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Pharmacology of ACEA-1416: a potent systemically active NMDA receptor glycine site antagonist

Victor I. Ilyin [a,1], Edward R. Whittemore [a], Minhtam Tran [a], Ke-Zhong Shen [a,2], Sui-Xiong Cai [a], Sunil M. Kher [b], John F.W. Keana [b], Eckard Weber [a], Richard M. Woodward [a,*]

[a] *Acea Pharmaceuticals Inc., a wholly owned subsidiary of CoCensys, Inc., 213 Technology Drive, Irvine, CA 92718, USA*
[b] *Department of Chemistry, University of Oregon, Eugene, OR 97403, USA*

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Abstract

Excitatory amino acid receptor antagonists show potential for the treatment of ischemic stroke and head trauma. In search of novel antagonists, a series of alkyl- and alkoxyl-substituted 1,4-dihydro-2,3-quinoxalinediones were synthesized and assayed for inhibition of glutamate receptors. We report on the pharmacological characterization of one such compound, 7-chloro-6-methyl-5-nitro-1,4-dihydro-2,3-quinoxalinedione (ACEA-1416). Electrophysiological assays showed that ACEA-1416 is a potent antagonist of rat brain NMDA receptors expressed in *Xenopus* oocytes, and NMDA receptors expressed by cultured rat cortical neurons. Antagonism is via competitive inhibition at glycine co-agonist sites (K_b = 7.9 nM in oocytes, K_b = 11 nM in neurons). ACEA-1416 also antagonizes AMPA receptors, though potency is considerably lower (K_b = 3.5 μM in oocytes, K_b = 1.6 μM in neurons). Oocyte assays indicated that ACEA-1416 is weak or inactive as an antagonist at NMDA receptor glutamate binding sites (K_b > 5.9 μM) and metabotropic glutamate receptors (K_b > 57 μM). Many NMDA receptor glycine site antagonists show poor penetration of the blood-brain barrier. Systemic bioavailability of ACEA-1416 was assessed by measuring the ability of the compound to protect against electroshock-induced seizures in mice. Protective effects of ACEA-1416 had rapid onset following i.v. administration. Peak efficacy was at ~ 2 min and the biological half-time of protection was ~ 60 min. The ED_{50} measured at peak efficacy was ~ 1.5 mg/kg. Our results show that ACEA-1416 is a high potency systemically active NMDA receptor glycine site antagonist and a moderate potency AMPA receptor antagonist. Separate studies indicate that ACEA-1416 is efficacious as a neuroprotectant in a rat model of focal cerebral ischemia. Taken together, our results suggest that ACEA-1416 has potential for clinical development as a neuroprotectant.

Keywords: Anticonvulsant; AMPA receptor antagonist; Neuroprotection; NMDA receptor glycine site antagonist

1. Introduction

NMDA receptor glycine site antagonists have therapeutic potential in three major areas of neurological disease: (i) as neuroprotectants for the treatment of ischemic stroke and head trauma (McCulloch, 1992; Kemp and Leeson, 1993; Leeson and Iversen, 1994; Warner et al., 1995), (ii) as anticonvulsants for the treatment of epilepsy (Rogawski, 1992; Leeson and Iversen, 1994), and (iii) as analgesics for the treatment of chronic or neuropathic pain (Dickenson and Aydar, 1991; Millan and Seguin, 1993; Vaccarino et al., 1993; Lutfy et al., 1995). Interest in glycine site antagonists as therapeutic agents has intensified in recent years due to evidence from animal models that this class of inhibitor may have a better side-effect profile than NMDA receptor channel blockers and conventional competitive antagonists (Tricklebank et al., 1989; Koek and Colpaert, 1990; Bristow et al., 1993; Balster et al., 1995; but see also Tricklebank et al., 1994). In addition, glycine site antagonists do not appear to induce neuronal vacuolization in the retrosplenial and posterior cingulate cortices, a phenomenon that remains a cause for concern with some other classes of antagonist (Olney et al., 1989; Hargreaves et al., 1993).

* Corresponding author. Acea Pharmaceuticals Inc., 1003 Health Sciences Road West, Irvine, CA 92715, USA. Tel.: (714)-725-2765; fax: (714)-725-2727.

[1] Permanent address: Laboratory of Nerve Cell Biophysics, Institute of Cell Biophysics, Russian Academy of Sciences, Pushino, Moscow Region, 142292, Russia.

[2] Current address: Department of Pharmacology, Oregon Health Sciences University, Portland, OR 97201, USA.

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1,4-Dihydro-2,3-quinoxalinediones were initially characterized as selective antagonists for α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-preferring non-NMDA receptors (Honore et al., 1988). In general, these compounds showed poor penetration of the blood-brain barrier. Subsequent studies found that this class of compound also inhibits NMDA receptor glycine sites (Randle et al., 1992), and, moreover, that certain tri-substituted 1,4-dihydro-2,3-quinoxalinediones are highly potent and selective glycine site antagonists (Woodward et al., 1995a; Keana et al., 1995). Some of these drugs were found to have anticonvulsant activity in rodents following systemic administration, indicative of penetration into the CNS (Woodward et al., 1995b). Most importantly, the latter compounds also showed strong neuroprotective effects in rat middle cerebral artery occlusion models of ischemic stroke (Warner et al., 1995; P. Marek and E. Weber unpublished data), and in a rat model of acute subdural hematoma (Tsuchida and Bullock, 1995).

To further explore the pharmacological properties and therapeutic potential of 1,4-dihydro-2,3-quinoxalinediones a series of di- and tri-substituted molecules were synthesized combining nitro- and halo-substitutions on the benzene ring with alkyl and alkoxyl substituents (S.M. Kher, S.X. Cai and J.F.W. Keana, unpublished data). In the present study we report on the glutamate receptor pharmacology and anticonvulsant effects of the most potent of these compounds, 7-chloro-6-methyl-5-nitro-1,4-dihydro-2,3-quinoxalinedione (ACEA-1416).

2. Materials and methods

2.1. Electrophysiology in Xenopus oocytes

Isolation of rat cerebral cortex poly(A)$^+$RNA, preparation of *Xenopus laevis* oocytes and micro-injections of RNA were all as described previously (Woodward et al., 1992; Hawkinson et al., 1996). Individual oocytes were injected with ~ 50 ng of rat cortex poly(A)$^+$RNA. Membrane current responses for preliminary experiments were recorded in frog Ringer solution containing (in mM): NaCl, 115; KCl, 2; $CaCl_2$, 1.8; Hepes, 5; pH 7.4. For the detailed pharmacological assays recordings were made in a nominally zero-Ca^{2+} Ringer in which $CaCl_2$ was replaced by equimolar $BaCl_2$. The zero-Ca^{2+}/Ba^{2+} Ringer helped to reduce endogenous Ca^{2+}-gated Cl^- currents activated by Ca^{2+} entry through NMDA receptors (Leonard and Kelso, 1990). Drugs were applied from a linear array of microcapillary tubes (Hawkinson et al., 1996).

2.2. Culture of rat cortical neurons

Primary cultures of mixed cortical neurons were obtained from Sprague-Dawley (Charles River) rat embryos (embryonic day 16 or 17) using a modification of procedures described previously (Whittemore et al., 1995). Briefly, following dissection cortices were incubated for 8–10 min in 0.25% trypsin, 0.5 mM EDTA, at 37°C in a Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution containing (in mM): NaCl, 138; KCl, 5; KH_2PO_4, 0.3; $Na_2HPO_4 \cdot 7H_2O$ 0.3; D-glucose, 5.6; $NaHCO_3$, 4.2; pyruvate, 1; and Hepes, 20. Trypsin digestion was terminated by washing 3 times in an equivalent volume of Neurobasal medium (GIBCO) containing 10% fetal calf serum. Tissue was triturized and passed through a nylon mesh (Falcon) to remove clumps of undissociated cells and debris. Cells were plated at a density of $3–5 \times 10^4/cm^2$ into 35 mm culture dishes (Costar or Nunc) in Neurobasal medium supplemented with 5% fetal calf serum, 0.5 mM L-glutamine and 0.25 μM L-glutamate. Dishes had been pre-coated with poly-D-lysine. Neurons were maintained at 37°C in a humidified incubator (5% CO_2 : 95% air). After 4–5 days the plating medium was replaced with Neurobasal medium supplemented with 5% fetal calf serum and 0.5 mM L-glutamine. Cultures were fed with this medium twice weekly, and maintained for 1–2 weeks before electrical recordings.

2.3. Electrophysiology in cultured neurons

Membrane current responses were recorded in the whole-cell configuration. Drugs and intervening wash were applied from a microcapillary linear array. This controlled the solution surrounding the cell under examination. The external solution for all drug applications and wash was (in mM): 150, NaCl; 4, KCl; 1, $CaCl_2$; 10 mM Na-Hepes (pH 7.4); 400 nM tetrodotoxin (310 mOsm). In addition, the recording chamber was continuously superfused (~ 5 ml/min) by the same solution supplemented with 2 mM $MgCl_2$ and 10 mM glucose. The internal pipette solution was (in mM): 145, CsCl; 10, Cs-Hepes (pH 7.4); 0.5, $CaCl_2$; 10, EGTA, with 2, ATP and 1, GTP (290 mOsm). ATP and GTP was added fresh each day. The intracellular solution was stored on ice. Whole-cell recordings were made with an Axopatch 200A amplifier (Axon Instruments) using non-fire-polished pipettes pulled from thick-walled capillaries (6–10 MΩ). Currents were filtered at 2 kHz, stored digitally, and analyzed using software provided by the laboratory of Dr Ricardo Miledi (University of California, Irvine, CA, USA).

2.4. Mouse maximum electroshock-induced seizure (MES) assays

General procedures for mouse MES assays were as described previously (Swinyard, 1972). Male Swiss-Webster mice were used in all experiments (body weight 20–30 g). Seizures were induced via corneal electrodes. The seizure stimulus was: rectangular positive pulses, amplitude 50 mA, frequency 60–75 Hz, pulse width 0.8 ms, train length 200 ms (Ugo Basile ECT 7801). ACEA-1416

was administered intravenously via the tail vein. On each day of experiments a control group of 5–8 animals was injected with vehicle alone. The vehicle showed no measurable anticonvulsant activity.

2.5. Data analysis

Agonist concentration-response curves were analyzed as described in Woodward et al. (1995a). Briefly, concentration-response data were fit to a conventional four parameter logistic equation. Apparent antagonist dissociation constants (K_b values) were estimated from simultaneous fits of concentration-response curves shifted by fixed concentrations of antagonist using the equation:

$$I/I_{max} = 1/\left(1 + \left((10^{-pEC_{50}}) \times \left(1 + \left([\text{antagonist}]/(10^{-pK_b})\right)\right)/[\text{agonist}]\right)^n\right)$$

where I is the measured current, I_{max} is the current elicited by saturating concentrations of agonists, n is the slope factor, pEC_{50} is $-\log EC_{50}$, and pK_b is $-\log K_b$ (Sigmaplot: Jandel Scientific). Consistency with the competitive model of inhibition was assessed by F-test using the equation:

$$F_{df2-df1,df1} = ((SS_2 - SS_1)/(df_2 - df_1))/(SS_1/df_1)$$

where SS_1 and SS_2 are the sum of squared deviations for individual and simultaneous fits, respectively, and df_1 and df_2 are the respective degrees of freedom. For weak antagonism (e.g. NMDA receptor glutamate sites and metabotropic glutamate receptors) potency was gauged from the lower limits of IC_{50} values. Limits of maximum potency were estimated in terms of K_b values using a generalized form of the Cheng-Prussoff relationship (Leff and Dougall, 1993; Woodward et al., 1995a).

2.6. Drugs: synthesis and suppliers

ACEA-1416 (mp. 340°C) was prepared from 2-chloro-5-fluorotoluene in four steps: (i) nitration, (ii) nucleophilic substitution of the halogen *ortho* to the nitro group with sodium glycinate, (iii) reduction of the nitro group with concomitant cyclization, and (iv) regiospecific oxidative nitration. ACEA-1416 gave a satisfactory elemental analysis. Details of the synthesis and structural determination are presented elsewhere (Kher et al., 1995). For in vitro experiments quinoxalinediones were made up in DMSO and then diluted 1000–3000-fold into saline. For intravenous injection ACEA-1416 was dissolved in 50 mM tris(hydroxymethyl)-amino-methane at a concentration of 2 mg/ml. 5,7-Dichlorokynurenic acid was purchased from Research Biochemicals (Natick, MA, USA). (1*S*,3*R*)-1-Aminocyclopentane-1,3-dicarboxylic acid was from Tocris Cookson (St. Louis, MO, USA). Tris(hydroxymethyl)-amino-methane was from J.T. Baker (Philipsburg, NJ, USA). Other chemicals were from Sigma or GIBCO.

3. Results

3.1. Antagonism of rat brain NMDA receptors expressed in Xenopus oocytes

Inhibition of NMDA responses by ACEA-1416 was initially assayed in side-by-side comparisons with the previously characterized compounds ACEA-1031 (6,7-dibromo-5-nitro-1,4-dihydroquinoxaline-2,3-dione) and 5,7-dichloro-kynurenic acid (Woodward et al., 1995a,b). A typical experiment is illustrated in Fig. 1 (upper panel). These preliminary results were used to determine suitable concentrations of antagonist for more detailed pharmacological assays. Estimates of antagonist potency were made by measuring effects of ACEA-1416 on glycine concentration-response curves (NMDA fixed at 100 μM). For these experiments recordings were made in zero-Ca^{2+}/Ba^{2+} Ringer where NMDA response showed a mono-phasic time course (Williams, 1993). Inhibition of NMDA responses by ACEA-1416 was associated with dose-dependent rightward displacement of the glycine concentration-response curve (Fig. 1, middle panel). Inhibition was fully surmounted by increasing the glycine concentration. At 100 nM, ACEA-1416 caused an ~ 14-fold reduction in glycine affinity. Apparent antagonist dissociation constants (K_b values) were calculated by simultaneous fit of glycine concentration-response curves under control conditions, and in 10 and 100 nM ACEA-1416 (Table 1, upper panel). Antagonism was consistent with simple competitive inhibition at the glycine site ($F(3,78) = 1.2$). Maximum NMDA responses (100 μM NMDA and 10 μM glycine) in these experiments ranged from 50 to 97 nA (mean ± S.E.M. = 75 ± 12 nA, $n = 4$). Assays designed to detect antagonism at glutamate (NMDA) binding sites (i.e. inhibition of responses elicited by 4 μM NMDA and 1 mM glycine) indicated the IC_{50} for ACEA-1416 was > 5 μM (not illustrated). As expected, testing for antagonism at glutamate binding sites was limited by the encroachment of inhibition at glycine sites. Estimates for possible interactions at glutamate sites are only given as an upper limit of potency. Assuming a competitive mechanism, and using previously measured EC_{50} and slope values for NMDA, these experiments indicated that the K_b value for ACEA-1416 at glutamate binding sites was at least 5.9 μM, and was possibly much higher (Table 1, upper panel).

3.2. Antagonism of rat brain non-NMDA receptors expressed in Xenopus oocytes

AMPA responses were elicited using either AMPA or kainic acid as agonists (Woodward et al., 1995a,b). AMPA causes strong desensitization of AMPA receptors, whereas desensitization is much less pronounced with kainic acid (Patneau and Mayer, 1991). In the oocyte recordings this is reflected in the relative amplitudes of responses for the two agonists. Maximum steady-state responses for AMPA

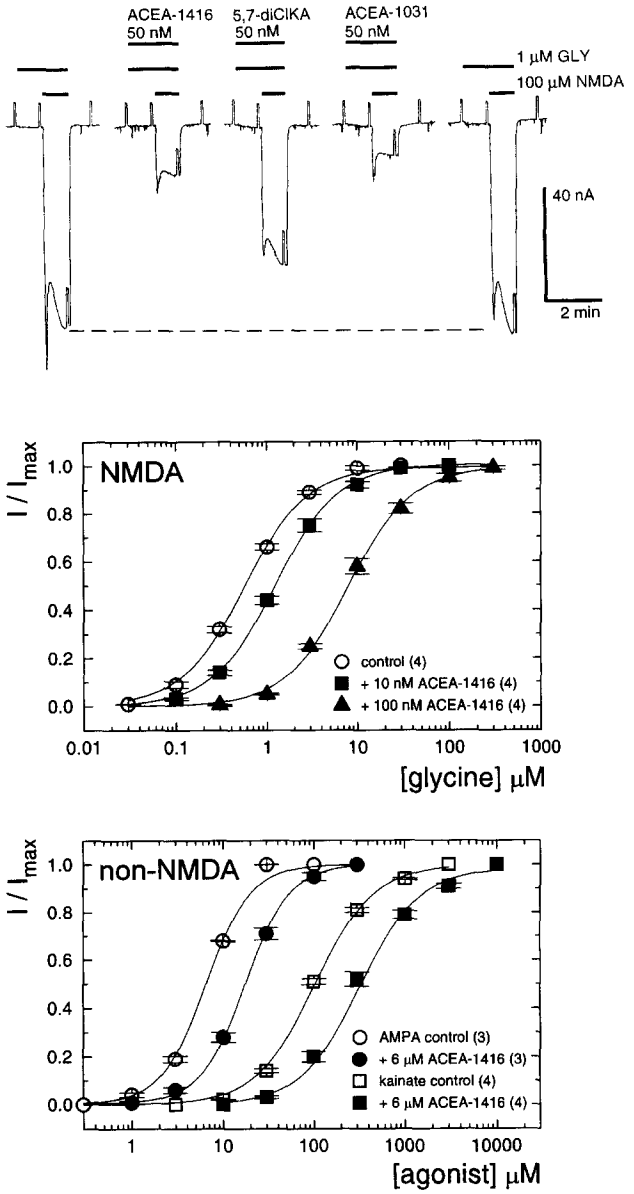

Fig. 1. Inhibition of rat brain ionotropic glutamate receptors expressed in *Xenopus* oocytes. Upper panel. Sample records of experiments comparing inhibition induced by ACEA-1416, 5,7-dichlorokynurenic acid (5,7-diClKA) and ACEA-1031. Recordings were made in normal Ringer. Inhibition was measured at the peak of the second/slow phase of the response. Holding potential was −70 mV periodically pulsed to −60 mV to help standardize timing of drug applications (bars). Downward deflection denotes inward current. The broken line is the estimated level of control current. Middle panel. Effect of 10 and 100 nM ACEA-1416 on the concentration-response relationship for glycine at NMDA receptors (NMDA = 100 μM). Lower panel. Effects of 6 μM ACEA-1416 on concentration-response relationships for AMPA and kainic acid at AMPA-preferring non-NMDA receptors. Data were normalized with respect to the maximum response. Smooth curves, best simultaneous logistic fits to the data. EC_{50} values for control curves and optimal slope values for the simultaneous fits are given in Table 1.

ranged between 36–49 nA (mean = 42 ± 5 nA, $n = 3$). Maximum steady-state responses for kainic acid were ~ 30 times larger, ranging between 1063–1630 nA (mean = 1366 ± 160 nA, $n = 4$). In oocytes, peak currents are not measurable when using AMPA as the agonist due to the slow speed of drug application. As described for NMDA receptors (Fig. 1, upper panel), inhibition of AMPA receptors by ACEA-1416 was first gauged using fixed agonist concentrations (not illustrated). These experiments indicated potency was significantly lower than at NMDA receptors. At 6 μM, ACEA-1416 induced rightward shifts in the concentration-response curves for AMPA and kainic acid (Fig. 1). The apparent affinity for each agonist was reduced ~ 3-fold. K_b values were calculated from simultaneous fits for the two pairs of curves (Table 1, upper panel). AMPA concentration-response curves were scaled as described previously (Woodward et al., 1995a). For both agonists, antagonism was consistent with competitive inhibition at glutamate binding sites (AMPA, $F(1,32) = 3.9$; kainic acid $F(1,45) = 1.2$).

3.3. Antagonism of rat brain metabotropic glutamate receptors expressed in Xenopus oocytes

Metabotropic glutamate receptors expressed in *Xenopus* oocytes couple to an endogenous intracellular receptor-channel coupling pathway mediated by hydrolysis of inositol phospholipids, release of Ca^{2+} from intracellular stores, and activation of Ca^{2+}-gated Cl^- channels (Gundersen et al., 1984; Oron et al., 1985). Activation of metabotropic glutamate receptors using the selective agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (10–30 μM) elicited the characteristic fluctuating Cl^- current (Manzoni et al., 1990). These responses were not appreciably blocked by equimolar ACEA-1416 (not illustrated), indicating the K_b value at metabotropic receptors was at least 57 μM (Table 1, upper panel).

3.4. Antagonism of NMDA receptors expressed by rat cortical neurons in culture

Neuronal NMDA responses were characterized by a rapid rising phase followed by desensitization to a steady-state level (Swartz et al., 1992). All pharmacological measurements were made under steady-state conditions on the plateau phase of the response. As described for NMDA receptors expressed in oocytes, ACEA-1416 showed potent antagonism of neuronal NMDA responses. Inhibition was due to rightward displacement of the glycine concentration-response curve (Fig. 2, upper panel). At 100 nM, ACEA-1416 induced a ~ 10-fold reduction in apparent affinity for glycine. The K_b value for ACEA-1416 was calculated by simultaneous fit of concentration-response data (Table 1, lower panel). Antagonism was consistent with competitive inhibition at glycine binding sites ($F(1,57) = 3.8$). Maximum responses (10 μM glycine, 100 μM NMDA) for control curves ranged between 945–1907 pA, mean = 1350 ± 193 ($n = 5$).

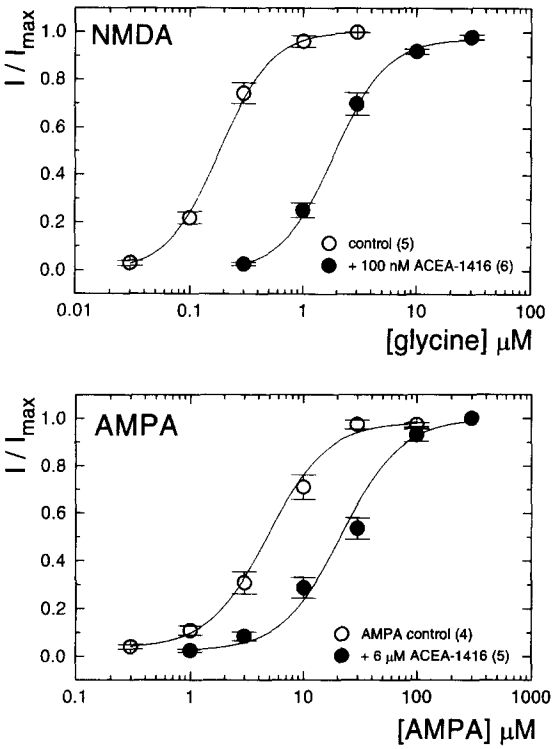

Fig. 2. Inhibition of glutamate receptors expressed by rat cortical neurons in culture. Upper panel. Effect of 100 nM ACEA-1416 on the concentration-response curve for glycine at neuronal NMDA receptors (NMDA = 100 μM). (*Lower panel*) Effect of 6 μM ACEA-1416 on concentration-response relationship for AMPA at AMPA-preferring non-NMDA receptors. Data presented as in Fig. 1. Optimum EC_{50} values for control curves and slopes for simultaneous fits are given in Table 1.

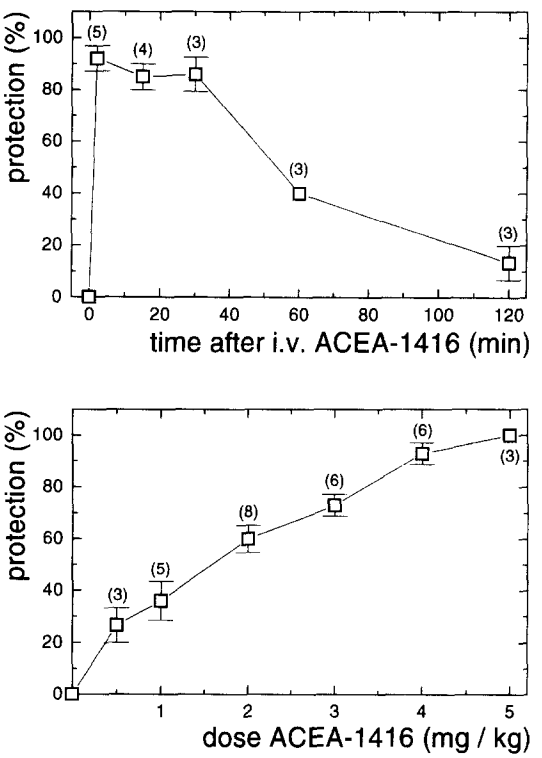

Fig. 3. Protection against maximum electroshock-induced seizures in mice. Upper panel. Time course of protection induced by an i.v. dose of 4 mg/kg ACEA-1416. Lower panel. Dose-response relationship for ACEA-1416 assayed 2 min after i.v. injection (approximate peak efficacy in the time course). Data are the means ± S.E.M. Values in brackets indicate the number of separate experiments. For each experiment percentage protection was determined in groups of 5 mice.

3.5. Antagonism of AMPA receptors expressed by rat cortical neurons in culture

Inhibition of AMPA receptor currents were measured on steady-state desensitized responses (Swartz et al., 1992). Antagonism was distinctly weaker at AMPA receptors than at NMDA receptors. At 6 μM, ACEA-1416 caused a modest rightward displacement of the AMPA concentration-response curve, decreasing the apparent AMPA affinity ~ 4.5-fold (Fig. 2, lower panel). K_b values were calculated from the simultaneous fit (Table 1, lower panel), and antagonism was consistent with competitive inhibition

Table 1
Potency of ACEA-1416 at rat brain glutamate receptors

Oocyte recordings Receptor/agonist	EC_{50} agonist [a] (μM)	Slope C/R curve	K_b ACEA-1416 [b] (μM)	K_b receptor/K_b NMDA [c]	n [d]
NMDA/glycine	0.57 (0.50–0.60) [e]	1.2 (1.1–1.3)	0.0079 (0.0072–0.0087)	–	4
NMDA/NMDA	27 (23–31) [f]	1.2 (0.8–1.7) [f]	> 5.9	> 750	3 [f]
Non-NMDA/AMPA	6.5 (6.1–6.9)	1.8 (1.7–1.9)	3.5 (3.1–4.0)	440	3
Non-NMDA/kainic acid	100 (90–110)	1.3 (1.2–1.4)	2.8 (2.3–3.4)	350	4
Metabotropic/ACPD	28 (24–32) [f]	1.8 (1.4–2.3) [f]	> 57	> 7200	3 [f]
Neuron recordings					
Receptor/agonist	EC_{50} agonist (μM)	Slope C/R curve	K_b ACEA-1416 (μM)	K_b receptor/K_b NMDA	n
NMDA/glycine	0.18 (0.17–0.20)	1.9 (1.7–2.1)	0.011 (0.009–0.012)	–	5
Non-NMDA/AMPA	5.1 (4.4–5.9)	1.4 (1.2–1.5)	1.6 (1.3–2.2)	150	4

[a] EC_{50} and slope values for agonist concentration-response curves measured under control conditions. Optimum logistic fits of data in Fig. 1 and Fig. 2. [b] K_b values calculated by simultaneous fits of concentration-response data as described in Methods. [c] Steady-state selectivity indices estimated from the ratio of K_b/K_b NMDA-glycine. [d] Number of independent experiments (cells examined). [e] Bracketed numbers are 95% confidence intervals adjusted to the linear scale. [f] Values from a previous study (Woodward et al., 1995a) used in assessment of potency limits at NMDA glutamate sites and metabotropic glutamate receptors.

at glutamate binding sites ($F(1,44) = 0.71$). Maximum steady-state currents (100 μM AMPA) for control curves ranged between 221–428 pA, mean = 314 ± 45 pA, $n = 4$.

3.6. Mouse maximum electroshock-induced seizure studies

Systemic bioavailability of ACEA-1416 was assessed by measuring anticonvulsant effects in a mouse maximum electroshock-induced seizure model (Swinyard, 1972). Onset of protection was rapid after intravenous injection of 4 mg/kg ACEA-1416, reaching maximum efficacy at ~2 min (Fig. 3, upper panel). The protective effect of ACEA-1416 then subsided over the following 120 min with a biological half-time of approximately 60 min. The dose-response curve for ACEA-1416 was measured at peak efficacy (2 min) (Fig. 3, lower panel). The ED_{50} was between 1–2 mg/kg. Protection was 100% in three out of five groups tested at 4 mg/kg. Protection measured at 0.5 mg/kg was significant at the level $P < 0.05$.

4. Discussion

Our experiments show that ACEA-1416, a novel alkyl-substituted 1,4-dihydro-2,3-quinoxalinedione, is a systemically active NMDA receptor antagonist with potency in the low nanomolar range. Antagonism is due to competitive inhibition at glycine co-agonist binding sites. Like other quinoxalinediones (Honore et al., 1988; Randle et al., 1992), ACEA-1416 also inhibits AMPA-preferring non-NMDA receptors. In this case, steady-state potency is 150–450 times weaker than at NMDA receptors and antagonism is due to competitive inhibition at glutamate binding sites. ACEA-1416 is weak or inactive as a ligand for NMDA receptor glutamate binding sites and metabotropic glutamate receptors.

Potency of ACEA-1416 at NMDA receptor glycine sites and selectivity of inhibition with respect to AMPA receptors are similar to those of the structurally related compound 6,7-dichloro-5-nitro-1,4-dihydro-2,3-quinoxalinedione (ACEA-1021) (Woodward et al., 1995a). The only difference between these two molecules is the substitution at position 6; methyl for ACEA-1416, and chloro for ACEA-1021 (Fig. 4). Using the same assay system, K_b values for ACEA-1021 are 6.8 nM for NMDA receptor glycine sites, and 1.5-1.8 μM for AMPA receptors, depending on whether AMPA or kainate is used as the agonist (Woodward et al., 1995b).

With regard to structure activity relationships in the 1,4-dihydro-2,3-quinoxalinedione series, when a nitro substituent is present at position 5 and a chloro at position 7, changing chloro for methyl at position 6 does not overtly alter affinities for NMDA glycine sites or for AMPA glutamate sites (Fig. 4). Compared to the unsubstituted 1,4-dihydro-2,3-quinoxalinedione, ACEA-1416 and ACEA-1021 are approximately 2000-fold more potent at

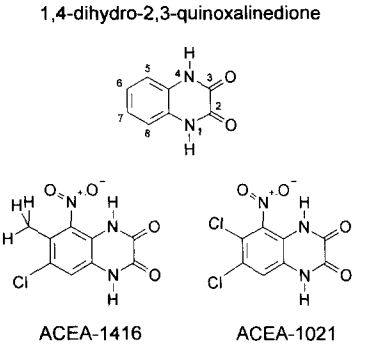

Fig. 4. Structures of unsubstituted 1,4-dihydro-2,3-quinoxalinedione (with numbering of the bicyclic ring system), ACEA-1416 and ACEA-1021.

glycine sites and 30–50-fold more potent at AMPA receptors. The K_b values for the unsubstituted 1,4-dihydro-2,3-quinoxalinedione for NMDA and AMPA receptors are 17 and 98 μM, respectively (Keana et al., 1995). Compared to the di-substituted 5-nitro 7-chloro molecule, ACEA-1416 and ACEA-1021 are ~50 times more potent at glycine sites and ~5 times more potent at AMPA receptors. The K_b values for 5-nitro-7-chloro-1,4-dihydro-2,3-quinoxalinedione for NMDA and AMPA receptors are 0.51 and 8.0 μM, respectively (Keana et al., 1995). In the case of ACEA-1021, at least three possible explanations for the increase in potency specifically conferred by the 6 chloro substituent can be considered: (i) additional electron withdrawing effects contributing to acidification of amides in the heterocyclic ring; (ii) increased hydrophobic interactions with the receptor, and (iii) steric hindrance of the nitro group causing it to twist out of the plane of the ring thereby permitting additional hydrogen bonding with the receptor (Keana et al., 1995). The high potency of ACEA-1416 shows that an electron-donating methyl substituent at position 6 does little to reduce potency, hence hydrophobic interactions and steric factors are the most likely determinants of affinity at this position. The same reasoning extends to antagonist interactions at AMPA receptors, but in this case the difference in potency between the di- and tri-substituted quinoxalinediones is not as pronounced.

Though ACEA-1416 is a potent NMDA receptor antagonist it is also a moderately potent inhibitor of AMPA receptors. The steady-state selectivity indices are 350–450-fold in favor of NMDA receptor inhibition in oocyte assays and approximately 150-fold in the neuronal assays. This would suggest that the anticonvulsant and neuroprotective effects of ACEA-1416 are primarily due to antagonism of NMDA receptors. Nevertheless, a steady-state selectivity index may be inappropriate for assessing selectivity in vivo where levels of inhibition will show dependence on relative agonist concentrations and on the kinetics of antagonist binding (Woodward et al., 1995a). Indeed, studies with related quinoxalinediones suggest that levels of functional selectivity for ACEA-1416 will be significantly reduced when measured in terms of synaptic

pharmacology (Randle et al., 1992). Furthermore, recent in vivo electrophysiological characterization of ACEA-1021 suggests that the selectivity index between NMDA and AMPA receptor antagonism measured in situ is < 10-fold (Lingenhohl et al., 1995). Given the similarity in profile between ACEA-1416 and ACEA-1021 it is likely that ACEA-1416 will also show diminished NMDA versus AMPA receptor selectivity when measured in vivo. This is of relevance because AMPA receptor antagonists, such as 2,3-dihydro-6-nitro-7-sulfamoylbenzo(*F*)quinoxaline (NBQX), have robust neuroprotective effects in animal models of global and focal ischemia (Sheardown et al., 1990; Buchan et al., 1993). We cannot, therefore, exclude the possibility that AMPA receptor antagonism contributes to the neuroprotective effects of ACEA-1416 in rat models of focal cerebral ischemia (P. Marek and E. Weber, unpublished data). In particular, AMPA receptor antagonism may become a factor within a developing ischemic infarct where integrity of the blood-brain barrier is compromised and antagonist concentrations are likely to be highest. It should be stressed, however, that numerous wholly selective NMDA receptor antagonists have anticonvulsant and neuroprotective properties (Parke et al., 1988; McCulloch, 1992; Rogawski, 1992), so invoking AMPA receptor antagonism is by no means necessary to explain the in vivo actions of a drug such as ACEA-1416.

The only conspicuous difference between ACEA-1416 and ACEA-1021 was seen in the mouse MES studies. Here, ACEA-1416 was consistently 2–5 times more potent than ACEA-1021 (Woodward et al., 1995b). Though there are numerous possible explanations for this difference in potency the most likely is that ACEA-1416 has improved bioavailability. This could be due to increased penetration of the blood brain barrier, or simply to a higher active plasma concentration of the drug due, for example, to less binding to plasma proteins. Whatever the reason, the increase in potency in vivo, relative to ACEA-1021, is related to the methyl substitution at position 6, suggesting that alkyl-substituted 1,4-dihydro-2,3-quinoxalinediones may have advantages over their halo-substituted analogues for in vivo studies. Many types of NMDA receptor glycine site antagonist are, like 1,4-dihydro-2,3-quinoxalinediones, based on fused heterocyclic ring structures. Examples include: kynurenic acids, 2-carboxy-1,2,3,4-tetrahydroquinolines, dihydro-2,5-dioxo-3-hydroxy-1*H*-benzazepines, indole-2-carboxylic acids, and 3-acyl-4-hydroxy-, 3-nitro-3,4-dihydro- and 3-phenyl-4-hydroxy-quinolin-2(1*H*)-ones (Kemp and Leeson, 1993; Leeson and Iversen, 1994). We suspect that select methyl substitutions onto the benzene ring, common to all these molecules, may bestow favorable in vivo properties across a wide range of chemotypes.

NMDA receptor antagonists need to satisfy at least six basic criteria to have clinical potential as neuroprotectants: (i) sufficient solubility and stability for i.v. formulation, (ii) high or moderate potency at the receptor, (iii) in vivo bioavailability following systemic administration, (iv) efficacy in animal models of stroke and head trauma, (v) a lack of overt behavioral side effects and neurotoxicity, and (vi) acceptable toxicology. The present study indicates that ACEA-1416 fulfills the first three criteria. Parallel studies indicate that ACEA-1416 has robust neuroprotective effects in a rat middle cerebral artery model of stroke (P. Marek and E. Weber, unpublished data). Studies on the sister compound ACEA-1021 indicate that this type of antagonist does not evoke phencyclidine-like behaviors (Balster et al., 1995), and does not have neurotoxic effects (K.H. Huber, unpublished data). Taken together, these results suggest that ACEA-1416 is a promising candidate for clinical development as a neuroprotectant.

In conclusion, ACEA-1416 is a combined high potency NMDA receptor glycine site antagonist and moderate potency AMPA receptor antagonist that shows systemic bioavailability in rodents. Our results suggest that methyl substitutions onto the benzene ring of 1,4-dihydro-2,3-quinoxalinediones, and by implication many other types of NMDA receptor glycine antagonist, are well tolerated and could lead to compounds with improved pharmacological and therapeutic profiles.

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