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Small Molecule Anticonvulsant Agents with Potent In Vitro Neuroprotection

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Abstract

Severe seizure activity is associated with recurring cycles of excitotoxicity and oxidative stress that result in progressive neuronal damage and death. Intervention to halt these pathological processes is a compelling disease-modifying strategy for the treatment of seizure disorders. In the present study, a core small molecule with anticonvulsant activity has been structurally optimized for neuroprotection. Phenotypic screening of rat hippocampal cultures with nutrient medium depleted of antioxidants was utilized as a disease model. Increased cell death and decreased neuronal viability produced by acute treatment with glutamate or hydrogen peroxide were prevented by our novel molecules. The neuroprotection associated with this chemical series has marked structure activity relationships that focus on modification of the benzylic position of a 2-phenyl-2-hydroxyethyl sulfamide core structure. Complete separation between anticonvulsant activity and neuroprotective action was dependent on substitution at the benzylic carbon. Chiral selectivity was evident in that the *S*-enantiomer of the benzylic hydroxy group had neither neuroprotective nor anticonvulsant activity, while the *R*-enantiomer of the lead compound had full neuroprotective action at 40 nM and antiseizure activity in three animal models. These studies

indicate that potent, multifunctional neuroprotective anticonvulsants are feasible within a single molecular entity.

Keywords

Neuroprotection; Glutamate toxicity; Oxidative stress; Hippocampal cultures; Epilepsy; Anticonvulsant

Introduction

Although effective anticonvulsant drugs have been available since the early 1900s, the single most significant unmet medical need remains disease modification to slow or stop the progression of seizure disorders. No existing commercial antiepileptic drug is disease-modifying. A widely proposed solution to this therapeutic need is that neuroprotection from seizure-related neural damage may be a key strategy for disease modification (Acharya et al. 2008). To date, this strategy has lacked an effective probe compound to test this hypothesis: a highly potent neuroprotective anticonvulsant. In the present study, the goal was to explore the possibility of devising a “proof of concept” small molecule that would exhibit both broad-spectrum anticonvulsant activity and high potency neuroprotection against toxic processes that are relevant to the pathology of seizure disorders.

The rationale for treating epileptogenesis through neuroprotection resides in our understanding of the multiple factors that contribute to neuropathology in this disease (Bengzon et al. 2002). These factors include genetic mutations, glutamate-induced excitotoxicity, mitochondrial dysfunction, oxidative stress, growth factor loss, and increases in cytokine concentration (Ferriero 2005). Further, intense seizure activity produces large increases in NMDA-mediated calcium influx (Van Den Pol et al. 1996). High levels of calcium lead to apoptotic cascades that result in acute neuronal cell death. Elevated calcium levels can also generate reactive oxygen species that can produce cell damage and death. With this background in mind, our neuroprotective anticonvulsant program focuses on structural optimization to provide potent protection from excitotoxicity and oxidative stress.

Previous studies employing animal models of seizure disorders have indicated neurodegenerative and behavioral abnormalities result from treatment with proconvulsant agents. For example, the cholinomimetic convulsant pilocarpine induced a status epilepticus that was accompanied by hippocampal damage and the development of spontaneous, recurrent seizures (Muller et al. 2009). In this epileptic model, increases in anxiety-related behavior and impaired performance on learning and memory tests were observed. Intrahippocampal injections of kainic acid produce both neural damage and alteration in cognitive performance (Groticke et al. 2008). Further, pentylenetetrazol kindling has been used to produce a convulsive component of epilepsy that resulted in impairment of learning of memory tests which included social discrimination, acoustic fear conditioning, water maze, and passive avoidance (Jia et al. 2006). Studies in human seizure disorder have shown that chronic uncontrolled epilepsy can result in neurodegeneration to specific brain areas (Houser 1992). Because seizure disorders have been associated with these indications of neural deficits and accompanying behavior problems, the concept has emerged that neuroprotection from these events might lead to disease modification to either slow or stop the progression of the disease.

The core compound selected for these exploratory studies was AND-171, a molecule that included a sulfamide moiety combined with features of a recently described anticonvulsant that had broad activity in animal models coupled with low-potency (mM) neuroprotection

(Deshpande et al. 2008). The fundamental choice of the sulfamide functionality was supported by reports on anticonvulsant compounds that indicated a sulfamide group with *N,N'*-disubstitution or *N,N,N'*-trisubstitution (Aeberli et al. 1967; Gavernet et al. 2007). Further, they reported that two primary sulfamides, PhCH₂NHSO₂NH₂ and BuNHSO₂NH₂, had anticonvulsant activity. Additionally, other groups have described anticonvulsant activity associated with sulfamides (Thiry et al. 2008; Parker et al. 2009). Initial testing of the core compound indicated both anticonvulsant activity and 30–100 nM potency for neuroprotection. Based on these initial findings, the present study was undertaken to explore this chemical scaffold and to optimize for neuroprotective anticonvulsant activity.

In the course of our work, we have discovered anticonvulsant agent AND-287 that can completely protect hippocampal neurons from excitotoxicity and oxidative stress with high potency, showing marked enantiomeric selectivity of action. We have developed a series-specific pharmacophore model that describes anticonvulsants that exhibited neuroprotection vs those that do not. These studies may be useful in the design of further optimized neuroprotective anticonvulsants.

Materials and Methods

Materials

The following reagents were obtained from Sigma-Aldrich (Milwaukee, WI): L-glutamic acid monosodium salt monohydrate (49621); hydrogen peroxide solution, 30 wt% (216763); propidium iodide solution (1.0 mg/mL in water; P4864); and 6-carboxyfluoresceine diacetate (CFDA, C5041).

Chemical Synthesis and Structure Verification

All reagents used in chemical synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI), Alfa Aesar (Ward Hill, MA), or Thermo Fisher Scientific (Pittsburgh, PA) and were used without further purification. ¹H-NMR spectra were obtained on a Varian Mercury 300-MHz NMR spectrometer. Chemical shifts were reported in parts per million (δ) using various solvents as internal standards (CDCl₃, δ 7.26 ppm; DMSO-d₆, δ 2.50 ppm). ¹H-NMR splitting patterns were designated as singlet, doublet, triplet, or quartet. Splitting patterns that could not be interpreted or easily visualized were recorded as multiplet or broad. Coupling constants were reported in hertz. UV Purity and electrospray mass spectral data were determined using a Waters Alliance 2695 HPLC/MS (Waters Symmetry C18, 4.6×75 mm, 3.5 μm) with a 2,996 diode array detector from 210 to 400 nm. All compounds were determined to be >95 % pure by HPLC at 225 nm. Enantiomeric purities were determined by R/S Tech-Prob Solutions, Palm Beach Gardens, FL using an Agilent 1200 HPLC equipped with a 25-cm×4.6-mm Chiralpak AS-H column with an isocratic mobile phase of heptane (with 0.1 % TFA)/2-propanol (80:20), monitored at 254 nm.

Synthesis of 2-Hydroxy-2-arylethylsulfamides and 2-Aryl Ethylsulfamides

The synthetic details are summarized in Scheme 1. Aryl aldehydes **1** were treated with trimethylsilyl cyanide in the presence of zinc chloride followed by reduction with borane to give the 2-hydroxy-2-aryl ethylamines as their hydrochloride salts **2**, which were treated with tert-butyl (chlorosulfonyl) carbamate, formed in situ by the reaction of chlorosulfonyl isocyanate with tert-butyl alcohol, to give N-Boc-protected sulfamides **3**. Intermediates **3** were then deprotected under acidic conditions to afford 2-hydroxy-2-arylethylsulfamides **4**. The 2-aryl ethylsulfamides **6** were prepared by heating the corresponding 2-arylethylamines **5** with sulfamide in 1,4-dioxane to give the desired products. Single enantiomers AND-287 and AND-286 were prepared from AND-171 as shown in Scheme 2. 2-(2-Fluorophenyl)-2-

hydroxyethylsulfamide AND-171 was coupled with (*S*)-methoxyphenylacetic acid using standard coupling conditions to give the corresponding diastereomeric pair of esters (**7**, **8**) which were separated by chromatography. The diastereomeric esters were then saponified with lithium hydroxide to give the desired sulfamides AND-287 and AND-286, whose enantiomeric excesses (e.e.) were determined to be 97.6 and >99.9 %, respectively. The absolute stereochemistry of AND-287 was determined to be (*R*) by X-ray crystallography.

Culture Model

Dissociated hippocampal cultures derived from embryonic day 18 rats were employed as the primary screening system to test for toxicity responses as well as neuroprotective actions. This cellular system was chosen because a limited number of compounds were to be tested thereby permitting the use of a low throughput screening system that is derived from a brain area that is highly susceptible to seizures. In brief, hippocampal tissue was obtained commercially through Brain Bits (Springfield, IL) and cultures prepared with slight modifications to methods previously described (Brewer et al. 1993). Tissue was dissociated with a papain-based kit from Worthington Biochemical Corporation (Lakewood, NJ) that is modeled after the method described by Huettnner and Baughman (1986). The hippocampal neurons were plated at low density (10,000 cell/well) in a 96-well format and maintained in serum-free medium consisting of neurobasal medium supplemented with B27 and GlutaMAX (Gibco). Precoated poly-L-lysine-coated plates (BD Biosciences, Franklin Lakes, NJ) were used because of the preferential adherence and survival of neurons on this matrix support. Prior to the initiation of experiments between days 11 and 21 in vitro, a complete change of medium was performed in a working volume of 100 μ L. This medium differed from the medium utilized for plating in that the B27-AO did not contain the five antioxidants present in the standard B27 (Gibco, product #10889). This change of medium was designed to decrease the background levels of antioxidants to facilitate the damage and death with glutamate or hydrogen peroxide treatment. The rationale for this change of medium was twofold: (1) because loss of antioxidant control may also be a component of epileptogenesis (Waldbaum and Patel 2010; Wu et al. 2010); and (2) because the goal was to obtain a significant and reproducible toxicity signal in hippocampal neurons. Thus, this decrease in the background antioxidant concentration was used to recapitulate a disease model for epilepsy as well as a means to produce neurotoxicity at the lowest concentration of toxin. Both the amount of glutamate and hydrogen peroxide used in the assays, as well as the time of treatment and duration of the experiment were designed to be relevant to the disease. Further, all time parameters employed in these studies were empirically determined to be within the limits of reversible toxic events. A critical feature in the glutamate studies was the duration of treatment of the hippocampal neurons. The rationale for using a 5-min treatment with glutamate was the previous observation that indicated 5 min glutamate exposure produced significant toxicity (Choi et al. 1987) as well as a demonstrated delay of increased intracellular calcium that overloaded neurons and produced cell death (Randall and Thayer 1992). The amount of glutamate employed in our screening (30 μ M) was selected for two reasons: (1) because this concentration was the basal level of glutamate observed in microdialysis measurements of the hippocampus from epileptogenic patients (Cavus et al. 2008) and (2) glutamate at this concentration produced a reliable and reproducible toxicity that could be prevented by pharmacological intervention in hippocampal cultures. The concentration of hydrogen peroxide employed (10 μ M) was also selected because this level was detected in the hippocampus of rats after kainate-induced status epilepticus (Jarrett et al. 2008).

Neuroprotection from Oxidative Stress

All the experimental compounds were dissolved to 10 mM stock solutions in Dulbecco's phosphate-buffered saline (DPBS; Sigma D-5780) prior to testing. To evaluate the

compounds for neuroprotection from hydrogen peroxide, day 11 hippocampal cultures were given a complete change of medium containing 100 μ L of neurobasal medium with B27 that contained no antioxidants. Twenty-four hours after the change in medium, the neuroprotective studies against hydrogen peroxide were started. The test compounds were added to the hippocampal cultures for a 4-h test period in concentrations that ranged from 1 pM to 300 μ M with eight replications. Immediately after treatment with test compound, 10 μ M of hydrogen peroxide was added for the 4-h test period.

Neuroprotection from Excitotoxicity

For the neuroprotection studies using glutamate excitotoxicity, several modifications were made from the method described for the hydrogen peroxide assay. For the glutamate neuroprotection assay, day 19 hippocampal cultures were given a complete change of medium containing 100 μ L of neurobasal medium with B27 that contained no antioxidants. Twenty-four hours after the change in medium, the glutamate neuroprotection studies are started. The day 20 cultures were treated for 5 min with 30 μ M glutamate dissolved in DPBS. For this treatment, a 900- μ M solution of glutamate was prepared and then 3.3 μ L of this solution added to the culture well containing 100 μ L of media. After this acute treatment, the medium containing the glutamate was removed from the cultures and fresh medium without antioxidants was added. The test compound was then added to the hippocampal cultures for a 4-h test period in concentrations that ranged from 1 pM to 300 μ M, with eight replications per concentration tested.

Fluorescence-Based Assays

At the conclusion of the test period, the cultures were evaluated with fluorescent dye-based assays for cell death (propidium iodide) and for neuronal viability (CFDA). These two standard assays were chosen because they could be measured as multiplexed determinations within a single well thereby monitoring both an increasing (cell death) parameter and a decreasing parameter (neuronal viability) after a toxic treatment of the hippocampal cultures. For the cell death assessment with the propidium iodide, slight modification to a method previously described was used (Sarafian et al. 2002). Propidium iodide (PI) stock solution of 1 mg/mL (1.5 mM) was diluted 1:30 in DPBS for a final working concentration of 50 μ M. After removal of the growth medium, 50 μ L of the 50 μ M PI solution was added to the cultures and allowed to incubate in the dark at room temperature for 15 min. On every plate, the wells without cells were used to provide a blank reading that was used to subtract background fluorescence. The cultures were assessed for fluorescence intensity at Ex536/Em590 nm in a CytoFluor fluorimeter (Perceptive Biosystems). Results were expressed in relative fluorescent units and EC₅₀s calculated from the dose-responses of the test compound and compared to values obtained from controls and wells treated with toxin alone.

After the assessment of cell death, cultures were then further assayed for neuronal viability by the CFDA method (Petroski and Geller 1994). For the neuronal viability assay, 1 mg of CFDA dye was dissolved in 100 mL of DPBS (Gibco D-5780) and kept in the dark until added to the hippocampal cultures. After a complete change of medium to rinse the cultures after the cell death assay, 100 μ L CFDA dye solution was added for 15 min of incubation at 37°C in the dark. At the conclusion of the incubation period, the dye was removed from the cultures and washed once with 100 μ L of DPBS. After removal of the first wash, a second wash of DPBS was added to the culture and then incubated for 30 min to allow the efflux of dye out of the glia in the cultures. At the conclusion of the 30-min efflux period, the culture efflux medium was removed and 100 μ L of 0.1 % Triton X-100 in water was added to the cultures before reading at Ex490/Em517 in a CytoFluor fluorimeter. Results were expressed in relative fluorescent units and EC₅₀s calculated from the dose-response of the test

compound. All statistical comparisons were made by ANOVA, with normality of values tested by the Shapiro–Wilk test followed by a multiple comparison of means test with the Holm–Sidak method as performed through SigmaPlot (v. 11).

Metabolic Stability Assay

The stability of test compound was determined by incubation with liver microsomes derived from rat, mouse, or human. All assays were conducted by Absorption Systems (Exton, PA) by methods previously described (Clarke and Jeffrey 2001). Test compound at 1 μ M was assayed at various times of incubation up to 60 min. Samples were analyzed using a LTQ–Orbitrap XL mass spectrometer. The assay was a single determination at each time point. As a metabolically labile control, 1 μ M testosterone was also tested.

Caco-2 Permeability Assay

The human colon adenocarcinoma cell line Caco-2 has been widely used as an in vitro absorption model. Caco-2 in vitro permeability correlates well with human drug absorption (Artursson et al. 2001). Bidirectional cell permeability was estimated from Caco-2 cells by methods described in a commercial protocol by Absorption Systems (Exton, PA). Assays were performed in duplicate in 25-day-old cultures, and test compounds were tested at 5 μ M using LC-MS/MS to determine concentrations.

Antiseizure Testing

All in vivo efficacy testing was conducted by the Anticonvulsant Screening Program of the National Institute of Neurological Disease and Stroke at the National Institutes of Health. As previously described (White 2002), compounds were evaluated in a series of antiseizure tests that are highly predictive of efficacy in human epilepsy. This testing began with the maximal electroshock test (MES) that is conducted in both mice and rats by methods previously described (Swinyard 1969; Rowley and White 2010). The route of administration was by intraperitoneal injection for the mouse MES and by oral gavage for the rat MES test. The MES testing was followed by the 6-Hz seizure test that further evaluated compounds in this model of psychomotor seizures (Barton et al. 2001). This model was used to detect seizures that may be useful for the treatment of therapy-resistant partial seizures. As a third test in these initial screens, the subcutaneous pentylenetetrazol (scPTZ) test was used as a model to identify compounds that raise seizure threshold (Swinyard 1969). For this model, the amount of test compound required to protect against threshold seizures (5 s of clonic activity) induced by subcutaneous injection of PTZ (85 mg/kg) was determined.

Results

In Vitro Neuroprotection

Neuroprotection from toxicity associated with glutamate or hydrogen peroxide treatment of hippocampal cultures was used as a primary screen to evaluate analogs of AND-171. Two multiplexed assays were used to assess cell culture viability: a PI assay used to estimate changes in the amount of cell death and a CFDA assay used to estimate changes in neuronal viability. With these multiplexed assays that were measured in the same culture well, neuroprotection was observed as an increase in fluorescence for CFDA and a decrease in fluorescence for the PI assay. Representative experiments for the most potent compound (AND-287) of this chemical series against glutamate toxicity are shown in Fig. 1a for the CFDA and PI assays. For experiments conducted in day 20 cultures, the 30- μ M glutamate treatment produced a decrease in the CFDA fluorescence to 64 ± 2 % of that for control cultures. This decrease in neuronal viability signal was contingent on the removal of antioxidant substances in the growth medium. With the PI assay, the increase in

fluorescence associated with cell death after treatment with 30 μM glutamate was 182 ± 10 % of that observed with control cultures. As shown in Fig. 1a, EC_{50}s for AND-287-mediated neuroprotection as determined by the two viability assays were very similar, 3–4 nM. With both assays, AND-287 treatment resulted in changes in fluorescence levels that were not different from that of control cultures (see dashed lines for respective control levels), indicating full efficacy protection under these conditions.

Representative experiments for the effects of AND-287 on neuroprotection from hydrogen peroxide are shown in Fig. 1b. For these experiments conducted in day 13 cultures, the 10- μM hydrogen peroxide treatment produced a decrease in the CFDA fluorescence to 67 ± 3 % of that for control cultures, very similar to the toxic signal observed with 30 μM glutamate in day 20 cultures (Fig. 1a). With the PI assay, the increase in fluorescence associated with cell death after treatment with 10 μM hydrogen peroxide was 202 ± 5 % of that observed with control cultures. AND-287 also exhibited potent neuroprotective activity hydrogen peroxide toxicity in the day 13 hippocampal cultures, with the dose–response shifted approximately tenfold to the right, with EC_{50}s of 30–40 nM observed from the two assays.

A summary of the multiplexed assays for neuroprotection against both 30 μM glutamate- and 10 μM hydrogen peroxide-associated toxicity for the compounds reported here is shown in Table 1. Of the 16 compounds synthesized, five were shown to exhibit significant neuroprotective activity in one or more of the assays. Racemic compound AND-171 was the core antiseizure compound for this study, and it exhibited significant neuroprotective activity in three of the four assays. A major finding of the present study was that the *R*-enantiomer (AND-287) of the core compound exhibited highly potent neuroprotective activity in all assays whereas the *S*-enantiomer (AND-286) did not exhibit any detectible neuroprotection up to the maximum tested concentration (100 μM). Thus, marked stereoselectivity was demonstrated for AND-287. Compared to the racemic compound (AND-171), the *R*-enantiomer was about tenfold more potent for neuroprotection against glutamate toxicity. Similar comparative studies for neuroprotection from hydrogen peroxide-mediated toxicity indicated at 3-fold increase in potency with the *R*-enantiomer vs the racemate. This bias is also seen for the *R*-enantiomer over the *S*-enantiomer for the 2-chloro analogs, AND-343 and AND-342, respectively.

In regard to the aryl substitutions of the *R*-enantiomers, it was apparent that the 2-fluorophenyl group was significantly more potent than the 2-chlorophenyl group (287 vs 343). Low-potency neuroprotection was observed with benzothiophene compounds 115 and 176. In general, these data indicate a dependence on the hydroxyl group at the benzylic carbon for neuroprotection, as any compound that did not have this component was not active.

In Vivo Anticonvulsant Activity

A summary comparison of anticonvulsant activity for study compounds is presented in Table 2. All of the animal evaluation for antiseizure activity was performed by the Anticonvulsant Screening Program of NINDS/NIH. In general, the most potent of the neuroprotective compounds were utilized to test for antiseizure activity. The core antiseizure compound, AND-171, exhibited a modest activity in the maximal electroshock model in both rats and mice. Similarly, this compound had only modest activity in the 6-Hz test and had no demonstrated activity in the mouse pentylenetetrazol model at 300 mg/kg i.p. However, the *R*-enantiomer (AND-287) of the core compound exhibited antiseizure activity in three of four assays, whereas the *S*-enantiomer (AND-286) had no demonstrable antiseizure activity in any of the animal models. Indeed, AND-287 had better activity in both the MES and 6-Hz tests as compared to the racemate AND-171. Importantly, AND-319, a compound without substitution at the benzylic carbon, had the best antiseizure

profile of all the study compounds, with all four models of antiseizure activity showing activity at similar ED₅₀s. None of these neuroprotective compounds were active in the mouse pentylenetetrazol model.

The time courses of antiseizure activity for two key compounds were compared in mice and rats for the MES test as shown in Fig. 2a, b. For this comparison, the most potent neuroprotective compound (AND-287) was compared with the best anticonvulsant compound that had no demonstrable neuroprotective activity (AND-319). For the mouse MES study shown in Fig. 2a, compounds were administered i.p. to measure their time to peak effect. The doses for the time to peak studies were chosen to be within the amounts that provided full protection and no apparent rotarod impairment. For AND-287, the time to peak effect was at 1 h as compared to 15 min for AND-319. Further, the neuroprotective anticonvulsant had a longer duration of action than that observed for non-protective anticonvulsant. The comparison of the time to peak effect for the MES test in the rat after oral administration is shown in Fig. 2b. In rats, the time to peak effect was 2 h for AND-287 and 30 min for AND-319. While the relative time to peak effect was similar to that of mice, the duration of action was much longer for the non-protective anticonvulsant (AND-319) as compared to the neuroprotective compound. In contrast to the dependence of neuroprotective activity on the benzylic carbon substituent, it is clear that anticonvulsant activity is not contingent on this structural feature.

Ancillary Pharmacology

In addition to measures of neuroprotective and anticonvulsive efficacy, several tests of early absorption, distribution, metabolism, and elimination (ADME) properties were conducted for the most potent and efficacious neuroprotective anticonvulsant of this chemical series: AND-287. These tests included: (1) estimating bidirectional permeability in the human colon adenocarcinoma cell line Caco-2 as an in vitro absorption model and (2) determination of metabolic stability in liver microsomes from various species. As shown in Table 3, AND-287 had high permeability in this absorption model, with no significant efflux. Known agents that act as positive controls were also tested, indicating that the model system was fully functional.

Because drug metabolism can be a significant factor in the agent evaluation process, the stability of AND-287 in liver microsomes from human, rat, and mice are compared in Fig. 3. In these experiments, the stability of the compound was monitored by mass spectrometry analysis over a time course of 1 h of incubation. As indicated, AND-287 was highly stable in this test, with >80 % of the compounds remaining unchanged during the test period with microsomes from all three species. Testosterone acted as positive control for this test, indicating that the model system was fully functional.

To further evaluate the best compound of the study (AND-287) and the anticonvulsant that was devoid of neuroprotective activity (AND-319), studies were conducted to provide an early assessment of the toxicological properties of these compounds by comparing their effects in a behavioral paradigm of an adverse effect: impairment on the rotarod test. As shown in Fig. 4, a comparative analysis of these compounds is shown with a dose-response measured 15 min after intraperitoneal injection. With a no effect dose for AND-319 being 88 mg/kg i.p., a progressive increase in the percentage of animals impaired was evident that reached an observed maximum at 300 mg/kg. Further observations conducted over a 4-h period at a dose of 300 mg/kg indicated continued impairment, with 25 % of the mice tested still exhibiting an inability to grasp the rotarod (data not shown). In contrast, there was no evidence of rotarod impairment for AND-287 from 125 to 500 mg/kg i.p. at 15 min. Further, there was no evidence of a delay in impairment of rotarod performance in mice administered 500 mg/kg i.p. and tested at 30 min, 1, 2, 4, 8, and 24 h (data not shown). These data

indicate a significant difference in the response between the neuroprotection anticonvulsant (AND-287) and its related non-neuroprotective anticonvulsant (AND-319), with the former showing no impairment up to 500 mg/kg i.p. over a 24-h period and the latter showing significant impairment lasting from 15 min to 4 h.

Discussion

Epilepsy is a common chronic neurological condition that affects at least 50 million people worldwide. It has been estimated that 25 % of people suffering from epilepsy receive no effective treatment for their seizures from available drugs. Further, no currently marketed antiepileptic drug has been shown to be antiepileptogenic, indicating that the available therapies do not address the underlying causes of the disease, only the symptomatic effect of seizures. The long-term purpose of our studies was to develop new antiepileptic drugs that are both effective in treating patients which are refractory to current treatments and that are disease-modifying by addressing the long-term causes of reoccurring cycles of seizure episodes. Our underlying hypothesis is that neurotoxicity associated with elevated glutamate and oxidative stress are at the center of the neural damage (Bonilha et al. 2010; Proper et al. 2000) and the neuronal cell death (Mathern et al. 1995) that contributes to the neuropathology and etiology of epilepsy. Neuroprotection from these damaging and cyclical processes was the strategy for discovering disease-modifying agents, along with the possibility of providing an alternative strategy for seizure control through multiple mechanisms. With these unmet medical needs in mind, the central concept was to discover a proof of concept agent that provided both broad-spectrum antiseizure activity and potent neuroprotection from excitotoxicity and oxidative stress within a single molecular entity.

Commercial antiepileptic drugs have been reported to have neuroprotective properties as summarized in a review of progress for neuroprotective strategies in preventing epilepsy (Acharya et al. 2008), which suggests that the extent of the neuroprotective and antiepileptogenic effects of current individual antiepileptic drugs is still unclear in part due to a lack of long-term animal studies as well as clinical trials to fully evaluate the impact of putatively neuroprotective compounds. Among the drugs with reported low-potency neuroprotective properties are valproate (Yamauchi et al. 2008), topiramate (Noh et al. 2006), felbamate (Longo et al. 1995), gabapentin (Cilio et al. 2001), lamotrigine (Papazisis et al. 2008), carbamazepine (Costa et al. 2006), and vigabatrin (Andre et al. 2001). In all cases, the neuroprotection was apparently incidental to the antiseizure properties in the chemical design of the anticonvulsants. The end result has been that anticonvulsants in general possess low-potency neuroprotective activity. In the present study, optimization of neuroprotective activity was the primary goal and design strategy for the antiseizure molecule.

With neuroprotection set as the defining strategy for the anticonvulsant lead, the central objective of all neuroprotective assays was their relevancy to excitotoxicity and oxidative stress. Both the amount of glutamate and hydrogen peroxide used in the assays, as well as the time of treatment and duration of the experiment, were designed to be relevant to the disease. Time parameters employed in these studies were empirically determined to be within limits (4 h) that would permit preventable amounts of neural damage and death. To produce neural damage and death with these amounts of glutamate and hydrogen peroxide, the cultures were changed to a medium with significant depletion of antioxidant components in the defined medium supplement B-27. This was performed to obtain a significant and reproducible toxic signal in the hippocampal neurons and because loss of antioxidant control may be a component of epileptogenesis (Waldbaum and Patel 2010; Wu et al. 2010). In particular, this removal of medium antioxidants was hypothesized to be a disease model for epilepsy.

With excitotoxicity associated with excessive glutamate as a focus for epilepsy-related damage and death, the details of neuroprotection from this toxic process are important to the rationale of the screening process. A critical feature of the glutamate-related studies was the duration of exposure to the hippocampal neurons. The rationale for using a short 5-min exposure with glutamate was based on the observation of Randall and Thayer (1992). This short-term incubation with glutamate produced a delayed but substantial increase in intracellular calcium that overloaded neurons and produced cell death. This intense burst of glutamate and resulting calcium overload may be relevant to seizures and therefore represents an important feature to be captured in in vitro assays.

A clear advantage for drug discovery against seizure disorders is the demonstrated validity of animal models that are predictive of efficacy for various types of epilepsy in humans (Rowley and White 2010). This in vivo advantage is particularly important in that validated molecular targets which permit screening of compounds in vitro for antiseizure properties remain elusive. Based on the predictive quality of the maximal electroshock model, it is likely that the core compound AND-171 and the lead compound AND-287 would be active against generalized tonic-clonic seizures (Swinyard 1969). Although AND-287 was not active in the pentylenetetrazol test, a model that identifies compounds that raise seizure threshold, this compound was active in the 6-Hz psychomotor test, a model that predicts activity against complex partial seizures. Further, because the 6-Hz test possesses a differential pharmacological profile in comparison to the MES and scPTZ tests, this screen may be useful in identifying compounds that could be useful for pharmacoresistant epilepsy (Rowley and White 2010). Additional testing will be required to determine if the potent neuroprotective compound AND-287 has such utility for the treatment of refractory epilepsy.

The present study does allow for some structure-activity relationships to be drawn for the novel core structure and related homologues. More importantly, a simple pharmacophore model for the neuroprotective anticonvulsants reported here is shown in Fig. 5. In this model, there are three design elements: (1) an aryl modulatory component that mediates potency, stability, and efficacy; (2) benzylic carbon substitution that is critical for neuroprotection and bioavailability; and (3) the sulfamide message that is important for both anticonvulsant and neuroprotective activities. In regard to the aryl component, data on the preference of F vs Cl in the two positions were evident from our studies in regard to neuroprotective potency. In comparison the responses of AND-343 and AND-287, about a 1,000-fold increase in potency was observed by the propidium iodide assay after glutamate treatment. This preference for F vs Cl substitution was evident across all assays for compounds containing the sulfamide and the benzylic *R*-hydroxy group. Substitution of the 2-fluorophenyl group with a benzothiophene also markedly decreased the potency. Perhaps the single most important finding is the clear chiral preference of the hydroxy group at the benzylic carbon for the *R*-enantiomer, as the *S*-enantiomer had no neuroprotective or antiseizure activity. Further, deletion of the hydroxy group at the benzylic carbon rendered AND-319 devoid of neuroprotective activity, but with antiseizure activity that was more potent than that of AND-287.

Although neuroprotective and antiseizure activity has been captured within a single molecular entity (AND-287), the drug-like properties of this lead compound are in an early stage of evaluation for its ADME/toxicological profile. MES studies on AND-287 in rat show that this compound is orally active, with duration of action of 4 h, and Caco-2 testing suggests good cellular permeability with a low efflux ratio. In addition, microsomal stability studies support that conclusion that this compound is highly stable to oxidative metabolism. Importantly, early studies on the toxicology properties showed no adverse effect with the rotarod test up to 500 mg/kg i.p.

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Fig. 1. Effect of AND-287 on neuroprotection from glutamate (**a**) and hydrogen peroxide (**b**) in hippocampal cultures tested in nutrient medium without antioxidants. For all neuroprotective studies, fluorescent assays for neuronal viability [6-carboxyfluorescein diacetate (*CFDA*), *closed circles*] and cell death (propidium iodide, *open circles*) were measured in the same culture well. For each of the fluorescent assays, the relative fluorescence units (RFU) are displayed, as well as the excitation and emission wavelengths that were utilized. For protection assays against 30 μ M glutamate, day 20 hippocampal cultures were utilized. Glutamate treatment was acute with a 5-min incubation followed by removal of the

glutamate and replacement with growth medium. Treatment with AND-287 was then initiated 4 h, followed by the viability assays. For protective assays against 10 μ M hydrogen peroxide, day 13 hippocampal cultures were utilized. Cultures were treated with various concentrations of AND-287 followed by the addition of hydrogen peroxide (HP). The duration of co-treatment with drug and HP was for 4 h followed by the viability assays. Each point for all assays is the mean of eight determinations from two separate experiments. The *error bars* are the S.E.M. The *dotted lines* for all assays represent the fluorescence levels from respective control cultures. In **a**, significant changes from cultures treated only with glutamate (* $P<0.001$) for propidium iodide and those measured with CFDA (** $P<0.001$). Similarly in **b**, significant changes from cultures treated only with hydrogen peroxide (* $P<0.001$) for propidium iodide and those measured with CFDA (** $P<0.001$)

Fig. 2.

Comparison of AND-287 and AND-319 in the MES (**a** mice; **b** rat). The time course of activity in the maximal electroshock model (MES) is compared in mice (**a**) and rats (**b**) for a neuroprotective anticonvulsant (*AND-287*) and a compound (*AND-319*) that exhibited only anticonvulsant activity. Mice were administered compounds by intraperitoneal injection and rats were administered agents by oral gavage at the doses indicated. All data are expressed as the percentage of animals (total tested at each time point was $N=4$) exhibiting MES activity

Fig. 3.

Time course of metabolic stability for AND-287. A comparison of metabolic stability was made after incubation of 1 μ M AND-287 with liver microsomes derived from human, rat, or mouse. As determined by a LTQ-Orbitrap XL mass spectrometer, the percentage of compound remaining as unchanged was measured at each time point as a single determination. As a metabolically labile control, 1 μ M testosterone was also tested

Fig. 4. Behavioral toxicity: comparison of impairment of rotarod performance for AND-319 and AND-287. All measures of rotarod performance were performed 15 min after intraperitoneal administration of compound. Each value is the percentage of mice that exhibited impairment of performance with an $N=8$ tested at each concentration

Fig. 5.

Diagram of design elements for program neuroprotective anticonvulsants. *Element 1* the aryl modulatory component for potency, stability, and efficacy. *Element 2* the benzyl carbon substituent for neuroprotection and bioavailability. *Element 3* the sulfamide “Message” for antiseizure and neuroprotective activity

Scheme 1.

Synthesis of 2-hydroxy-2-arylethylsulfamides and 2-aryl ethylsulfamides

Scheme 2.
Separation of the enantiomers of AND-287 and AND-286

Comparison of compounds for neuroprotection against acute glutamate or hydrogen peroxide toxicity in hippocampal cultures after 4 h treatment

Table 1

AND ID #	Aryl Group (Ar)	R	CFDA ^a Glutamate	Pt ^b Glutamate	CFDA ^a HP	Pt ^b HP

96	2-chlorophenyl	H	> 100 μM	> 100 μM	> 100 μM	> 100 μM
106	3-chlorophenyl	OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
107	4-chlorophenyl	OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
118	2,5-dichlorophenyl	OH	> 100 μM	30 μM	> 100 μM	> 100 μM
112	2,4-dichlorophenyl	OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
170	2,3-dichlorophenyl	OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
342	2-chlorophenyl	(S)-OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
343	2-chlorophenyl	(R)-OH	1 μM	6 μM	> 100 μM	> 100 μM
319	2-fluorophenyl	H	> 100 μM	> 100 μM	100 μM	> 100 μM
171	2-fluorophenyl	(R,S)-OH	30 nM	40 nM	> 100 μM	100 nM
286	2-fluorophenyl	(S)-OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM
287	2-fluorophenyl	(R)-OH	3 nM	4 nM	40 nM	30 nM
116	benzo[b]thiophen-3-yl	H	> 100 μM	> 100 μM	> 100 μM	> 100 μM
176	benzo[b]thiophen-3-yl	OH	> 100 μM	> 100 μM	> 100 μM	5 μM
115	benzo[b]thiophen-2-yl	OH	30 μM ^c	8 μM ^c	60 μM	30 μM
177	benzofuran-3-yl	OH	> 100 μM	> 100 μM	> 100 μM	> 100 μM

CFDA carboxyfluorescein diacetate, Pt propidium iodide

^aLow efficacy

Table 2

Comparison of anticonvulsant activity of test compounds in four animal models

Compound	2-OH	Rat MES (oral)	Mouse MES (i.p.)	Mouse 6-Hz test (i.p.)	Mouse scPTZ (s.c. EC50)
AND-171	<i>R,S</i>	1/4 at 30 mg/kg	1/3 at 100 mg/kg	1/4 at 100 mg/kg	>300 mg/kg
AND-287	<i>R</i>	ED50, 50±2 mg/kg	ED50, 75±1 mg/kg	3/4 at 100 mg/kg	>250 mg/kg
AND-286	<i>S</i>	Not determined ^a	ED50 >300 mg/kg	Not determined ^a	>300 mg/kg
AND-319	none	ED50, 36±1 mg/kg	ED50, 45±2 mg/kg	ED50, 50±1 mg/kg	ED50, 74±3 mg/kg

^aThe lack of efficacy in the mouse MES and scPTZ models precluded testing in these models by the anticonvulsant screening program

Table 3

Effect of AND-287 on Caco-2 bidirectional permeability

Test compound	P_{app} ($\times 10^{-6}$ cm/s)	Permeability classification		Efflux ratio ^a	Significant efflux
		A-B	B-A		
AND-287	11.8	7.8	High	0.7	No
Digoxin control	0.62	12.5	Low	20.2	Yes
Propranolol control	17.1	-	High		
Atenolol control	0.23	-	Low		

^a Efflux ratio = (P_{app} B-A / P_{app} A-B)