bath or until appearance of symptoms. The time to onset of seizures was recorded. Zero time was defined as the end of the 4 min heat-stress session. For rats seizing while in the water, the remaining time to the end of the 4 min session was expressed as a negative number. A positive time indicated the seizure occurred after removal from the water. Seizure scoring was done according to Racine (1972). At the end of the 4-min period involving immersion or immediately after appearance of seizures, the animals were dried (incubator maintained at 28°C, ~30 min) and returned to the home cages. Groups of rats were tested 2 days per week over a 2-week period for a total of four immersions per rat. Group size was 12, unless otherwise noted. The following parameters of the experiment were analyzed:

1. body weights taken on the respective test days;
2. maximum seizure grade or score;
3. time to seizure onset in seconds;
4. seizure duration in seconds, and
5. percent of animals seizing per testing session.

Statistical comparisons for all parameters, except the number of rats seizing, were made using an ANOVA with repeated measures and where significant differences were recorded, the post hoc Newman Keuls analysis was applied. The Z test was used to compare the difference between groups (treated versus controls) in the proportion of animals seizing in a population (Zar, 1984).

MES Tolerance Test

Mice. Mice were divided into three groups of between 50 and 60 animals each. One group received an p.o. dose of AR-R 15896AR (45 mg/kg, s.i.d.) for 4 days administered in water as vehicle. A second group received water, s.i.d. A third group was not dosed (to evaluate for effects of stress). On day 5 a dose-response curve (doses were 10, 12.5, 15, 20, 25, and 30 mg/kg, $n = 10$ mice per dose) was run on each group to determine the individual oral ED_{50} values for protection in the MES test. An increase in the ED_{50} value in the AR-R 15896AR-treated mice would indicate tolerance following a subchronic course of administration (Palmer et al., 1995). This testing paradigm has been used by us in the past to show tolerance to the low-affinity

uncompetitive NMDA antagonist remacemide hydrochloride as well as phenytoin (Palmer and Hutchison, 1997).

Rats. Separate groups of rats (50 per group) received either water or AR-R 15896AR (24 mg/kg, s.i.d., represents $6 \times$ oral ED_{50} value) for 4 days. On day 5 a dose-response curve (doses were 1, 3, 6, 9, and 12 mg/kg, $n = 10$ rats per dose) was run on each group at 60 min postdose to determine the resultant ED_{50} values for protection in the MES test. An increase in the ED_{50} for protection in the MES test is suggestive of tolerance (Garske et al., 1991).

Proconvulsant Test in Mice

The metrazol seizure threshold test measures the minimal seizure threshold and is used to determine possible proconvulsant properties of compounds (Palmer et al., 1995).

Mice were pretreated with AR-R 15896AR, i.p. and 30 min later received controlled i.v. infusions of metrazol. The selected doses of AR-R 15896AR correspond to the i.p. ED_{98} value (19 mg/kg) obtained from the MES test and a TD_3 value of 40 mg/kg obtained from the inverted screen test (see below for description of the latter test). To eliminate bias due to stress, the infusion needle with attached tubing was inserted into the tail vein, taped in place, the mouse then placed into a plastic cage (24 cm long $\times$ 24 cm wide $\times$ 16 cm high), and allowed to move about freely for 30 s. The convulsant solution (0.5% metrazol/0.9% NaCl plus 10 μg/ml heparin sodium) was infused at a constant rate of 0.34 ml/min by means of a Harvard model 901A syringe infusion pump. The time was recorded when the mouse exhibited the first twitch (time to first twitch) and the infusion continued until the mouse experienced 3 s of persistent clonus (time to clonus). The data were expressed as mean time in s $\pm$ S.E. to first twitch and to clonus. Comparisons were made to controls using the Student's two-tailed t test. An equal number of controls to experimental animals were run on a given day. Drug-treated animals were alternated with controls during the runs.

Therapeutic Index/Acute Safety

Mice. The inverted screen test was used to determine the acute TD_{50} value in mice. The TD_{50} value was the calculated endpoint at which 50% of the mice failed the task (Palmer et al., 1995). The apparatus consisted of six 13-cm square platforms of 0.6-cm wire mesh supported by metal bars, which in turn were mounted on a steel rod. The rod was supported at both ends and was inverted through an arc of 180°. Mice unable to climb to an upright position within 1 min were rated as failures. Testing occurred at 30 min following p.o. drug administration.

Rats. The gangplank escape test was used to determine the acute TD_{50} following 60 min of p.o. administration of test compounds in rats (Garske et al., 1991). The apparatus consisted of a narrow board 1.25 cm wide $\times$ 63 cm long mounted 49 cm above the bench top. At one end there is an entry cubicle (13 $\times$ 13 $\times$ 15 cm high) open at the top and illuminated by a 52-W bulb suspended immediately overhead (21 cm above the plank). A larger box covering the plank was progressively darkened to an identical, but darkened, escape cubicle at the other end. The rat received a familiarization trial before drug testing. The escape cubicle takes advantage of the natural tendency of rats to seek a darker environment. A rat was rated as impaired if it fell from the plank

Screening for PCP Liability. The method was identical with that used previously (Hudzik et al., 1996). Compounds were administered to rats as multiples of the oral ED_{50} value obtained from the MES test (5 naïve rats per dose). Animals were then placed in individual shoe box, Plexiglas cages and observed for approximately 5 h for the appearance of any of these five signs: ataxia, circling, head weaving, hyperactivity and retropulsion. Cumulative incidence scores (a maximum of 25, or all five signs in all five rats) were recorded. Phencyclidine was used as a reference standard and run side by side with each compound.

Pharmacokinetics

Dosing and Sampling Schedule. For the p.o. and i.v. pharmacokinetic study, in-dwelling catheters were surgically inserted into the jugular vein of anesthetized (ketamine cocktail) male rats. On the following day, AR-R 15896AR was administered as a single p.o. or i.v. (tail vein) dose of 5 mg/kg. For the i.v.-dosed rats, blood was taken from the jugular vein catheter at 2, 5, 10, 20, and 40 min and 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. For the p.o.-dosed rats, blood was taken at 5, 15, 30, 40, 50, min and 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. The blood was placed in tubes containing anticoagulant and the plasma was obtained by low-speed centrifugation. Plasma samples from 10 rats were pooled from each time, and the concentration of AR-R 15896AR determined as described below.

For the study to determine brain concentrations, AR-R 15896AR was given i.v. at 10 mg/kg to rats via the tail vein. At 5 and 20 min and 1, 2, 4, and 6 h, the animals were sacrificed (3 rats per experimental time point) and samples of blood immediately taken from the posterior vena cavae. Following a 30-s perfusion of the left cardiac ventricle with saline, the brains were removed, immediately frozen on dry ice, and stored at -70°C. Plasma was obtained by low-speed centrifugation of the blood from which a portion was removed to obtain protein-free ultrafiltrate for determination of plasma-free drug concentrations.

Rats used in the MES test were sacrificed within 5 min following electroshock, i.e., 65 min after p.o. administration of AR-R 15896AR or 185 min after i.v. administration of compound. The brain and plasma samples were stored frozen at -70°C until assay.

Plasma and Brain Levels. Plasma samples were analyzed by liquid-liquid extraction. For sample analysis, 50 μl of solution containing 500 ng of internal standard (α-methyl-α-phenyl-2-pyridine-ethanamine free base) was 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 subsequent to 10 min low-speed centrifugation. The organic phase was removed and added to tubes containing 125 μl of 0.1 M HCl followed by mixing, centrifugation ($822 \times g$) and removal of the organic layer. Added to the tubes was 15 μl of 1.0 M NaOH, followed by brief mixing and transfer to an HPLC autosampler. Standards were prepared in plasma from naïve rats at concentrations between 5 and 10,000 ng/ml. The method was specific and the peak height ratios versus concentration were 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 KH_2PO_4, pH 3.75, and 0.2% triethylamine (final pH adjusted to 3.75 with 85% H_3PO_4). The variable wavelength detector was setup at 260 nm with a 250- $\times$ 4-mm column (Lichrospher 60 RP-select B 5-μM particle size) heated to 40°C. Plasma-free samples were analyzed directly using similar HPLC conditions. The detection limit for AR-R 15035AR and its isomers is 5.0 ng/ml in plasma. Compound levels are reported as the free base.

The brains were thawed, weighed, and transferred to centrifuge tubes containing 20 μg free base/ml of internal standard followed by addition of 3 ml of 0.2 N NaOH and homogenization for 30 s using a Tissuremizer (Tekmar). Butanol:Hexane (10:90) was added to the homogenate followed by mixing for 10 min. The extract was separated by centrifugation, the aqueous layer frozen (dry ice acetone bath), later thawed, neutralized with 0.2 N NaOH, and analyzed using HPLC. The organic layer was transferred to centrifuge tubes containing 0.5 ml of 0.1 N HCl, mixed, and the subsequent aqueous phase likewise analyzed by HPLC.

Results

MES Testing

Mice. The respective ED_{50} values for [S]-AR-R 15896AR and [RS]-AR-R 15035AR for protection of mice against MES were roughly similar, 5.7 to 8.6 mg/kg (i.v.), to 17.3 to 17.5

mg/kg (p.o.), to 13.0 to 13.4 mg/kg (i.p.), to 9.4 to 8.6 mg/kg (s.c.). In contrast, the [*R*]-isomer, AR-R 15895AR, was consistently two- to threefold less potent ($P < 0.0001$; Student's two-tailed t test) when administered i.p., p.o. or i.v., but not following the s.c. route, where potency was only slightly less ($P < 0.025$) than for the racemate or the [*S*] isomer (Table 1).

Rats. The ED_{50} values for [*S*]-AR-R 15896AR in the rat MES test were similar (2.5–4 mg/kg) with all four routes of drug administration. AR-R 15896AR tended to exhibit greater potency than the racemate, AR-R 15035AR, but none of the ED_{50} values for the separate routes of administration were significantly different (Student's two-tailed t test). Both [*S*]-AR-R 15896AR and [*RS*]-AR-R 15035AR were considerably more potent than [*R*]-AR-R 15895AR in protecting rats from MES. For example, after p.o. dosing AR-R 15896AR was fivefold more potent ($P < 0.0001$) than AR-R 15895 (comparable ED_{50} values were 3.7 versus 20.3 mg/kg, respectively). After i.v. administration both AR-R 15896AR and the racemate were twofold more potent (respective P values < 0.05 and < 0.02) than AR-R 15895AR (comparable ED_{50} values were 2.6 and 2.7 versus 6.0 mg/kg, respectively). Moreover, following s.c. treatment, potencies for AR-R 15896AR and AR-R 15035AR were from two- to sixfold greater (P values < 0.0001) than for AR-R 15895AR (comparable ED_{50} values were 2.5 and 5.9 versus 15.0, respectively) (see Table 2).

In order to observe for possible gender differences in response to protection in the MES test, AR-R 15896AR was administered p.o. in male and female rats. At 1 h postdose the rats were tested using a balanced design, direct side-by-side comparison. The resultant ED_{50} values were males = 3.1 [95% confidence limits = 2 to 5]; females = 4.5 [95% confidence limits = 3 to 11] (comparisons were not significant, Student's two-tailed t test).

Mice: Time Course for Protection against MES. After receiving single p.o. doses of the approximate ED_{98} value of the three compounds (AR-R 15896AR = 45 mg/kg, AR-R 15895AR = 115 mg/kg, and AR-R 15035AR = 50 mg/kg), mice were tested for protection against MES for up to 6 h (Table 3). For statistical comparisons of the proportion of animals seizing between the three compounds, the *Z* test ($P > 0.05$) (Zar, 1984) did not indicate marked differences among the respective time course profiles for protection. For all three compounds, maximal protection (100%) was observed from 30 to 90 min after dosing. By 2 h, protection was reduced from 100 to 40% for AR-R 15035AR, from 100 to 70%

TABLE 2
Antiseizure profiles (ED_{50} values) in rats of [*S*]-AR-R 15896AR, [*R*]-AR-R 15895AR, and [*R,S*]-AR-R 15035AR following various routes of administration

Model	[*S*]-AR-R 15896AR	[*R*]-AR-R 15895AR*	[*R,S*]-AR-R 15035AR
MES, i.p.	4.0 [3–5]	Not done	5 [4–7]
MES, p.o.	3.7 [3–6]	20.2 [10–35]	9.9 [4–17]
MES, i.v.	2.6 [0.6–4.8]	6.0 [4–8]	2.7 [1.5–4]
MES, s.c.	2.5 [1.6–3.5]	15.0 [13–17]	5.9 [4–8]

ED_{50} values are in mg/kg; values in brackets are 95% confidence limits.
* Values for AR-15895AR are significantly different from AR-15896AR and AR-R 15035AR (Student's two-tailed t test). Animals received MES at 60 min after i.p., p.o., and s.c. dosing and 15 min after i.v. administration of compounds.

TABLE 3
Time course of percent protection in mice against MES-induced seizures following single oral doses of the approximate ED_{98} value for the three compounds

Time of Test (h Postdose)	[*S*]-AR-R 15896AR (45 mg/kg)	[*R*]-AR-R 15895AR (115 mg/kg)	[*R,S*]-AR-R 15035AR (50 mg/kg)
0.5	100	100	100
1.5	100	90	90
2.0	70	80	40
4.0	30	50	20
6.0	20	20	40

n = 10 animals per dose.

for AR-R 15896AR, and from 100 to 80% for AR-R 15895AR. At 6 h, protection ranged from 20 to 40% for all three compounds.

Rats: Time Course for Protection against MES. Single doses of approximately three times the oral ED_{50} value of AR-R 15035AR and its enantiomers were compared side by side in rats for protection against MES over a course of 24 h (Table 4). The percentage of animals protected ranged from 60 to 100% during the initial 4 h, but by 8 h protection dropped to 30% for all three compounds. No protection was evident at 24 h. For comparison of the proportion of animals seizing in a population, the *Z* test (Zar, 1984) revealed no significant differences in the degree of protection among the three compounds. Similar studies were conducted with AR-R 15896AR alone using i.v., s.c., and i.p. routes of administration. With i.v. (7.5 mg/kg) and i.p. (12 mg/kg) dosing, 90 to 100% protection was observed over 4 h, decreasing to 70% (i.v.) and 50% (i.p.) protection at 6 h. Administration of AR-R 15896AR by the s.c. route yielded the shortest time course of protection: 100% at 30 min, 80 to 60% from 2 to 4 h, and 10% at 6 h. Another study directly compared male and female rats

TABLE 1
Antiseizure profiles (ED_{50} values) in mice of [*S*]-AR-R 15896AR, [*R*]-AR-R 15895AR, and [*R,S*]-AR-R 15035AR

Model	[*S*]-AR-R 15896AR	[*R*]-AR-R 15895AR*	[*R,S*]-AR-R 15035AR
MES, i.p.	13.0 [12–14]	26.9 [25–29]	13.4 [12–15]
MES, p.o.	17.3 [15–19]	57.3 [49–69]	17.5 [14–21]
MES, i.v.	5.7 [5–6]	13.8 [13–15]	8.6 [7–10]
MES, s.c.	9.4 [9–12]	13.1 [13–15]	8.6 [8–9]
NMDA seizures, i.p.	54.0 [38–112]	>75	42.0 [25–54]
NMDA lethality, i.p.	23.3 [18–30]	>75	20.7 [16–26]
Kainate seizures, i.p.	~50	>75 [40%]***	30.6 [24–163]
Kainate lethality, i.p.	29.7 [24–36]	>75	27.8
4-Aminopyridine, i.p.	17.1 [15–19]	32.4 [28–37]	22.0 [18–28]
Bicuculline tonic seizures, p.o.	9.8 [6–9]	43.4 [33–55]	29.0** [19–35]
Bicuculline clonic seizures, p.o.	IA	IA	IA
Bicuculline lethality, p.o.	13.0 [8–19]	69.5 [55–98]	43.0** [36–55]

Values in brackets are 95% confidence limits; IA = inactive; ED_{50} values are in mg/kg.
* Values in this column were significantly different from AR-R 15896AR and AR-R 15035AR; ** Significantly different from AR-R 15896AR (Student's two-tailed t test).
*** 40% protection at 75 mg/kg. Anticonvulsant testing occurred at 30 min after compound administration (except for i.v. dosing when testing occurred at 15 min).

TABLE 4

Time course of percent protection in rats against MES-induced seizures following single oral doses of compounds administered at approximately three times the ED_{50} value

Time of Test (h Postdose)	[S]-AR-R 15896AR (12 mg/kg)	[R]-AR-R 15895AR (60 mg/kg)	[R,S]-AR-R 15035AR (30 mg/kg)
2	100	60	80
4	70	60	100
8	30	30	30
24	0	0	0

$n = 10$ animals per dose.

using 12 mg/kg of AR-R 15896AR administered p.o. Protection at 2 h was 100% for males and 90% for females; at 4 h protection was 90% for males and 100% for females; and at 6 h protection was 80% for both sexes. The proportional breakdown by gender of animals seizing was not significant (Z test, see Zar, 1984).

Chemically Induced Seizures in Mice

4-Aminopyridine. The test for efficacy against 4-aminopyridine seizures, following i.p. dosing of AR-R 15896AR and AR-R 15035AR yielded ED_{50} values in the same range as were demonstrated to be effective against MES (Table 1). Based on the ED_{50} value, the potency for AR-R 15895AR (32.4 mg/kg) was slightly less than half that for AR-R 15896AR (17.1 mg/kg) ($P < 0.0001$, Student' two-tailed t test). The differences in ED_{50} values between AR-R 15895AR and the racemate (ED_{50} value = 22 mg/kg) were also significant ($P < 0.025$). Because compounds active in the MES test are effective against 4-aminopyridine-induced seizures (Yamaguchi and Rogawski, 1992), phenytoin was tested as a positive control. The ED_{50} value for i.p. dosed phenytoin was 7.5 mg/kg [95% confidence limits = 4 to 9].

Bicuculline. The γ-aminobutyric acid receptor antagonist, bicuculline (Kupferberg, 1989) produced clonic seizures followed by tonic extension and death. [S]-AR-R 15896AR, [RS]-AR-R 15035AR, and [R]-AR-R 15895AR (administered p.o.) were inactive in preventing clonic seizures; however, the compounds did inhibit tonic seizures in the following order of potency: AR-R 15896AR (ED_{50} value = 9.8 mg/kg) ≫ AR-R 15035AR (ED_{50} value = 29 mg/kg) > AR-R 15895AR (ED_{50} value = 43.4 mg/kg) (see Table 1). Statistical analyses (Student's two-tailed t test) of the data showed that the inhibitory effects of AR-R 15896AR were more potent ($P < 0.0001$) when ED_{50} values were compared to either AR-R 15895AR or AR-R 15035AR. Bicuculline-induced mortality was likewise prevented following pretreatment with AR-R 15896AR (ED_{50} = 13 mg/kg), AR-R 15035AR (ED_{50} = 43 mg/kg), and AR-R 15895AR (ED_{50} = 69.5 mg/kg) (Table 1). The ED_{50} value for protection versus mortality by AR-R 15896AR (13 mg/kg) was statistically less ($P < 0.0001$) than the ED_{50} values for AR-R 15035AR (43 mg/kg) and AR-R 15895AR (69.5 mg/kg). Moreover, the ED_{50} value for protection against lethality by AR-R 15035AR was significantly less ($P < 0.05$) than AR-R 15895AR. The positive control for this test was phenobarbital, which inhibited both the clonic and tonic components of bicuculline. The oral ED_{50} value for phenobarbital was 21 mg/kg [95% confidence limits = 13 to 22].

NMDLA or Kainate. Following administration of either NMDLA or kainate, the majority (>95%) of the seizures occurred within 2 to 3 min. Pretreating mice with AR-R 15035AR and AR-R 15896AR (i.p.) resulted in prevention of NMDLA-induced seizures as well as the subsequent mortality. The respective ED_{50} values were 42 and 54 mg/kg versus convulsions and 20.7 and 23.3 mg/kg versus mortality (Table 1). AR-R 15895AR was ineffective at the screening dose (75 mg/kg, i.p.). Both AR-R 15896AR and AR-R 15035AR were somewhat effective in suppressing kainate-induced seizures (respective ED_{50} values = ~50 and 30.6 mg/kg) and the ensuing lethality (respective ED_{50} values = 29.7 and 27.8 mg/kg). AR-R 15895AR was partially active in the kainate test—40% of the animals exhibited no seizures at a dose of 75 mg/kg (i.p.); however, this appeared to be a threshold response because higher doses were less effective. This threshold response was also noticed with AR-R 15896AR (versus convulsions) and AR-R 15035AR (versus mortality) (Table 1). The potent uncompetitive NMDA receptor antagonist, phencyclidine, served as a positive reference standard: ED_{50} values for prevention of NMDLA-elicited seizures and lethality were 6.3 mg/kg [95% confidence limits = 5 to 8] and 2.8 mg/kg [95% confidence limits = 1.6 to 3.8], respectively.

PTZ, Strychnine, or Picrotoxin. AR-R 15896AR was tested further for possible protection against seizures elicited by PTZ, strychnine, or picrotoxin. At the screening dose, p.o., of 40 mg/kg, mice were not protected from convulsions elicited by either PTZ or picrotoxin. The only protection noticed was after administration of high p.o. doses (50–100 mg/kg) in the strychnine test. The time to tonic hind limb extension induced by strychnine was modestly increased by 52% from 5.9 min in the controls to 9.1 min following pretreatment, p.o., with 75 to 100 mg/kg AR-R 15896AR. Moreover, the time to death was likewise increased by ~100% from 6.7 min in the controls to 13.4 min in the treatment group ($P < 0.001$, ANOVA followed by Newman Keuls analysis). Oral doses from 25 to 100 mg/kg AR-R 15896AR failed to protect mice from the clonic spinal seizures elicited by strychnine. At relatively high doses, the reference compound, phenobarbital, inhibited strychnine-induced clonic convulsions with an oral ED_{50} value of 142 mg/kg [95% confidence limits = 124 to 165]. With the other two tests, oral efficacies ED_{50} values for phenobarbital were PTZ = 17 mg/kg [95% confidence limits = 14 to 21] and picrotoxin = 25 mg/kg [95% confidence limits = 17 to 33].

Kindled Seizures in Rats

Established Bicorneal Kindled Seizures. Once seizures were fully kindled, animals were administered, p.o., AR-R 15896AR or phenobarbital on the day of testing using either 6 or 10 times the ED_{50} value from the MES test. Similarly valproate was administered at 1 or 3 times the ED_{50} value. (ED_{50} values for valproate and phenobarbital in the MES test are given in Table 7.) One hour after dosing, the subthreshold electrical stimulus was applied. AR-R 15896AR (24 and 40 mg/kg) and the lower dose of valproate (260 mg/kg) were ineffective in this test (Table 5). Seizures were significantly prevented with the higher dose of valproate (780 mg/kg, Mann-Whitney U test; Zar, 1984) and the two doses of phenobarbital (24 and 45 mg/kg).

Development of Bicorneal Kindled Seizures. Subchronic p.o. administration of AR-R 15896AR (12 or 24 mg/kg for 5 days) did not retard the development of kindled seizures. Valproate, when administered p.o. at either 260 or 780 mg/kg significantly impeded the formation of kindled seizures. The valproate treatment effects from the 2 by 10

TABLE 5

Effects comparison of orally administered [*S*]-AR-R 15896AR, phenobarbital, and valproate on established kindled seizures in rats

Treatment	Dose (mg/kg)	Mean Seizure Score	*p* Value, Mann-Whitney
Control	0	4.5 [4.1–4.8]	
AR-R 15896AR	24	4.1 [3.2–5.0]	NS
AR-R 15896AR	40	3.7 [3.4–4.0]	NS
Phenobarbital	24	1.5 [0.4–2.5]	0.01
Phenobarbital	45	0.9 [0.7–1.1]	0.01
Valproate	260	4.5 [4.1–4.9]	NS
Valproate	780	0.4 [0.04–0.8]	0.001

Values in brackets are 95% confidence limits; NS = not significant; n = 8–10 rats per treatment group. All compounds were administered 1 h prior to application of electroshock.

ANOVA were: 260 mg/kg − F(1, 14) = 24.56, P = 0.0002; 780 mg/kg − F(1, 14) = 151.6, P = 0.00001. Moreover, at the higher dose, the seizure score after the valproate washout period (days 6 and 7) remained significantly lower than the controls ($P < 0.0008$) (Fig. 2).

Kindled Febrile Seizures in Weanling Rats. Seizures generally occurred when the animal was in the water bath; the onset consisted of tremors with the animal curling into a ball at which point the rat was removed from the bath and allowed to recover. In those animals exhibiting seizures, the time to onset was fairly consistent, occurring within ±30 s from the end of the 4 min exposure to the water bath. Seizure duration became more prolonged following repeated exposures to the bath. There were no differences between males (n = 14) or females (n = 10) regarding the time to seizure onset and seizure duration. The effect of heat stress significantly elevated the body temperatures of both females and males to approximately 43°C. This elevated body temperature did not fluctuate significantly following either single or
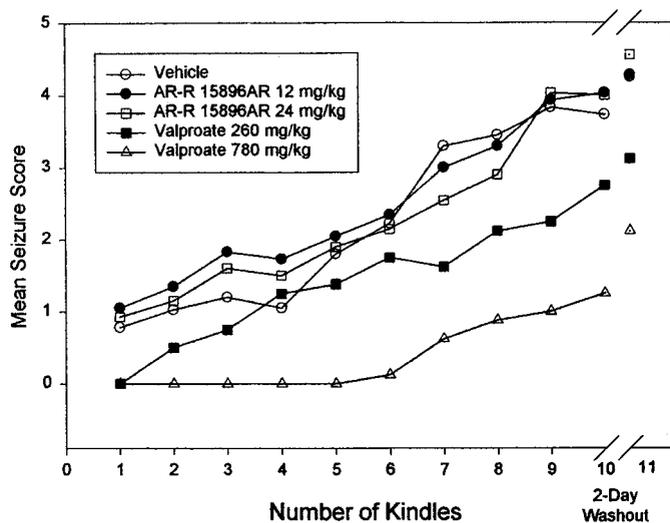
multiple exposures and no differences between heat-stressed males and females were observed.

The percentage of animals seizing during the four testing periods did not differ remarkably between controls and those dosed with AR-R 15896AR. During the first testing session 66% of the controls and 83% of AR-R 15896AR-treated animals convulsed, however, during the second to fourth sessions the controls ranged from 93 to 99% seizing, whereas 100% of the AR-R 15896AR-treated rats experienced seizures. This contrasts with MK801 (0.11 mg/kg) run in conjunction with the experiment and reported previously (see Palmer et al., 1998). The percent of animals seizing in the presence of MK801 ranged from 36 to 42% ($P < 0.05$, Z test) during the initial three sessions and was 67% during the fourth session. Neither drug treatment influenced body temperature before or after exposure to heat stress.

AR-R 15896AR did not influence body weight of the animals over the 2-week course of the investigation (Fig. 3). Likewise, the scores for time to seizure onset and maximal seizure grade were generally unaffected by pretreatment with AR-R 15896AR, the exception occurring during the second testing session when seizure grade was significantly worsened ($P < 0.05$, ANOVA followed by Newman Keuls
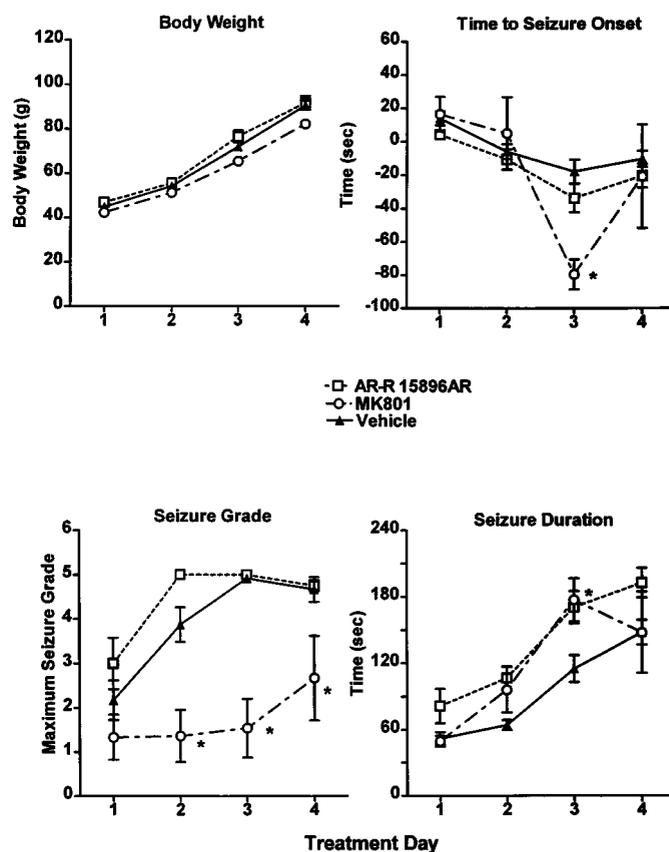

Fig. 2. Effect of AR-R 15896AR or valproate on the development of bicorneal kindling in rats. Animals were dosed orally (s.i.d./day/5 days) and 1 h later received the first subthreshold stimulation (8 mA, 60 Hz for 2 s), the second subthreshold stimulus was given 4 h after dosing. Stimulation was continued (two times per day) for a total of 5 days (10 kindles) at which time a drug free "washout period" was allowed for 2 days and on day 8 (11th kindle) the stimulus was given in the absence of drug. Seizures were scored using the criteria of Racine (1972). The two curves for valproate are significantly different than the control curve ($P < 0.0002$ for the low dose and $P < 0.00001$ for the high dose; ANOVA followed by Newman Keuls range test, n = 8 animals/dose).

Fig. 3. Effect of p.o. administered AR-R 15896AR (4 mg/kg) [–□ –] or MK801 (0.11 mg/kg) [–○–] or vehicle [–▲–] on heat-stress induced seizures in weanling rats. Measurable end points are influence on body weight, time to seizure onset, maximum seizure grade and seizure duration. Animals (n = 12/dose) were pretreated at 60 min before heat stress, which consisted of exposure to a 45°C water bath for 4 min 2 days/week for 2 weeks (4 exposures per rat). Seizures were scored using the criteria of Racine (1972). Results were compared using an ANOVA followed by the Newman Keuls range test, the asterisk (*) denotes significance from control ($P < 0.05$). The comparative data for MK801 have been previously published (Palmer et al., 1998).

range test). For the other measurable parameter, AR-R 15896AR tended to prolong seizure duration during the third testing session. No effects of the compound with regard to other observable behavioral signs were evident. For comparison and as reported previously (Palmer et al., 1998), the MES ED_{50} value for MK801 (0.11 mg/kg) was highly effective in reducing the maximal seizure grade during treatment sessions 2 to 4. During the third testing session, MK801 prolonged the duration of seizures and decreased the time to seizure onset ($P < 0.05$, ANOVA followed by Newman Keuls range test). However, treatment with MK801 increased ataxia and spontaneous motor activity before and up to 60 min after heat-stress exposure.

Proconvulsant Test in Mice

Shortening the latencies to first twitch and to clonus indicate the ability of a compound to manifest proconvulsant properties by lowering the threshold for metrazol seizures. Pretreating mice with AR-R 15896AR (19 or 40 mg/kg, i.p.) did not change the effects of i.v. infusion of metrazol regarding times to first twitch and full clonus ($P > 0.05$, Student's two-tailed t test) (Table 6).

Tolerance

Mice. There were no differences in the ED_{50} values from the MES tests when comparisons were made among the three different treatment groups of mice evaluated on day 5 for possible indications of tolerance to AR-R 15896AR (Student's two-tailed t test). The resultant ED_{50} values in mg/kg (in brackets below, 95% confidence limits), slopes $\pm$ S.E. of the dose-response curves were:

- Group I (naïve mice no treatment)—14.4 [11 to 19], 4.7 $\pm$ 1.2;
- Group II (saline p.o. for 4 days)—16.9 [14 to 20], 6.1 $\pm$ 1.5;
- Group III (AR-R 15896AR p.o. for 4 days)—18.9 [16 to 22], 7.0 $\pm$ 1.7

Rats. Following p.o. administration of AR-R 15896AR daily for 4 days at 6 times the ED_{50} value, there was a nonsignificant trend ($P = 0.1$; Student's two-tailed t test,) for the ED_{50} value in the MES test to increase on day 5. The resultant ED_{50} values in mg/kg (in brackets below, 95% confidence limits), slopes $\pm$ S.E. were:

1. Controls (saline daily 4 days)—3.4 [2 to 5], 2.2 $\pm$ 0.6;
2. AR-R 15896AR (24 mg/kg/daily 4 days)—6.6 [4 to 10], 2.6 $\pm$ 0.8

TABLE 6
Mouse proconvulsant test: Effects of i.p. administration of [*S*]-AR-R 15896AR on the seizure threshold during metrazol infusion

Treatment	Dose (mg/kg)	Time to First Twitch (s)	Time to First Clonus (s)
Group I			
Control	0	27.8 $\pm$ 1.1	35.0 $\pm$ 1.2
AR-R 15896AR	19	26.3 $\pm$ 1.0	40.1 $\pm$ 2.0
Group II			
Control	0	30.9 $\pm$ 1.0	45.1 $\pm$ 2.0
AR-R 15896AR	40	27.5 $\pm$ 1.2	47.6 $\pm$ 3.3

Values expressed as mean $\pm$ S.E.; $n = 15$ mice per treatment group. Mice were pretreated with AR-R 15896AR 30 min before beginning the metrazol infusion.

Therapeutic Index/Acute Safety

Mice. The p.o. toxic dose-50 (TD_{50}) values and i.v. median estimated lethal dose (MELD) values for AR-R 15896AR, AR-R 15895AR, and AR-R 15035AR, from the inverted screen and lethality tests, respectively, in mice fell within the overlapping 95% confidence limits of one another and therefore did not differ significantly (comparisons by the Student's two-tailed t test) (see Table 7). Respective TD_{50} values in mg/kg were: AR-R 15896AR = 193.3 [95% confidence limits = 145 to 235], AR-R 15895AR = 142 [95% confidence limits = 80 to 226], and AR-R 15035AR [95% confidence limits = 139 to 359]. The therapeutic indices, i.e., TD_{50}/ED_{50} from the MES test were greater for AR-R 15035AR and AR-R 15896AR than for AR-R 15895AR (TI values of 14, 11.2, and 2.5, respectively). However, statistical comparisons of the TI values using Fieller's Theorem (Zar, 1984) revealed no significant differences between ratios. Table 7 also provides comparative data for clinically useful compounds that have been evaluated at Astra Arcus USA. For example, p.o. administered lamotrigine is the most potent compound regarding protection of mice in the MES test (ED_{50} value = 6.1 mg/kg) and valproate is the least potent (ED_{50} value = 631 mg/kg). The TI values for felbamate, lamotrigine, phenytoin, flunarizine, AR-R 15035AR and AR-R 15896AR ranged from 10 to 22. The compounds with the lowest TI values in mice (range 2–5) were AR-R 15895AR, valproate and phenobarbital.

The MELDs, in mg/kg following bolus, single dose i.v administration of the three test compounds were [*S*]-AR-R 15896AR = 44.1 [95% confidence limits = 42 to 47], [*R*]-AR-R 15895AR = 33.8 [95% confidence limits = 32 to 36], and [*RS*]-AR-R 15035AR = 41.8 [95% confidence limits = 38 to 45]. The resultant safety margin (MELD/ED_{50} from the MES test) for i.v. administration was more favorable for AR-R 15896AR than for either AR-R 15035AR or AR-R 15895AR. These safety margins were: 7.7, 4.9, and 2.5, respectively. Statistical analyses of the safety margins indicated that the difference between AR-R 15896AR and AR-R 15895AR was highly significant ($P < 0.0001$, Fieller's Theorem; Zar, 1984).

Rats. TD_{50} values for rats following single p.o. dosing regimens were determined from the gangplank escape test. At 1 h after dosing, the TD_{50} values for AR-R 15035AR and AR-R 15895AR were greater than 1000 mg/kg, whereas that for AR-R 15896AR was 932.5 mg/kg [95% confidence limits 712 to 2361]. The resultant TI value for AR-R 15896AR was 252, whereas the estimated TI values for AR-R 15035AR and AR-R 15895AR were >100 and >49.5, respectively (Table 7). Comparative data for clinically useful drugs are also included in Table 7. With the exception of valproate, the therapeutic indices for these reference compounds were more favorable in rats than in mice.

Twenty-four hours after single bolus injections (i.v.), MELD values for AR-R 15896AR and AR-R 15035AR were similar: 58.5 [95% confidence limits = 40 to 74] and 59 [95% confidence limits = 50 to 66] mg/kg, respectively), while that for AR-R 15895AR was less (45 [95% confidence limits = 42 to 50] mg/kg). The resultant safety margin of 7.5 for AR-R 15895AR was almost one-third that observed for either AR-R 15896 and AR-R 15035AR (22.5 and 21.9, respectively), however, the ratios were not significantly different (Fieller's Theorem).

TABLE 7

Comparison of acute oral efficacy (MES, mg/kg) and safety (TD_{50}-inverted screen for mice and plank walk for rats) of [S]-AR-R 15896AR, [R]-AR-R 15895AR, and [R,S]-AR-R 15035AR to known antiepileptic drugs

Compound	Mice			Rats		
	MES ED_{50}	TD_{50}	TI	MES ED_{50}	TD_{50}	TI
[S]-AR-R 15896AR	17.3 [15–19]	193 [145–235]	11.2	3.7 [3–6]	933 [712–2361]	252
[R]-AR-R 15895AR	57.3 [49–69]	142 [80–226]	2.5	20.2 [10–35]	>1000	>49.5
[R,S]-AR-R 15035AR	17.5 [14–21]	245 [139–359]	14.0	10 [4–17]	>1000	>100
Carbamazepine*, **	13.0 [12–16]	137 [122–153]	10.2	4.0 [3–6]	571 [421–775]	143
Phenytoin**	16.8 [15–19]	234 [131–354]	13.9	13.3 [11–16]	>2000	>150
Valproate*, **	631 [522–757]	>2000	>3	261 [216–301]	578 [482–752]	2.2
Phenobarbital**	21.2 [19–25]	108 [93–125]	5.1	3.4 [2–5]	71 [63–80]	20.9
Lamotrigine	6.1 [5–7]	71.6 [49–98]	11.7	1.2 [1–2]	>60	>50
Felbamate	45.6 [43–53]	>1000	>22	26.7 [20–34]	>600	>22.5
Flunarizine	35.6 [30–42]	>500	>14	6.9 [5–10]	ND	ND

* Mouse data from Stagnitto et al. (1990).
** Rat data from Garske et al. (1991). Prior to testing for anticonvulsant efficacy or safety, mice and rats were pretreated with test compounds at 30 and 60 min, respectively (except flunarizine in rats the time of test was 5 h). ED_{50} and TD_{50} values are in mg/kg, values in brackets are 95% confidence limits.

PCP-like Actions. The three compounds were administered to rats in p.o. doses as multiples of the ED_{50} values from the MES test. Doses 20 times (AR-R 15035AR), 30 times (AR-R 15895AR), and 50 times (Hudzik et al., 1996, for previously published findings for AR-R 15896AR) the respective ED_{50} values produced no evidence of the five characteristic PCP-like behaviors (PCP behavioral scores were 0 out of a possible 25). Phencyclidine-dosed rats readily produced progressive behavioral signs: $1 \times ED_{50}$ value (score = 3 of 25); at $3 \times ED_{50}$ value (score = 8 of 25); at $6 \times ED_{50}$ value (score = 16 of 25); and at $10 \times ED_{50}$ value (score = 25 of 25). The previously determined ED_{50} value for PCP in the MES test was 1.24 mg/kg, p.o. (Hudzik et al., 1996).

Pharmacokinetics of AR-R 15896AR

Following an i.v. bolus dose (5 mg/kg) in the rat, the disposition of AR-R 15896AR was best described by a two-compartment model with a distributive phase that was complete within 2 h postdose (Fig. 4). The terminal half-life ($t_{1/2}$) was estimated to be 4.75 h with a plasma clearance of 0.64 liters/h/kg (Table 8). The compound was distributed into a moderate volume (V_1 = 1.89 liters/kg and V_{dss} = 3.97 liters/kg) indicating significant tissue disposition. In rat, the oral bioavailability following 5 mg/kg AR-R 15896AR was 83% with a C_{max} and t_{max} of 720 ng/ml and 1.05 h, respectively (Fig. 4, Table 8).

Following an i.v. bolus, AR-R 15896AR entered the brain rapidly and was in equilibrium with the plasma within 5 minutes after dosing (Fig. 5). The ratio of brain to plasma
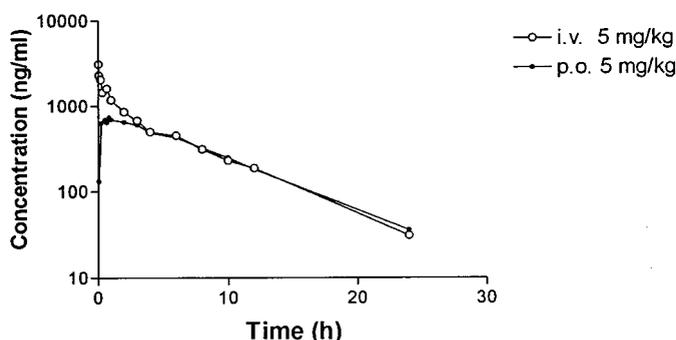
TABLE 8

Pharmacokinetic parameters in rats for AR-R 15896AR after a 5 mg/kg oral or i.v. dose

Parameter	AR-R 15896AR	
	i.v.	p.o.
Dose (mg/kg)	5	5
$T_{1/2}$ (h)	4.75	5.42
Cl (L/h/kg)	0.64	NC^a
V_1 (L/Kg)	1.89	NC^a
V_{dss} (L/Kg)	3.97	NC^a
t_{max} (h)	NA (immediate)	1.05
C_{max} (ng/ml)	NA (immediate)	720
AUC ($\mu g \times$ h/ml)	7.8	6.5
Bioavailability	assume 100%	83%

NA = not applicable; NC^a = not calculated.

Pharmacokinetic parameters were estimated using the nonlinear, least squares computer program PCNOLIN, version 4 (SCI Software, Statistical Consultants, Inc., Lexington, KY). Total drug concentration time data from the 5 mg/kg oral study was fitted to the one-compartment first-order input and output. Oral bioavailability (F) was calculated using the 5 mg/kg i.v. dose by:

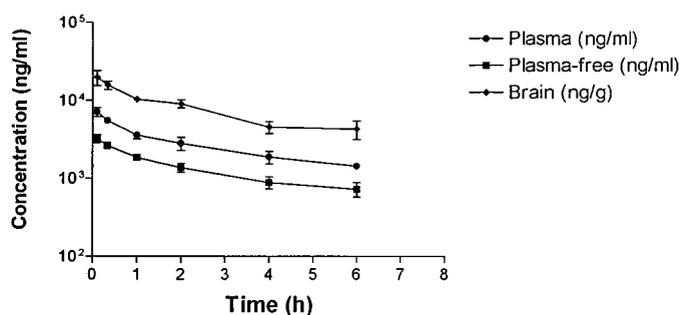$$F = (AUC_{p.o.}/AUC_{i.v.})(Dose_{i.v.}/Dose_{p.o.})$$

Fig. 5. The brain, plasma, and plasma-free kinetics of AR-R 15896AR in the rat after i.v. dosing of 10 mg/kg into the tail vein. The values are the mean concentration of compound ± S.E. from 3 rats per time point.

Fig. 4. The plasma kinetics of AR-R 15896AR in the rat after p.o. and i.v. dosing of 5 mg/kg. Plasma samples from 10 rats were pooled for each time point.

concentration (i.v. brain uptake index = 109%) and brain to plasma-free concentration remained constant over time.

In order to determine whether efficacy of AR-R 15896AR in the MES test was directly correlated with a predictable pharmacokinetic profile and whether differences between i.v. or p.o. dosing would be evident, a separate study was performed. Rats received either p.o. or i.v. injections of AR-R 15896AR and were evaluated for protection in the MES test

at 1 h or 3 h, respectively. The 1-h time point was selected for the p.o.-dosed rats because this time is near the t_{max} for AR-R 15896AR. For the rats dosed i.v., the 3-h time point was picked for testing because at this dose the plasma concentrations approximated the concentrations of AR-R 15896AR after an equal p.o. dose. The resultant concentrations of AR-R 15896AR in plasma and brain were proportional to the dose administered (Fig. 6). The dose-response curves were similar for both the i.v. and p.o. routes of administration. The calculated ED_{50} values were 3.3 mg/kg [95% confidence limits = 2.1 to 4.4] for p.o. dosing and 3.9 mg/kg [95% confidence limits = 1.7 to 6.2] for i.v. dosing (Fig. 7, top). In addition, the brain concentrations of drug over the range of doses administered were similar for both the i.v. and p.o. dosed animals (Fig. 7, bottom). The EC_{50} value for MES protection based on total brain concentration was approximately 25 μM.

Discussion

The present investigations with the low-affinity uncompetitive NMDA receptor antagonist, [*RS*]-AR-R 15035AR, described the acute preclinical in vivo studies using animal models of seizures and safety, as well as pharmacodynamic profiling that contributed to the identification and selection of the [*S*] enantiomer, AR-R 15896AR, over the [*R*]-enantiomer, AR-R 15895AR, as the most favorable candidate for further consideration as an antiepileptic drug.

Anti-seizure Efficacy. Uncompetitive NMDA receptor antagonists are potent anticonvulsants, especially in the
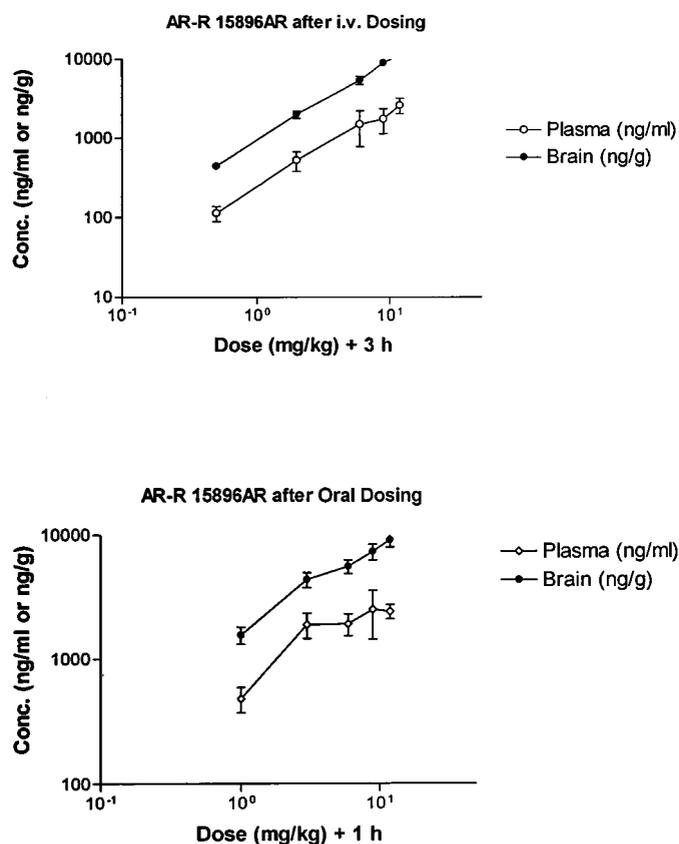
Fig. 6. Brain and plasma levels of AR-R 15896AR in rats sacrificed within 5 min after being tested for protection against MES. MES was administered 3 h after i.v. (top) and 1 h after p.o. (bottom) dosing (values are the mean ± S.E.; n = 10).
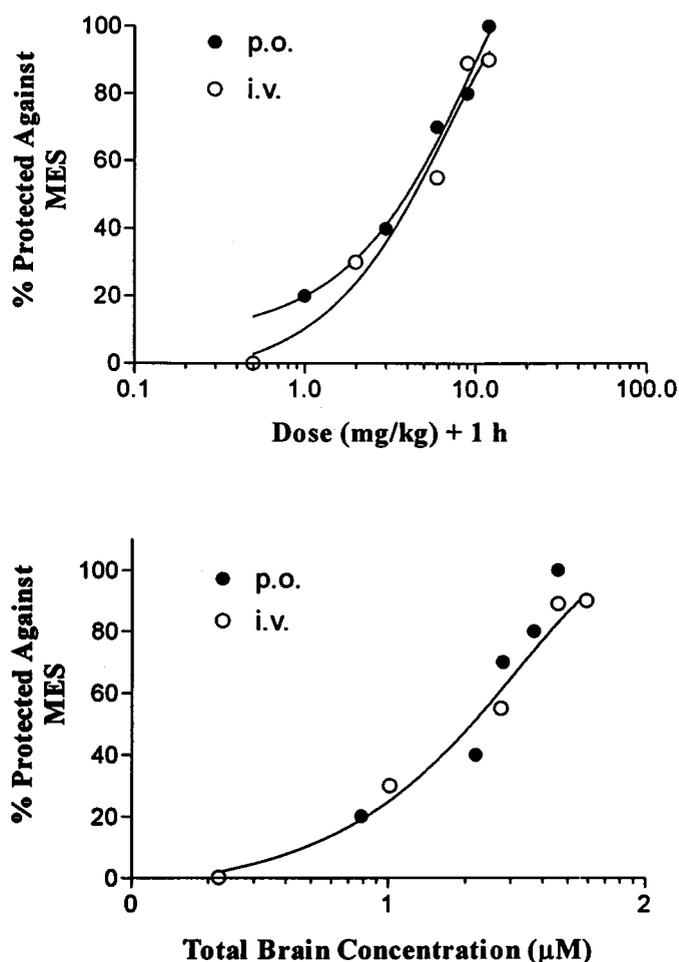
Fig. 7. Dose-response data for male rats for protection against MES-induced seizures (n = 10 rats/data point). The rats were tested 1 h after p.o. or 3 h after i.v. dosing. The calculated ED_{50} values were 3.3 mg/kg [95% confidence limits = 2.1 to 4.4] for p.o. and 3.9 mg/kg [95% confidence limits = 1.7 to 6.2] for i.v.-dosed animals (top). The EC_{50} value for MES protection based on total brain concentration was approximately 25 μM (bottom).

MES and NMDLA seizure tests (Czuczwar et al., 1985; Chapman et al., 1990; Rogawski et al., 1991; Rogawski, 1992; Bradford, 1995). These testing paradigms were used as rapid screens to evaluate efficacy of potential compounds previously identified from their respective receptor ligand binding profiles (displacement of labeled MK801) in rat synaptosomal preparations. The ability of AR-R 15035AR and respective isomers to prevent tonic hind limb extension in rodent seizure tests as a consequence of MES application or injections of 4-aminopyridine, bicuculline, and, to a lesser extent, strychnine, indicated the compounds act in part to suppress seizure spread. This phenomenon has been demonstrated with clinically useful drugs, namely, phenytoin, carbamazepine, phenobarbital, valproate, felbamate, lamotrigine, and flunarizine (Swinyard and Woodhead, 1982; Loscher and Schmidt, 1988; Kupferberg, 1989; see Table 7). The potency and acute safety profile for AR-R 15896AR, compared favorably to compounds currently in the clinic (comparisons of data generated at Astra Arcus USA are given in Table 7; see also Swinyard and Woodhead, 1982; Loscher and Schmidt, 1988; Kupferberg, 1989).

The action of a compound to suppress the phenomenon of

seizure spread has been attributed to an ability of an agent to limit neuronal firing at fast Na^+ channels (Macdonald and Kelly, 1995). This property is shared by uncompetitive NMDA receptor antagonists (Wamil and McLean, 1993). However, two studies namely, limitation of sustained repetitive firing and the measured rates of slow inactivation of Na^+ channels in isolated neurons in culture, have not demonstrated Na^+ channel activity for AR-R 15896AR (Palmer et al., 1996). Of further interest, the mechanism for seizure genesis by 4-aminopyridine is inhibition of sustained voltage-dependent K^+ channels and this activity may likewise promote the phenomenon of seizure spread (Yamaguchi and Rogawski, 1992). No electrophysiological studies have been conducted to date with AR-R 15896AR on neuronal K^+ channels.

Bicuculline, PTZ, and picrotoxin possess mechanisms of action associated with the neuronal γ-aminobutyric acid receptor/chloride channel. Injections of these compounds into mice produced clonic seizures (Loscher and Schmidt, 1988; Kupferberg, 1989), an event not prevented with AR-R 15896AR. Moreover, preliminary biochemical screening investigations did not indicate any affinity of the racemate, AR-R 15035AR, at central γ-aminobutyric acid or benzodiazepine receptors. AR-R 15896AR likewise did not prevent the clonic components of spinal seizures elicited by the glycine antagonist, strychnine (Swinyard and Woodhead, 1982). On the other hand, strychnine-induced tonic seizures and the ensuing mortality were prevented by rather large doses of AR-R 15896AR.

Pharmacokinetics. Predictable pharmacokinetic profiles are a desirable characteristic of a clinically useful anticonvulsant (Kupferberg, 1989). The pharmacokinetic evaluation of AR-R 15896AR did indeed reveal a favorable pharmacokinetic profile. Linear plasma levels of the compound were rapidly attained following either i.v. or p.o. administration in rats (Fig. 4). In fact, levels of AR-R 15896AR in rat brains exceeded that of plasma, whereas the ratio of brain-to-plasma concentration and brain-to-plasma-free concentration remained constant over time (Figs. 5 and 6). Moreover, there was a direct correlation between brain levels and efficacy of AR-R 15896AR in the MES test following either i.v. or p.o. dosing (Figs. 6 and 7), a result indicating a paucity of barrier mechanisms (absorption, distribution, excretion, first pass metabolism, etc.) limiting the bioavailability of p.o. administered AR-R 15896AR.

NMDA Receptor-mediated Events. The inhibition by AR-R 15896AR and its racemate, AR-R 15035AR, of NMDLA- and to a lesser extent kainate-elicited convulsions/subsequent mortality, provided a functional link to the original in vitro receptor ligand binding parameters for the compounds (Black et al., 1995). [*RS*]-AR-R 15035AR, [*S*]-AR-R 15896AR, and [*R*]-AR-R 15895AR all exhibited low affinities at the ion channel site of the NMDA receptor with K_i values of 3.5, 1.3, and 6.9 μM, respectively, for displacement of radiolabeled MK801 (MK801 $K_i = 0.014$ μM) (Black et al., 1995; see Palmer et al., 1992 for MK801 data).

In an incubated preparation of rat hippocampal slices, AR-R 15896AR limited the depolarizing actions of added NMDA or glutamate. In contrast, the [*R*]-isomer, AR-R 15895AR, was not active at concentrations as high as 300 μM (Palmer et al., 1996). Glutamate- or NMDA-triggered entry of Ca^{++} and the associated cell death in cultured rat cortical neurons were also prevented with addition of AR-R 15896AR

into the medium (Black et al., 1995, 1996). Cellular protection exhibited by AR-R 15896AR appeared to be specific for the NMDA receptor because in hippocampal cell cultures, cell death was not evident following exposure to either kainic acid or AMPA (α-amino-3-OH-5-methyl-4-isoxazole propionate) (Palmer et al., 1996). Therefore, the in vivo and in vitro findings described above indicate that the primary mechanism of anticonvulsant action for the compound is attributed to uncompetitive antagonism at the NMDA receptor.

Kindling. Glutamate is responsible for at least three phenomena associated with experimental epilepsy, namely, seizure initiation, seizure spread, and seizure kindling (Bradford, 1995). According to Bradford (1995), there appears to be a chronic tendency to an exaggerated release of glutamate occurring in the kindled epileptic focus during both epileptogenesis and full seizure expression. In general, uncompetitive NMDA receptor antagonists, including AR-R 15896AR (present study), are not markedly effective regarding inhibition of fully established kindled seizures but have been demonstrated to retard the development of seizures during the kindling process, an action also shared with valproate (present study), and clonazepam (McNamara et al., 1988; Rogawski et al., 1991; Palmer et al., 1992, 1995, 1998; Applegate et al., 1997). Even though both doses of valproate retarded the development of the kindling process, it was more active at the highest concentration representing three times the ED_{50} value for efficacy in the MES test; and at this dose, side effects were evident. Moreover, at this dose alone valproate inhibited established kindled seizures, whereas phenobarbital was highly effective at the $3 \times ED_{50}$ dose. It's unlikely that AR-R 15896AR would be effective at doses higher than 40 mg/kg (10 times the ED_{50} value) because in an earlier study with febrile convulsions in rats, the low-affinity uncompetitive NMDA receptor antagonist, remacemide hydrochloride, was ineffective at doses as high as 24 times the ED_{50} value for the MES test (Palmer et al., 1998).

In the present experiments, AR-R 15896AR affected the initial two processes of kindling, namely, seizure initiation (NMDLA-induced convulsions) and seizure spread (MES) with no effect on generalized forms of kindling (e.g., bicorneal stimulation or heat stress). It has not been determined whether AR-R 15896AR affects kindling via stimulation of a specific locus such as the amygdala or the hippocampus. Development of this type of kindling has been shown to be inhibited by other low-affinity NMDA antagonists, such as ADCI (Rogawski et al., 1991) and remacemide hydrochloride (Palmer and Hutchison, 1997).

Safety Issues. Safety as been the major problem with development of effective therapy to treat epilepsy (Kupferberg, 1989). Moreover, in the present study AR-R 15896AR exhibited a remarkable safety profile in the rat when compared with clinically useful compounds (see Table 7). Clinical trials for epilepsy using higher affinity uncompetitive NMDA antagonists, MK801, and dextromethorphan were discontinued because of unacceptable psychotomimetic effects (Willetts et al., 1990; Rogawski, 1992; Muller et al., 1995; Grant et al., 1996). Neither AR-R 15035AR nor its respective [*R*]- and [*S*]-enantiomers, at doses an order of magnitude or higher than their respective MES ED_{50} values, produced acute PCP-like behaviors in rats. In addition, AR-R 15896AR was not proconvulsant (present study) and had no effect on

learning acquisition (Hudzik and Palmer, 1995) or motor behaviors (Palmer et al., 1996). In contrast to PCP and cocaine, rats did not self-administer AR-R 15896AR (Hudzik et al., 1996). In the present evaluations for acute safety, the therapeutic indices for AR-R 15896AR compared favorably to antiepilepsy drugs currently in the clinic (Table 7).

Conclusions. [*S*]-AR-R 15896AR has proven thus far to be a safe, low-affinity uncompetitive NMDA receptor antagonist with a favorable pharmacokinetic profile that exhibited efficacy in animal models of epilepsy. Extensive investigations with various animal models of stroke/injury, including focal and global ischemia, revealed neuroprotection by AR-R 15896AR (Green et al., 1996; Palmer et al., 1996; Cregan et al., 1997). The successful outcome of these studies led to the decision to proceed with a 30-day toxicology evaluation and eventually into Phase I clinical trials in which acute i.v. doses up to 160 mg were well-tolerated in humans (Palmer et al., 1996). Phase IIa clinical trials for treatment of acute stroke indicated the compound was well tolerated (unpublished findings). Phase IIb stroke trials are ongoing. The present experiments describing the anticonvulsant efficacy, acute safety, and pharmacokinetic properties of AR-R 15896AR support continued development of the compound as a possible candidate to treat epilepsy.

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