Adenosine Kinase Inhibitors as a Novel Approach to Anticonvulsant Therapy

JAMES B. WIESNER, BHEEMARAO G. UGARKAR, ANGELO J. CASTELLINO, WESBAR, HARRY E. GRUBER, ALAN C. FOSTER, and MARK D. ERION

Metabasis Therapeutics, Inc. (a Subsidiary of Gensia Sicor Inc.), San Diego, California

ABSTRACT

Adenosine levels increase at seizure foci as part of a postulated endogenous negative feedback mechanism that controls seizure activity through activation of A1 adenosine receptors. Agents that amplify this site- and event-specific surge of adenosine could provide antiseizure activity similar to that of adenosine receptor agonists but with fewer dose-limiting side effects. Inhibitors of adenosine kinase (AK) were examined because AK is normally the primary route of adenosine metabolism. The AK inhibitors 5'-amino-5'-deoxyadenosine, 5'-iodotubercidin, and 5'-deoxy-5'-iodotubercidin inhibited maximal electroshock (MES) seizures in rats. Several structural classes of novel AK inhibitors were identified and shown to exhibit similar activity, including a prototype inhibitor, 4-[(N-phenylamino)-5-phenyl-7-(5'-deoxyribofuranosyl)pyrrolo[2,3-d]pyrimidine (GP683; MES ED50 = 1.1 mg/kg). AK inhibitors also reduced epileptiform discharges induced by removal of Mg2+ in a rat neocortical preparation. Overall, inhibitors of adenosine deaminase or of adenosine transport were less effective. The antiseizure activities of GP683 in the in vivo and in vitro preparations were reversed by the adenosine receptor antagonists theophylline and 8-(p-sulfophenyl)theophylline. GP683 showed little or no hypotension or bradycardia and minimal hypothermic effect at anticonvulsant doses. This improved side effect profile contrasts markedly with the profound hypotension, bradycardia, and hypothermia and greater inhibition of motor function observed with the adenosine receptor agonist N6-cyclopentyladenosine and opens the way to clinical evaluation of AK inhibitors as a novel, adenosine-based approach to anticonvulsant therapy.

Adenosine has been described as the brain’s natural anticonvulsant (Dragunow, 1986). This hypothesis is supported by findings that stimulation of adenosine receptors exerts anticonvulsant activity in animal models of seizures and that adenosine receptor antagonists are proconvulsant, suggesting tonic suppression of epileptic activity by adenosine (for review, see Knutsen and Murray, 1997). Moreover, local levels of endogenous adenosine increase during seizure activity, purportedly as a feedback signal to curtail epileptic activity (During and Spencer, 1992). This inhibitory activity is mediated by both pre- and postsynaptic mechanisms (Greene and Haas, 1991; Wu and Saggau, 1994). In the hippocampus, the A1 adenosine receptors are present on nerve terminals that release glutamate but not on those that release γ-aminobutyric acid, suggesting that excitatory transmission is regulated selectively (Thompson et al., 1992). Many epileptic patients, particularly those with certain seizure types (e.g., partial complex seizures), are not adequately treated with current anticonvulsant therapies; thus, considerable need exists for new therapeutic approaches. Increased efficacy in these seizure types may be gained through novel therapies that act through mechanisms other than those utilized by known anticonvulsant drugs. One attractive approach is the modulation of excitatory neurotransmission through activation of adenosine receptors. As indicated above, adenosine receptor agonists are effective in various animal seizure models, including limbic seizures, which may be analogous to the partial complex seizure class in humans. However, the side effect profile of these agonists,
including profound reductions in blood pressure and heart rate, pronounced hypothermia, and motor depression (Dun-widdie and Worth, 1982; Phillis and Wu, 1983; Malhotra and Gupta, 1997), is daunting. An alternative strategy is to enhance the natural adenosinergic feedback mechanism associated with seizures. This can theoretically be achieved by preventing the uptake and metabolism of adenosine by means of adenosine-regulating agents (ARAs; Foster et al., 1995). Because adenosine release is enhanced locally during seizure activity and the released adenosine has a short half-life (e.g., on the order of seconds in blood), ARAs may exert substantial selectivity by altering this site- and event-specific surge of adenosine. Consequently, ARAs are predicted to have fewer side effects than adenosine receptor agonists, which activate adenosine receptors indiscriminately throughout the body.

Accordingly, we examined the activity of ARAs in in vivo and in vitro models of epileptic activity. We have found evidence indicating that inhibitors of the enzyme adenosine kinase (AK), one of the principal adenosine-catabolizing enzymes (Fig. 1), are potent antiepileptic agents with an improved side effect profile compared with adenosine receptor agonists. The AK inhibitors profiled include the known AK inhibitors 5'-amino-5'-deoxyadenosine (5'-ADO), 5'-idotubercidin (ITU), and 5'-deoxy-ITU (5'-dITU) and novel compounds from various diverse structural classes. Certain of these novel compounds appear to be devoid of toxicities and potency issues limiting the utility of earlier AK inhibitors.

**Experimental Procedures**

**Materials.** 5'-ADO, diprydamole, nitrobenzylthioinosine (NBTA), erythro-9-(2-hydroxy-3-sonlyladenine (EHNA), and theophylline were purchased from Sigma Chemical Co. (St. Louis, MO). ITU, 8-[(sulphophenyl)theophylline (8-SPT), and N6-cyclopentad-enosine (CPA) were purchased from Sigma-Aldrich Research Biochemicals Inc. (Natick, MA). 2'-Deoxycoformycin (DCF), 5'-dITU, and other AK inhibitors (see Table 1) were synthesized at Metabasis Therapeutics, Inc. (B.G.U., A.J.C., J. DaRe, J. Kepcho, C.E. Browne III, J. Schanzer, D.P.D., J.B.W., and M.D.E., manuscripts in preparation). Radioligands were obtained from NEN Life Science Products (Boston, MA).

**AK Inhibition.** Human AK was purified from an *Escherichia coli* expression system as described by Sypycha et al. (1996). Activity was measured essentially as described by Yamada et al. (1981), with minor modifications. Assay mixtures contained 50 mM Tris-maleate buffer (pH 7.0), 0.1% BSA, 1 mM ATP, 1 mM MgCl2, 1.0 μM [U-14C]adenosine (400–600 mCi/mmol; Moravek Biochemicals, Brea, CA), and varying concentrations of inhibitor. [14C]AMP (Am-ersham Life Sciences, Arlington Heights, IL) was separated from unreacted [14C]adenosine by absorption to anion exchange paper (Whatman Inc., Clifton, NJ) and quantified by scintillation counting.

**Inhibition of Adenosine and AMP Deaminases.** For evaluation of inhibition of calf intestinal mucosal adenosine deaminase (ADA) and porcine heart adenosine monophosphate deaminase, assay conditions were essentially as described previously (Erion et al., 1998).

**Cell AK Inhibition.** To assess AK activity in intact cells, cultured bovine microvascular endothelial cells were grown to confluence in 35-mm diameter plates (Corning Inc., Corning, NY) at 1.0 × 10^6 cells/plate, washed in RPMI 1640 medium, and incubated in 500 μl of serum-free RPMI 1640 with 50 μM DCF for 60 min at 37°C to block endogenous ADA activity. Test compounds dissolved in di-methyl sulfoxide (DMSO) were then added to the cells to final concentrations of 0 to 1000 nM (1% final concentration of DMSO in all wells) for an additional 15-min incubation. Adenosine (2 μl of a 250 μM) (2.5,8-8H)adenosine (5 μl of a 1-mCi/ml solution, 32.5 Ci/mmol solution; Moravek Biochemicals) were added to the wells, and the incubation was continued for an additional 10 min. The cells were then quickly washed in PBS, extracted with 100 μl of 0.4 M perchloric acid, and neutralized with tri-n-octylamine/Freon mixture. The adenosine nucleotide pools were separated by thin-layer chromatography as previously described (Barankiewicz et al., 1990); these pools contained virtually all of the radiolabel, which was measured with a Beckman model LS-1701 counter (Beckman Instru-ments, Fullerton, CA). Experiments were performed in triplicate.

**Radioligand-Binding Assays.** Radioligand-binding assays for adenosine A1 and A2 receptors and for the NBTA transporter site were performed as described by Bruns et al. (1980, 1986) and Marangoz et al. (1982), respectively. Briefly, A1 receptor binding was determined in membranes of rat whole brain using the A1 receptor agonist [3H]cyclohexyladenosine (CHA). Binding to the NBTA transport site was determined in whole rat brain membranes with [3H]NBTA. A2 receptor binding was determined in rat striatal membranes with the nonselective agonist [3H]5'-N-ethylcarboxamidoadenosine (NECA) in the presence of unlabeled CPA, which selectively inhibits binding to the A1 receptor. [3H]NECA was used at a concentration of 4 nM, which is near the Kᵢ for A2A receptors but 28× below the Kᵢ for rat A1 receptors and 78 to 650× below the EC5₀ for activity in functional A2B assays with various species, including rat (Brownhill et al., 1996; Muller and Stein, 1996; Feoktistov and Biaggioni, 1997).

With this method for determination of A2 binding (Bruns et al., 1986), the activity of purinergic compounds correlates highly with their activity in assays with ligands highly specific for A2A receptors (Jarvis et al., 1989).

All steps including centrifugation (10 min at 50,000 × g) were at 4°C unless otherwise indicated; homogenization, suspensions, and incubations of membranes were in 50 mM Tris-HCl buffer at pH 7.4 (A1 and NBTA binding) or pH 7.7 (A2 binding). To prepare mem-branes from rat whole brains, frozen brains were thawed and ho-mogenized, washed, and resuspended in buffer containing ADA (2 U/ml) for a 15-min incubation at 37°C to remove endogenous adenosine. For A2 binding, striata were dissected from the thawed brains, weighed, and homogenized. Membrane suspensions were centrifuged and the pellets frozen (−20°C) for a maximum of 2 weeks before resuspension for incubation with radioligands in glass tubes at final
TABLE 1
Structure and anticonvulsant potency of representative nucleoside AK inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R’</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>ED$_{50}$ [95% CI]$^{a}$ (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-adADO</td>
<td>CH$_2$OH</td>
<td>H</td>
<td>NH$_2$</td>
<td>I</td>
<td>CH</td>
<td>153 [71.6 – 326]</td>
</tr>
<tr>
<td>ITU</td>
<td>H</td>
<td>H</td>
<td>NH$_2$</td>
<td>I</td>
<td>CH</td>
<td>6.2 [3.5 – 10.1]</td>
</tr>
<tr>
<td>5’-dITU</td>
<td>CH$_3$</td>
<td>H</td>
<td>NH$_2$</td>
<td>I</td>
<td>CH</td>
<td>0.3 [0.0 – 0.6]</td>
</tr>
<tr>
<td>GP836</td>
<td>CH$_2$NH$_2$</td>
<td>H</td>
<td>NH$_2$</td>
<td>I</td>
<td>CH</td>
<td>7.1 [5.9 – 13.0]</td>
</tr>
<tr>
<td>GP683</td>
<td>CH$_3$</td>
<td>H</td>
<td>NHPh</td>
<td>Ph</td>
<td>CH</td>
<td>1.1 [0.6 – 2.1]</td>
</tr>
<tr>
<td>GP947</td>
<td>H</td>
<td>CH$_3$</td>
<td>NHPh</td>
<td>Ph</td>
<td>CH</td>
<td>0.2 [0.1 – 0.3]</td>
</tr>
<tr>
<td>GP790</td>
<td>H</td>
<td>CH$_2$OH</td>
<td>NHPh</td>
<td>Ph</td>
<td>CH</td>
<td>1.6 [0.8 – 3.0]</td>
</tr>
<tr>
<td>GP515</td>
<td>CH$_2$NH$_2$</td>
<td>H</td>
<td>NH$_2$</td>
<td>Br</td>
<td>N</td>
<td>15.9 [10.2 – 24.8]</td>
</tr>
<tr>
<td>GP665</td>
<td>CH$_3$</td>
<td>H</td>
<td>NHPh</td>
<td>Ph</td>
<td>N</td>
<td>11.7 [6.1 – 22.6]</td>
</tr>
<tr>
<td>GP547</td>
<td>CH$_2$NH$_2$</td>
<td>H</td>
<td>NH$_2$</td>
<td>I</td>
<td>N</td>
<td>9.7 [3.8 – 24.7]</td>
</tr>
</tbody>
</table>

$^{a}$ ED$_{50}$ values and 95% confidence intervals (95% CI) for inhibition of MES seizures in rats at 1 h after i.p. administration (see Fig. 2 and 3).

volumes of 0.5 ml. For A$_1$ binding, incubation was with 1 nM $^{3}$$^3$H[CHA (20–50 Ci/mmol) for 60 min at 37°C; nonspecific binding was determined by inclusion of 20 μCi unlabeled CHA. For A$_2$ binding, membranes were incubated for 60 min at 25°C in buffer containing 4 nM $^{3}$$^3$H[NECA (15–30 Ci/mm), 10 mM MgCl$_2$, 0.1 U/ml ADA, and 50 nM CPA; nonspecific binding was determined by inclusion of 10 μM NECA. For NBTI binding, incubation was with 0.5 nM $^{3}$$^3$H[NBTI (15–30 Ci/mm) for 30 min at 25°C; nonspecific binding was determined by inclusion of 5 μM NBTI. Membranes were collected onto Whatman GF/B filters with a Brandel Cell Harvester (Brandell Instruments, Gaithersburg, MD) and washed three times with ice-cold buffer. Radioactivity was determined by scintillation counting with a Beckman beta counter. Specific radioligand binding (total binding in absence of test compound minus nonspecific binding) was calculated as (total binding − (binding in presence of test compound)) × 100/(specific binding in absence of test compound). Test compounds were initially dissolved in 80% DMSO and serially diluted in buffer to concentrations ranging from $10^{-11}$ to $10^{-4}$ or $10^{-3}$ M, in duplicate. IC$_{50}$ values were determined via Graphpad Prism (Graphpad Software, Inc., San Diego, CA). In most cases, three or more determinations were made, although specific IC$_{50}$ values are listed only if <10,000 nM.

**General Receptor/Enzyme Screen.** GP683 was tested in radioligand-binding assays for 35 receptor types for neurotransmitters and neuromodulators and activity assays for five enzymes (cyclooxygenase, 5-lipoxygenase, 15-lipoxygenase, phospholipase A$_2$, and protein kinase C) by Panlabs Pharmacology Services (Bothell, WA) at a concentration of 10 μM.

**In Vitro Rat Neocortex Recordings.** In vitro recordings were made with a greased-gap technique (O’Shaughnessy et al., 1988; Aram and Lodge, 1988). Brains were rapidly removed from male Simonsen albino rats (300–350 g; Simonsen, Gilroy, CA) after decapitation, and a 3- to 4-mm-thick coronal section was cut and immersed in continuously gassed (95% O$_2$/5% CO$_2$) artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 2 mM MgSO$_4$, 2 mM KCl, 1.25 mM CaCl$_2$, 11 mM glucose) at room temperature. Coronal sections, 500-μm thick, were cut with a TPI Vibratome (Series 1000; Technical Products International, Inc., St. Louis, MO) and dissected into 1-mm-wide wedges consisting of cortex, white matter, and striatal tissue. The wedges were mounted in a two-compartment chamber such that the cortical tissue lay in one compartment and the white matter and striatal tissue in the other. The cortical tissue chamber was continuously perfused with aCSF at 1.5 to 2 ml/min. The d.c. potential between the two compartments was monitored on a Kipp & Zonen dual-channel recorder (Bohemia, NY) via conventional Ag/AgCl electrodes. The slices were allowed to equilibrate for 1 h, and then Mg$_{2+}$ was omitted from the aCSF. Spontaneous epileptiform events developed within 30 to 60 min. Subsequently baseline responses were obtained for 60 min, before test substances were introduced into perfusate medium. Stock solutions (10 mM) of 5’-dITU and GP683 (in DMSO), GP515 (in saline), and 8-SPT (in water) were added to the aCSF to yield the final concentrations used. Cumulative concentration-response curves were generated by exposing slices ($n = 3–7$) to ascending concentrations for 60 min each. Treatment effects were quantified as the total number of epileptiform events during the last 30 min of exposure to each treatment, expressed as a percentage of the appropriate baseline. For the experiment with 8-SPT, 8-SPT was superfused alone before addition of GP683, and the effect of GP683 was calculated relative to the baseline in the presence of 8-SPT alone.

**In Vivo Assays.** Experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee. Simonsen albino rats were maintained on a 12/12 h light/dark cycle in a temperature-controlled facility. The in vivo assays, conducted during the light part of the cycle, utilized males weighing 100 to 150 g, or 180 to 200 g for hemodynamic studies. Test substances or corresponding vehicles were administered by i.p. injection (1 ml/kg) or p.o. (oral) gavage (4 ml/kg). Rats were fasted overnight before p.o. administration but otherwise had free access to food and water. For i.p. administration, DCF, dipryridamide, 5’-adADO, GP515, theophylline, and GP547 were dissolved in saline; ITU, 5’-dITU, and CPA were dissolved in 20 to 30% polyethylene glycol; and NBTI, GP683, GP836, GP947, GP790, and GP665 were dissolved in DMSO or a solution of 8% DMSO in polyethylene glycol 400. For p.o. administration, GP683 was dissolved in polyethylene glycol 400. Control groups of animals receiving vehicle were tested concurrently with each experiment.

**MES Seizures.** The maximal electroshock (MES) seizure model was used as described by Swinyard et al. (1989). An electrolyte solution (2% lidocaine in 0.9% sodium chloride) was applied to the eyes, and MES seizures were induced by administering a 60-Hz current of 150 mA for 0.2 s via conical electrodes with a Wahlquist model H stimulator (Wahlquist Instrument Co., Salt Lake City, UT). The endpoint measured was suppression of hindlimb tonic extension (HTE), which is expressed as the percentage of animals in which the response was inhibited. At this supramaximal stimulation level, virtually 100% of control (vehicle-treated) animals showed HTE. Except for time course experiments, the test was conducted at 60 min after administration of AK inhibitors or 20 min after administration of CPA. ED$_{50}$ values and 95% confidence intervals (CIs) were calculated from quantal dose-response curves (4–8 animals/dose) via probit analysis (Tallarida and Murray, 1987).

**Locomotor Activity.** Locomotor activity was measured with a Photobeam Activity System (San Diego Instruments, San Diego, CA) consisting of multiple activity arenas fitted with a lower set of 3 photobeams for recording horizontal movements (ambulation) and an upper set of 20 photobeams for recording vertical movements (rearing). Activity counts were totaled over a 15-min test period. The effects of test compounds are shown as percentage of inhibition of activity relative to the mean of a control group of animals that received vehicle administration.

**Hemodynamic Measurements.** For hemodynamic measurements, rats were instrumented with a carotid cannula (Intramedic PE50 polyethylene tubing; Becton Dickinson, Sparks, MD) while anesthetized with 2% halothane (Halocarbon Labs, River Edge, NJ) and allowed to recover. Heart rate and mean arterial pressure were measured in the conscious, freely moving rats with an isolated pre-
tor agonist CPA inhibited MES seizures with an ED \(_{50}\) of 2.3 mg/kg (95% CI, 1.6–3.3). In these experiments, substantial dose-related mortality was observed in rats treated with ITU, and 5'-adADO. At 60 min after i.p. administration of compounds, electrical current was delivered via corneal electrodes; % inhibition is expressed as percentage of animals in which the endpoint of HTE was inhibited. Quantal dose-response curves (n = 4–8/dose) were constructed via probit analysis (Tallarida and Murray, 1987).

### Statistical Analysis
Results are shown as means ± S.D. or S.E.M., except for the quantal values of percentage of inhibition in the MES test. The methods of Tallarida and Murray (1987) were used to calculate 1) ED\(_{50}\) and 95% CI values for inhibition of MES seizures or of locomotor activity and 2) the LD\(_{50}\) value of ITU. Hypothemic and hemodynamic effects were analyzed by ANOVA followed by post hoc comparisons to baseline values via Dunnett’s method.

### Inhibition of MES Seizures by Known AK Inhibitors and CPA
Three previously known AK inhibitors, 5'-adADO, ITU, and 5'-dITU inhibited MES seizures in a dose-related fashion (Fig. 2) with respective ED\(_{50}\) values of 153, 6.2, and 0.3 mg/kg (349, 15.8, and 0.8 \(\mu\)g/ml) determined at 1 h after administration. In comparison, the A\(_1\) adenosine receptor agonist CPA inhibited MES seizures with an ED\(_{50}\) of 2.3 mg/kg (95% CI, 1.6–3.3). In these experiments, substantial dose-related mortality was observed in rats treated with ITU, exhibiting an LD\(_{50}\) of ~14 mg/kg, with mortality occurring over a period of 72 h after the i.p. administration.

### Identification of Novel AK Inhibitors
Analogues of the known AK inhibitors were prepared (Table 1) with modifications that would be expected to possess increased specificity for AK (Table 2). To eliminate the possibility of in vivo 5'-O-phosphorylation, the 5'-OH group was replaced with H, NH\(_2\), halogen, or various alkyl groups. The NH\(_2\) group proved to be one of the preferred substituents, based on enzyme-inhibition potencies (e.g., GP386, IC\(_{50}\) 3 pM). Other ribose modifications were explored, most of which were not well tolerated by the enzyme (e.g., substitutions of the 2' or 3' hydroxyls or replacement of the ribose ring oxygen with carbon). One exception was replacement of the ribose with lyxofuranosyl sugars, which resulted in analogs with potent inhibitory activity (e.g., GP947 and GP790; IC\(_{50}\), 0.46 and 0.80 nM, respectively); these are expected to possess enhanced specificity for AK because most nucleoside binding sites do not recognize lyxofuranosyl-containing compounds. In addition to sugar modifications, various heterocyclic bases and ring substituents were explored. Substituted pyrrolo[2,3-d]pyrimidines were substantially more potent AK inhibitors than their purine analogs. Replacement of the pyrrolypyrimidine heterocycle with a pyrazolopyrimidine led to compounds with similar biological activity (e.g., GP515, GP665, and GP547). Various analogs were synthesized with substituents attached to the 2-, 4-, 5-, and 6-positions of the pyrrolypyrimidine or the 3-, 4-, and 6-positions of the pyrazolopyrimidine heterocycles. Substituents at the 4- and 5-positions of the pyrrolypyrimidines and the 3- and 4-positions of the pyrazolopyrimidines were especially important for activity. Replacement of the iodo group of 5'dITU with H, alkyl, or thio alkyl groups resulted in a dramatic loss in potency, whereas replacement with aryl groups led to potent AK inhibitors. Diarylated compounds are very lipophilic and readily entered cells in vitro and the central nervous system in vivo, as indicated by inhibition of AK activity; moreover, certain of these compounds such as GP683 (see below) have exhibited less adverse pharmacology than the previous lead compounds.

### Selectivity for AK Inhibition
To assess the specificity of the AK inhibitors for AK, the compounds were assayed for activity against ADA and adenosine monophosphate deaminase (the deaminases that also recognize adenosine or adenylates) and for affinity to the A\(_1\) and A\(_2\) (A\(_2\)A) receptors that may exert similar pharmacological effects. No activity was detected against the deaminases, and radioligand-binding assays showed only weak (micromolar) affinity for the A\(_1\) and A\(_2\) receptors (Table 2). The compounds were also tested for affinity to the NBTI-sensitive adenosine transport site. IC\(_{50}\) values for all compounds exceeded 1 \(\mu\)M, with the 5' amino analogs (ITU, GP386, GP515, and GP547) exhibiting particularly weak potencies (IC\(_{50}\) >100 \(\mu\)M; Table 2). An additional screen including radioligand binding at 35 receptor types and activity at 5 enzymes (cyclooxygenase, 5-lipoxygenase, 15-lipoxygenase, phospholipase A\(_2\), and protein kinase C)
shown no activity at a concentration of 10 μM (data not shown).

**Inhibition of MES Seizures by Novel AK Inhibitors.**

The novel AK inhibitors exhibited potent anticonvulsant activity in the rat MES test. These agents inhibited maximal seizures with 100% efficacy in a dose-related fashion (Fig. 3), and ED_{50} values ranged from 0.2 to 15.9 mg/kg (0.5–41.7 μmol/kg; Table 1). One of the most promising inhibitors was GP683, which exhibited potent inhibition of MES seizures without overt toxicity (no mortality after administration of doses up to 100 mg/kg i.p. or 300 mg/kg p.o.) Time-response data with GP683 indicated a time of peak activity 1 h after either i.p. or p.o. administration (Fig. 4). Analysis of plasma concentrations achieved at 1 h after p.o. administration showed dose-related absorption and a plasma EC_{50} of ~195 ng/ml (Table 3). Comparison of plasma levels after p.o. and i.v. administration (not shown) indicated an oral bioavailability of 17%.

**Antagonism by Theophylline.**

The mechanism of the antiseizure activity was evaluated with the adenosine receptor antagonist theophylline, administered 10 min before GP683. With an ED_{50} dose of GP683 (3 mg/kg, i.p.), HTE was inhibited in 8 of 10 rats in the group administered the theophylline vehicle, whereas HTE was inhibited in 0 of 10 rats in the group receiving theophylline (10 mg/kg i.p.). The effect of orally administered GP683 was also antagonized by theophylline (20 mg/kg i.p.), as shown by the dose-response data listed in Table 4.

**Hemodynamic, Hypothermic, and Motor Effects.**

GP683, administered at doses up to 40 mg/kg i.p. (36 times its ED_{50} of 1.1 mg/kg for inhibition of MES seizures) had no effect on mean arterial blood pressure or heart rate in the conscious rat (Fig. 5A). GP683 was administered at either 1 mg/kg i.p. (n = 15/dose) or 5 mg/kg p.o. (n = 8/dose). Percentage of inhibition is expressed as percentage of animals in which the endpoint of HTE was inhibited.

### Table 2

**Affinity and selectivity of AK inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell AK IC_{50} M</th>
<th>IC_{50} nM</th>
<th>A_{1}</th>
<th>A_{2}</th>
<th>NBTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-adADO</td>
<td>86 ± 55</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>ITU</td>
<td>1.6 ± 1.3</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>5′-dITU</td>
<td>3.7 ± 1.8</td>
<td>&gt;10,000</td>
<td>&gt;100,000</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>GP836</td>
<td>12 ± 5</td>
<td>&gt;10,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>GP683</td>
<td>0.10 ± 0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GP790</td>
<td>1.1 ± 0.4</td>
<td>&gt;10,000</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GP947</td>
<td>0.08 ± 0.01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GP547</td>
<td>20 ± 8.4</td>
<td>100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>GP790</td>
<td>0.4 ± 0.1</td>
<td>&gt;10,000</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GP547</td>
<td>0.5 ± 0.1</td>
<td>&gt;10,000</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Means ± S.D. of 5 determinations. ND, not determined.

{\textsuperscript{a}} A_{1} binding by method used (Bruns et al., 1986) primarily reflects binding to A_{2A} receptors (see Experimental Procedures).

{\textsuperscript{b}} Single IC_{50} determination; all other values calculated from ≥3 determinations. ND, not determined.

---

**Fig. 3.** Inhibition of MES seizures by novel AK inhibitors described in Table 1. At 60 min after i.p. administration of compounds, electrical current was delivered via corneal electrodes; % inhibition is expressed as percentage of animals in which the endpoint of HTE was inhibited. Quantal dose-response curves (n = 6–8/dose) were constructed via probit analysis (Tallarida and Murray, 1987).

**Fig. 4.** Time courses of anticonvulsant effect of known AK inhibitors 5′-dADO and 5′-dITU and of novel AK inhibitor GP683. 5′-dADO was administered at 300 mg/kg i.p. (n = 4–7/dose); 5′-dITU was administered at 0.3 mg/kg i.p. (n = 6/dose); GP683 was administered at either 1 mg/kg i.p. (n = 15/dose) or 5 mg/kg p.o. (n = 8/dose). Percentage of inhibition is expressed as percentage of animals in which the endpoint of HTE was inhibited.

**Table 3**

Dose-response and plasma concentrations after p.o. administration of GP683

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>MES Inhibition</th>
<th>Plasma Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>20</td>
<td>75</td>
<td>380 ± 80</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>960 ± 110</td>
</tr>
</tbody>
</table>

*Mean ± S.E.M. for eight animals.
Anticonvulsant Activity of Other Classes of ARAs.
The effects of AK inhibitors in the MES seizure model can be compared to those of other classes of ARAs, specifically inhibitors of ADA and of adenosine transport. The potent and irreversible ADA inhibitor DCF was inactive (0% inhibition) at a dose (1 mg/kg i.p.) reported to produce near-complete inhibition of ADA in the brain (Geiger et al., 1987). NBTI, a potent and selective adenosine transport inhibitor, was also ineffective (0% inhibition) after doses of 10 or 30 mg/kg i.p. Dipyridamole, an inhibitor of NBTI-sensitive and -insensitive adenosine transport, was ineffective (0% inhibition) at doses of 10, 30, and 100 mg/kg i.p.

Antiepileptic Effects of ARAs In Vitro.
The antiepileptic effect of AK inhibitors and other ARAs was evaluated in an in vitro model of epileptic activity. Rat neocortical wedges, perfused with aCSF, exhibited spontaneous potentials when Mg²⁺ was removed from the superfusate. The known AK inhibitor 5'-dITU and the novel AK inhibitors GP683 and GP515 inhibited the spontaneous events in a concentration-dependent manner up to a maximum of 30 to 49% (Fig. 7). The inhibitory effect of GP683 was abolished in the presence of the adenosine receptor antagonist 8-SPT (100 μM). 8-SPT alone (before addition of GP683) increased the frequency of spontaneous events by 139 ± 46% over baseline, and this elevated rate was used as a new baseline for calculating the effect of adding GP683. Neocortical preparations treated

<table>
<thead>
<tr>
<th>Dose of GP683</th>
<th>% Inhibition MES seizures</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>29.6</td>
</tr>
<tr>
<td>2.2</td>
<td>42.9</td>
</tr>
<tr>
<td>5.8</td>
<td>100</td>
</tr>
<tr>
<td>12.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

"Twenty mg/kg i.p. administered 10 min before GP683; n = 7.

Fig. 5. Effect of GP683 and CPA on mean arterial blood pressure (MAP) and heart rate (A) and rectal temperature (B). Doses provided are in milligrams per kilogram i.p. administered at time 0. For the blood pressure/heart rate experiments, the dose of CPA used is equal to its ED₅₀ of 2.3 mg/kg for inhibition of MES seizures; the dose of GP683 shown is 40 mg/kg i.p. (36 times its ED₅₀ of 1.1 mg/kg against MES seizures). Data are means ± S.E.M. For the blood pressure/heart rate experiment (n = 5), there were no significant effects of GP683 (p > .36). For the rectal temperature experiment (n = 4–5), p < .05 for all doses of GP683 at 1 h.

Fig. 6. Inhibition of MES seizures and of locomotor activity by i.p. doses of GP683 or CPA. Locomotor activity is expressed as ambulation (AMB) and rearing. Tests were conducted at 60 min after administration of GP683 (n = 6–8/point) or 20 to 35 min after administration of CPA (n = 8–32/point). Percentage of inhibition of MES seizures is expressed as percentage of animals in which HTE was inhibited. Locomotor activities were totaled over a 15-min period, compared to mean of vehicle-treated control group to calculate percentage of inhibition, and expressed as means ± S.E.M.

Fig. 7. Inhibition of spontaneous epileptiform events recorded in rat neocortical preparations in absence of Mg²⁺. Left, cumulative concentration response curves shown for 5'-dITU, GP515, and GP683. Right, antagonism by adenosine receptor antagonist 8-SPT (100 μM). Inhibition is expressed as percentage of the number of events recorded during baseline recording period. Effect of GP683 combined with 8-SPT was calculated relative to baseline with 8-SPT alone. n = 3–7.
with the ADA inhibitors DCF or EHNA (Schaeffer and Schwender, 1974) exhibited an increased frequency of spontaneous events (Table 5). Adenosine transport inhibitors had variable effects, with NBTI eliciting no concentration-related change in the frequency of spontaneous events and dipyridamole inhibiting a maximum of 32% at 10 μM.

**Discussion**

These studies have demonstrated anticonvulsant activity of AK inhibitors in the rat MES seizure model. This activity was observed with known AK inhibitors and various novel AK inhibitors from several structural classes. In addition, antiepileptic activity of AK inhibitors was demonstrated in vitro in a rat neocortical preparation. These findings extend the observations of Zhang et al. (1993), who demonstrated inhibition of bicuculline-induced limbic seizures by 5’-adADO and ITU injected directly into the prepyriform cortex of the rat. Our report also provides evidence linking the antiseizure activity with AK inhibition and adenosine receptor activation.

Before this work, 5’-adADO, ITU, and 5’-dITU were the most potent AK inhibitors known. The utility of these compounds as a therapeutic approach was limited because of the toxicities described above and in previous reports (Davies et al., 1986; Cottam et al., 1993) or the modest potency and short half-life in the case of 5’-adADO (Fig. 4). These limitations could be at least partly related to the significant structural similarity of the compounds to adenosine and, accordingly, to their ability to bind with protein-binding sites that recognize adenosine or adenylylates. Therefore, novel analogs of these lead compounds were prepared (see also Erion et al., 1997), including the most potent AK inhibitor reported to date (GP836) and a compound (GP683) with substantial in vivo potency and no overt toxicity at doses up to 100 mg/kg i.p., or 91 times its ED50 in this seizure model.

GP683 was chosen as a prototype AK inhibitor for further exploration. Its inhibitory effect on MES seizures was antagonized by the nonselective adenosine receptor antagonist theophylline, indicating that the effect is mediated via the action of adenosine on its receptors. Similar antagonism by theophylline has been observed with several of the AK inhibitors synthesized (data not shown). Theophylline is known to exert proconvulsant effects, although the proconvulsant effect could not be demonstrated in MES seizures because the induction of seizures in this model is at a maximal level. Thus, although the antagonism is consistent with an adenosine-mediated mechanism of action, other mechanisms are not unambiguously eliminated by these studies. The inhibitory effect of GP683 in the rat cortical slice preparation was also antagonized by an adenosine receptor antagonist (8-SPT). In this model, the effect of GP683 was calculated relative to the baseline with 8-SPT alone, thus eliminating the possibility of indirect pharmacological antagonism and supporting the hypothesized mechanism of action. Additional support for this mechanism arises from the breadth of structural classes of the AK inhibitors active in the MES model, because AK inhibition is probably the only pharmacological feature shared uniformly among these compounds. The compounds were not active at other enzymatic steps (ADA, adenosine monophosphate deaminase) in the metabolic pathway that recognize adenosine or adenylylates, and affinity to adenosine receptors or to the NBTI transport site was apparent only at micromolar concentrations. That inhibitors of AK would exert such profound control over the regulation of extracellular adenosine levels is not surprising, because AK is known to play a primary role in regulating the utilization of adenosine under conditions of adequate oxygen and glucose supply (Lloyd and Fredholm, 1995).

The clinical relevance of the MES model is supported by observations that drugs active in this model have shown therapeutic efficacy in generalized tonic-clonic seizures and partial seizures (Upton, 1994; White, 1997). However, despite the activity of currently used drugs against MES, kindled, and other seizures in animals, some epileptic conditions remain refractory to these drugs, perhaps because of different etiologies. In lieu of a comprehensively predictive model, drugs that act via novel mechanisms such as adenosine receptor stimulation should be evaluated. The AK inhibitors investigated in this study exhibited potencies in the MES model similar to those of the most potent adenosine receptor agonists, exemplified by CPA. However, as illustrated by GP683, the AK inhibitors possess a substantially improved side-effect profile. GP683 did not reduce mean arterial pressure and had little effect on heart rate, even at a dose 36-fold above its ED50 for inhibition of MES seizures. In contrast, CPA reduced blood pressure and heart rate to one-third of baseline level when administered at its ED50 for MES seizures. Moreover, the hypothermic effect observed with adenosine receptor agonists in rats was largely absent with GP683, and the inhibition of locomotor activity by GP683 was reduced compared with CPA. It can be hypothesized that this improved profile results from the amplification of the surge of adenosine levels associated with epileptiform activity within seizure foci (Schultz and Lowenstein, 1978; Winn et al., 1980; During and Spencer, 1992). Although AK inhibition is expected to raise basal levels of adenosine, the increase from basal levels (well below 1 μM) is not enough to produce pharmacological activity such as hypotension. In contrast, augmentation of the seizure-related elevation of adenosine would have a greater effect because concentrations would be within the pharmacologically active concentration range. Thus, the impact of AK inhibition would be greatest within the seizure focus during seizure activity, with relatively little consequence on other systems. In contrast, an exogenously
administered adenosine agonist would directly stimulate adenosine receptors in the periphery and other neuronal areas, leading to a greater spectrum of effects.

The epileptiform potentials evoked by Mg$^{2+}$-free conditions in the neocortical preparation are inhibited by conventional anticonvulsant drugs (Aram and Lodge, 1988) and by adenosine receptor activation, and their frequency is tonically controlled by endogenous adenosine (O'Shaughnessy et al., 1988; Kostopoulos et al., 1989). The inhibitory activity of AK inhibitors in this preparation is consistent with these findings. However, the inhibition was incomplete compared with the actions of adenosine and adenosine receptor agonists, which can produce ≥90% reduction in the frequency of the spontaneous events (O'Shaughnessy et al., 1988, and A.C.F. and L. Jelovich, unpublished observations). Moreover, the concentrations of the AK inhibitors required for efficacy were generally 10- to 100-fold higher than their respective IC$_{50}$ values at the isolated enzymes and in cultured cells. It is possible that substantial (e.g., >90%) inhibition of AK activity is required before significant elevation of extracellular adenosine occurs in this preparation. In addition, these compounds typically show low aqueous solubility and high protein binding, factors that could limit their activity in this preparation. Nevertheless, the consistent, concentration-dependent inhibitory activity supports in vivo results that showed AK inhibitors to be completely efficacious against both MES seizures (above) and limbic seizures (Zhang et al., 1993).

In the MES model, DCF was inactive in the dose range previously shown to result in near-complete, irreversible inhibition of ADA in the brain (Geiger et al., 1987). In the neocortical preparation, both DCF and EHNA exerted an excitatory effect; the reason for this effect is not clear, but failure of these agents to mimic adenosine in this model and in the MES model is consistent with previous findings that ADA is not the primary route of adenosine metabolism under normal energy supply (Lloyd and Fredholm, 1995). NBTI and dipryridamole, inhibitors of adenosine transport sites, were ineffective in MES seizures and had a variable effect in the spontaneous events (O'Shaughnessy et al., 1988, and A.C.F. and L. Jelovich, unpublished observations). However, the inhibition was complete compared with the actions of adenosine and adenosine receptor agonists, which can produce ≥90% reduction in the frequency of the spontaneous events (O'Shaughnessy et al., 1988, and A.C.F. and L. Jelovich, unpublished observations). Moreover, the concentrations of the AK inhibitors required for efficacy were generally 10- to 100-fold higher than their respective IC$_{50}$ values at the isolated enzymes and in cultured cells. It is possible that substantial (e.g., >90%) inhibition of AK activity is required before significant elevation of extracellular adenosine occurs in this preparation. In addition, these compounds typically show low aqueous solubility and high protein binding, factors that could limit their activity in this preparation. Nevertheless, the consistent, concentration-dependent inhibitory activity supports in vivo results that showed AK inhibitors to be completely efficacious against both MES seizures (above) and limbic seizures (Zhang et al., 1993).

In conclusion, AK inhibitors are potent anticonvulsants in the rat MES model in vivo and exert antiepileptic effects in a rat neocortical preparation in vitro. Studies demonstrating antiseizure activity with various structurally diverse classes of AK inhibitors and studies demonstrating the reversal of this activity with an adenosine receptor antagonist strongly support the role of AK in anticonvulsant action. Among the ARA classes examined, AK inhibitors represented the best approach for antiepileptic activity. Furthermore, novel AK inhibitors exerted little hypotensive or bradycardic activity and resulted in minimal hypothermia and decreased suppression of motor activity compared with adenosine receptor agonists in the rat. AK inhibitors thus represent a novel approach to anticonvulsant therapy that, through amplification of adenosine feedback, may provide broad protection from seizures and a valuable addition to current anticonvulsant drugs.

Acknowledgments
We thank Stacy Zimring, Laura Jelovich, Christine Haywood, Susanne Bayat, Jay DaRe, Joe Kopcho, Michelle Ramirez-Weinhaus, and Juergen Schanzer for expert technical assistance.

References


Send reprint requests to: James Wiesner, Metabasis Therapeutics, Inc., 9390 Towne Center Drive, San Diego, CA 92121. E-mail: wiesner@mbasis.com