The Metabotropic Glutamate Receptor 7 Allosteric Modulator AMN082: A Monoaminergic Agent in Disguise?

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Received November 21, 2010; accepted April 12, 2011

ABSTRACT

Metabotropic glutamate receptor 7 (mGluR7) remains the most elusive of the eight known mGluRs primarily because of the limited availability of tool compounds to interrogate its potential therapeutic utility. The discovery of N,N’-dibenzhydrylethylene-1,2-diamine dihydrochloride (AMN082) as the first orally active, brain-penetrable, mGluR7-selective allosteric agonist by Mitsukawa and colleagues (Proc Natl Acad Sci USA 102:18712–18717, 2005) provides a means to investigate this receptor system directly. AMN082 demonstrates mGluR7 agonist activity in vitro and interestingly has a behavioral profile that supports utility across a broad spectrum of psychiatric disorders including anxiety and depression. The present studies were conducted to extend the in vitro and in vivo characterization of AMN082 by evaluating its pharmacokinetic and metabolite profile. Profiling of AMN082 in rat liver microsomes revealed rapid metabolism (t1/2 < 1 min) to a major metabolite, N-benzhydrylethylene-1,2-diamine (Met-1). In vitro selectivity profiling of Met-1 demonstrated physiologically relevant transporter binding affinity at serotonin transporter (SERT), dopamine transporter (DAT), and norepinephrine transporter (NET) (323, 3020, and 3410 nM, respectively); whereas the parent compound AMN082 had appreciable affinity at NET (1385 nM). AMN082 produced antidepressant-like activity and receptor occupancy at SERT up to 4 h postdose, a time point at which AMN082 is significantly reduced in brain and plasma while the concentration of Met-1 continues to increase in brain. Acute Met-1 administration produced antidepressant-like activity as would be expected from its in vitro profile as a mixed SERT, NET, DAT inhibitor. Taken together, these data suggest that the reported in vivo actions of AMN082 should be interpreted with caution, because they may involve other mechanisms in addition to mGluR7.

Introduction

Metabotropic glutamate receptors (mGluRs) are a diverse and critically important group of receptors involved in the regulation of neurotransmission in the CNS (Conn and Pinn, 1997; reviewed in Cartmell and Schoepp, 2000). Of the eight described receptor subtypes, mGluR7 has been proposed as one of the most important to affective states based on its wide distribution in the CNS and in brain regions relevant to anxiety and depression (e.g., amygdala, hippocampus, hypothalamus) (Kinoshita et al., 1998). It is noteworthy that mGlu7 receptors seem to be exclusively located near or within the active zone of presynaptic terminals on glutamate neurons, which suggests that mGluR7 is an autoreceptor serving a specific role in regulating glutamate release at the synapse (Glaum and Miller, 1997). mGlu7 receptors are also located on presynaptic γ-aminobutyric acid interneurons acting as heteroreceptors (reviewed in Cartmell and Schoepp, 2000), which may contribute significantly to how potential pharmacotherapeutic agents acting at mGluR7 might modulate neuropsychiatric disease.

ABBREVIATIONS: mGlu, metabotropic glutamate; mGluR, mGlu receptor; CNS, central nervous system; AMN082, N,N’-dibenzhydrylethylene-1,2-diamine dihydrochloride; Met-1, metabolite 1 (N-benzhydrylethylene-1,2-diamine); AUC0-t, area under the curve at last observed time point; t1/2, time to maximum concentration; Cmax, maximum concentration; LC, liquid chromatography; HPLC, high-performance LC; MS, mass spectrometry; SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; DASB, N,N-dimethyl-2-(2-amino-4-cyanophenyl)thiol benzylamine; SSR, selective serotonin reuptake inhibitor; ANOVA, analysis of variance; KO, knockout; ACN, acetonitrile; RLM, rat liver microsomes; CHO, Chinese hamster ovary; WIN 35428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; L-AP4, (2S)-2-amino-4-phosphonobutanoic acid; ESI, electrospray ionization; i.p., intraperitoneally; 5-HT, 5-hydroxytryptamine.
rotransmission. Furthermore, mGluR7 has a low affinity for glutamate (>100 μM), indicating it would only be engaged in situations where extracellular glutamate concentrations are elevated and implicating this receptor system as an ideal target for a number of pathological conditions that are thought to involve hyperactive glutamate neurotransmission such as anxiety and depression (Kuguya and Sanacora, 2005; Conn and Niswender, 2006; Hashimoto, 2009).

Prior to the availability of selective pharmacological tools for directly interrogating mGluR7, preclinical studies evaluating the consequence of mGluR7 deletion in knockout (KO) animals or by gene silencing demonstrated an antidepressant- and anxiolytic-like phenotype. Specifically, mGluR7 KO mice demonstrated that, relative to wild-type cohorts, KO mice exhibit an antidepressant-like phenotype in the forced swim test and the tail suspension test as well as an anxiolytic-like phenotype (Masugi et al., 1999; Cryan et al., 2006), which demonstrated that the antidepressant-like phenotype of mGluR7 KO mice in their studies was in direct correlation with increased levels of hippocampal brain-derived neurotrophic factor protein, a biochemical effect that has been demonstrated to occur after treatment with antidepressant agents (Thakker et al., 2005), demonstrating that short interference RNA knockdown of the mGlu7 receptor produces a similar phenotype, and by Mitsukawa et al. (2006), who demonstrated that the antidepressant-like phenotype of mGluR7 KO mice in their studies was in direct correlation with increased levels of hippocampal brain-derived neurotrophic factor protein, a biochemical effect that has been reported to occur after treatment with antidepressant agents (reviewed in Martinovich et al., 2007). Taken together, these data led to an initial hypothesis that novel agents acting as antagonists of mGluR7 would produce antidepressant- and anxiolytic-like activity.

The discovery of the first orally active, brain-penetrable mGluR7 allosteric agonist, N,N’-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082), by Mitsukawa et al. (2005) provided an extremely valuable tool for elucidating the consequences of directly activating this receptor. In vitro, AMN082 demonstrates potent agonist activity and is reported to be highly selective for mGluR7 relative to other glutamate receptor subtypes as well as having no reported interactions with a variety of CNS targets in a receptor binding analysis (Mitsukawa et al., 2005). AMN082 has been used extensively to interrogate the role of mGluR7 activation in vivo and has been reported to produce broad-spectrum activity across a number of preclinical models including anxiety and depression (Mitsukawa et al., 2005; Paluch et al., 2007; Dolan et al., 2009; Li et al., 2009; Greco et al., 2010), results that would not have been predicted based on the phenotype of the mGluR7 knockout mice. However, at the doses required to observe efficacy in a number of these preclinical models, nonspecific behaviors are also often reported such as tremors and reduced locomotor activity (Paluch et al., 2007), thereby suggesting possible off-target effects of AMN082 (see discussion in Salling et al., 2008). These conflicting reports, coupled to an initial hypothesis that antagonists of mGluR7 would be predicted to have anxiolytic- and antidepressant-like activity, prompted us to extend the in vitro and in vivo characterization of AMN082 to evaluate its pharmacokinetic and metabolite profiles to help elucidate the mechanism by which this compound might be producing efficacy in preclinical models predictive of antidepressant-like activity.

Materials and Methods

Animals. All experiments were performed in accordance with the specifications of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (revised 1996) and under the approval of the Institutional Animal Care and Use Committee. Animals were group-housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility upon arrival and maintained on a 12-h light/dark cycle (lights on at 6:00 AM) with food and water provided ad libitum. Upon arrival, animals were acclimated to the facility for a minimum of 5 days prior to experiments.

Materials. AMN082 was purchased from Ascent Scientific (Bristol, UK). After metabolite profiling and identification as described below, metabolite 1 (Met-1) (N-benzyldihydrolatan-1,2-diamine) was synthesized by Pfizer Chemical Sciences (Princeton, NJ) as the internal standard compounds used for assay controls. Ammonium acetate (GR ACS Crystals), acetonitrile (ACN; HPLC grade), and water (HPLC grade) were purchased by EMD Chemicals Inc. (Gibbstown, NJ). Drug-free EDTA plasma was obtained from Bioreclamation (Hicksville, NY). For in vivo binding experiments, [3H]DASB was synthesized by the Pfizer Radiosynthesis and Chemical Development Group (Pearl River, NY).

Microsomal Stability. Microsomal stability in rat liver microsomes (RLM) was determined as described previously (Di et al., 2006). In brief, compounds were dissolved in dimethyl sulfoxide/ACN and added directly to diluted rat liver microsomes. The reaction was initiated by adding NADPH-regenerating solution. The solutions were incubated at 37°C for 15 min. At the end of the incubation, cold ACN was added to stop the reaction. The solution was centrifuged, and the supernatant was transferred for LC-MS-MS analysis.

Metabolite Profiling of AMN082 in Rat Liver Microsomes. Male Sprague-Dawley RLM and NADPH reagents were purchased from BD Gentest (San Jose, CA). Potassium phosphate monobasic, dibasic sodium phosphate, formic acid, LC grade water, methanol (ACN), and ACN were obtained from EMD Chemicals. AMN082 (10 μM) was incubated with RLM (1.0 mg/ml) and a freshly prepared NADPH solution (1 mM) in potassium phosphate buffer (100 mM, pH 7.4) at 37°C for 15 min. The total incubation volume was 1.0 ml. The incubation reaction was initiated by the addition of a NADPH solution and stopped by the addition of 1 ml of ACN. After centrifugation (13,000 rpm for 10 min), supernatants were dried down under nitrogen and reconstituted in 50:50 of ACN/H2O for LC/MS and LC/MS/MS analysis. An Agilent 1100 series HPLC system (Hewlett-Packard GmbH, Waldbronn, Germany) was used in this study. The chromatographic separation was carried out using a Chromolith RP-18E (100 × 3.0 mm i.d.) (Merck, Darmstadt, Germany), maintained at 40°C. The mobile phase consisted of solvent A [0.1% formic acid in water-ACN (H2O/ACN = 95:5, v/v)] and B [0.1% formic acid in water-ACN (H2O/ACN = 95:5, v/v) and 0.1% formic acid in water-ACN (H2O/ACN = 95:5, v/v)]. The injection volume was 20 μl. The HPLC analysis started with 5% B for 0.5 min, followed by a gradient from 5 to 95% B in 4 min, and subsequently held at 95% B for 1 min. The flow rate was 1 ml/min. The HPLC flow was split before the MS and −0.2 ml/min effluent was directed into the electrospray ionization (ESI) source of the mass spectrometer. The HPLC system was interfaced to a Micromass Quattro Top tandem quadrupole mass spectrometer (Waters, Milford, MA) operated in positive ESI mode ([+]+ESI) with the ion source temperature of 150°C. The (+)ESI conditions were optimized to a desolvation temperature of 350°C, a spray voltage of 3.5 kV, and a cone voltage of 20 V. Nitrogen was used as both desolvation (1000 l/h) and nebulizer gas (fully open). The MS analysis was performed at a scan range from 100 to 800 Da. The MS/MS analyses were performed using argon as the collision gas with collision energy at 30 eV.
Receptor Selectivity Screening. Interaction of AMN082 and Met-1 with a selected panel of major neurotransmitter binding sites (16) was examined in competition binding experiments. Affinities for recombinantly expressed human SERT, DAT, and NET were determined in scintillation proximity assays with membrane preparations and selective radioligands. Human SERT was expressed in human embryonic kidney 293 cells and labeled with [3H]citalopram, whereas human DATs expressing CHO-K1 membranes were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA) and labeled with [3H]WIN 35428 (2S-carbomethoxy-3-carbonyl-4-fluorophenyl)tropane, and human NET was expressed in MDCCK cells and labeled with [3H]Hinokisxetine.

Activity at mGluR7. Functional activity of AMN082 and Met-1, as well as the control compounds (2S)-2-amino-4-phosphobutanolic acid (L-AP4) (agonist), 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-yl-isoxazololo[4,5-c]pyridin-4(H)-one (MMP1), and 3,4-methylenedioxy-N-isopropylamphetamine (MDIP), were determined by Euroscreen (Brussels, Belgium) in a mGluR7-CHO-K1 cell line (GenBank accession number NP_000835.1). Detached cells were assayed in suspension (2500 cells/well) for mGluR7-mediated inhibition of forskolin-stimulated (5 μM) adenylate cyclase activity in 96-well plates. 3-Isobutyl-1-methylxanthine (IBMX, 1 mM) was used as a phosphodiesterase inhibitor. Test compound was added to cells and incubated for 10 min, then cells were challenged with forskolin and either an EC50 of L-AP4 (1.18 mM) for negative allosteric modulator testing or an EC50 of L-AP4 (1.6 μM) for positive allosteric modulator testing. The plates were then incubated for 30 min at room temperature and lysed with the kit-supplied reagent. cAMP was detected using competitive immunoassay technology according to the manufacturer’s protocol (ITRF kit from Cisbio, Bedford, MA).

Pharmacokinetic Studies in Mice. The pharmacokinetics of AMN082 and its primary metabolite Met-1 were characterized in male Swiss-Webster mice (17–22 g) after a single intraperitoneal dose of 10 mg/kg. Blood and brain samples were collected at intervals (n = 3 animals per time point) and up to 6 h after AMN082 or Met-1 administration into tubes containing EDTA as the anticoagulant, and the content was diluted 1:1 with deionized water. Samples were treated for protein precipitation by the addition of acetonitrile containing an internal standard. The supernatants were transferred into clean test tubes and evaporated under a stream of N2 at 37°C followed by reconstitution in acetonitrile/deionized water [50:50 (v/v)] for LC/MS analysis. LC/MS analysis was carried on Agilent 1100 HPLC interfaced with a Sciex API-4000 (Thonhill, Toronto, Canada) for LC/MS analysis. The binding assay was initiated by the addition of 50 μl of tissue homogenate (1.25 mg wet wt./well) and incubated at room temperature for 1 h. The reaction was terminated by rapid vacuum filtration through presoaked (0.5% polyethylenimine) Whatman GF/B filter mats (Whatman, Clifton, NJ). Filters were washed with ice-cold 50 mM Tris, pH 7.5 and transferred to scintillation vials containing Ultima Gold LSC scintillation cocktail. Radioactivity was measured using the PerkinElmer TriCarb liquid scintillation counter. The inhibition of specific [3H]DASB binding, calculated as a percentage of vehicle-treated animals, was determined to provide an indication of receptor occupancy (percentage of receptor occupancy = (1 – (specific binding with compound)/specific binding with vehicle)) × 100).

Results

Metabolite Profiling of AMN082. The chemical structures of AMN082 and its metabolites are presented in Fig. 1. AMN082 was incubated with RLM in the presence of NADPH. Analysis of the incubation mixture by LC/MS and MS/MS revealed that the stability of AMN082 in RLM was poor (t1/2 < 1 min) with major formation of two N-dealkylated metabolites, which should result from C-N-bond cleavage from the initially formed monohydroxylated metabolites with oxidation on the α-carbon to adjoined nitrogen atoms on the molecule (Fig. 1). Minor metabolites included monohydroxylated and dihydroxy metabolites with oxidation on the phenyl rings.

Activity of AMN082 and Met-1 at mGluR7. AMN082 in combination with the EC20 of L-AP4 inhibited forskolin-induced adenylate cyclase activity through mGluR7 with an EC50 of 81 nM consistent with allosteric agonist activity (Fig. 2). The primary metabolite of AMN082, Met-1, produced similar allosteric agonist activity at mGluR7, how-
ever, with approximately 100-fold less potency (EC\textsubscript{50} = 5936 nM; Fig. 2).

**Selectivity Screening Binding Assay.** Results from binding assays across 16 different neurotransmitter receptor and transporter binding sites are presented in Table 1. In addition to its reported activity at mGluR7, AMN082 binds to the α1-adrenergic receptor and NET. Likewise, Met-1 has appreciable affinity at both NET and DAT (3410 and 3020 nM, respectively) and 10-fold higher affinity for SERT (3230 nM). The affinities of AMN082 and its primary metabolite at NET, DAT, and SERT are especially interesting in light of AMN082’s profile of anxiolytic-like and antidepressant-like activity, and the similar properties of the selective serotonin reuptake inhibitors (SSRIs) and mixed transporter inhibitors (serotonin/norepinephrine reuptake inhibitors, triple reuptake inhibitors).

**Antidepressant-Like Activity of AMN082 after a 60-min Pretreatment.** In the tail suspension test, acute 60-min pretreatment of AMN082 (1–10 mg/kg i.p.) to naive Swiss-Webster mice produced the expected antidepressant-like effect as indicated in previous literature reports. One-way ANOVA revealed a statistically significant effect of treatment relative to vehicle-treated control (Fig. 3A; \( F_{4,99} = 13.45; p < 0.001 \)). Post hoc analysis indicated significant reductions in immobility time by 42 and 50%, respectively, for 5.6 and 10 mg/kg, relative to vehicle-treated controls (\( p < 0.01 \) relative to vehicle treatment). In comparison, acute administration of the standard SSRI antidepressant fluoxetine (30–56 mg/kg i.p., 60-min pretreatment) produced the expected dose-dependent reductions in immobility time indicative of an antidepressant-like effect (Fig. 3A; \( F_{2,33} = 6.36; p < 0.01 \)). Post hoc analysis indicated a significant reduction in immobility time at the highest dose tested (56 mg/kg), which was a 44% reduction in immobility time relative to vehicle-treated controls (\( p < 0.01 \) relative to vehicle treatment).

### Table 1

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<td>Muscarinic, M2</td>
<td>&gt;10,000,&gt;10,000</td>
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[Fig. 1. The proposed metabolites of AMN082 and ion chromatogram after 15-min incubation in rat liver microsomes (10 μM). RT, retention time; MW molecular weight.]

[Fig. 2. Functional activity of AMN082 and its primary metabolite Met-1 in CHO-K1 cells overexpressing human mGluR7.]
Pharmacokinetic Properties of AMN082. Brain and plasma exposure levels after a single dose of AMN082 were determined in naive male Swiss-Webster mice (Fig. 3B). After a single injection of 10 mg/kg i.p., AMN082 was found to cross the blood-brain barrier with peak brain concentrations of 339 ng/g recorded at 30 min post-treatment. In plasma, peak exposure levels of 424 ng/ml were also observed at 30 min post-treatment. The brain/plasma ratio of 0.99 was based on AUC0-t 569 (h/18528 ng/g) in brain and 575 (h/18528 ng/ml) in plasma (Table 2). It is noteworthy that at the time point that corresponded with antidepressant-like efficacy of AMN082 in the tail suspension test (1 h) the mean concentration of AMN082 in brain was reduced to 177 ng/g, whereas plasma concentration at the 1-h time point was reduced to 165 ng/ml (Fig. 3B).

Antidepressant-Like Activity of Met-1. Acute 60-min pretreatment of Met-1 (3–10 mg/kg i.p.) to naive Swiss-Webster mice resulted in a dose-dependent reduction in immobility time in the tail suspension test (Fig. 4A) consistent with an antidepressant-like effect. One-way ANOVA revealed a significant effect of treatment relative to vehicle control ($F_{3,34} = 4.234; p < 0.05$). Post hoc analysis indicated a statistically significant reduction in immobility time at the 10 mg/kg dose, which was a 52% reduction relative to vehicle-treated controls ($p < 0.05$).

Pharmacokinetic Properties of Met-1. Brain and plasma exposure levels after a single dose of Met-1 were determined in male Swiss-Webster mice (Fig. 4B). After a single injection of 10 mg/kg i.p., Met-1 was found to robustly cross the blood-brain barrier with peak brain concentrations measured at 5 h post-treatment of 9267 ng/g and peak plasma concentrations measured at 30 min post-treatment of 1055 ng/ml. The brain/plasma ratio was 18.80 based on AUC0-t 46,715 (h/18528 ng/g) in brain and 2482 (h/18528 ng/ml) in plasma (Table 3). At the time point that corresponded with antidepressant-like efficacy of Met-1 in the tail suspension test (1 h), the mean concentration of Met-1 in brain was 6875 ng/g, whereas plasma concentration at the 1-h time point was reduced to 884 ng/ml (Fig. 4B).

Antidepressant-Like Activity of AMN082 after a 4-h Pretreatment. Four-hour pretreatment of AMN082 (5.6–10 mg/kg i.p.) to naive Swiss-Webster mice resulted in a dose-dependent reduction in immobility time in the tail suspension test (Fig. 4A). After a single injection of 10 mg/kg i.p., Met-1 was found to robustly cross the blood-brain barrier with peak brain concentrations measured at 5 h post-treatment of 9267 ng/g and peak plasma concentrations measured at 30 min post-treatment of 1055 ng/ml. The brain/plasma ratio was 18.80 based on AUC0-t 46,715 (h/18528 ng/g) in brain and 2482 (h/18528 ng/ml) in plasma (Table 3). At the time point that corresponded with antidepressant-like efficacy of Met-1 in the tail suspension test (1 h), the mean concentration of Met-1 in brain was 6875 ng/g, whereas plasma concentration at the 1-h time point was reduced to 884 ng/ml (Fig. 4B).

Fig. 3. Antidepressant-like activity of AMN082 and correlating brain and plasma exposure levels in Swiss-Webster mice. A, acute administration of AMN082 (60-min pretreatment, intraperitoneally) produced antidepressant-like activity in the tail suspension test with reductions in immobility time comparable with the standard SSRI antidepressant agent fluoxetine. *, $p < 0.05$ versus appropriate vehicle-treated control. B, at the correlating efficacy time point (60 min) AMN082 levels in brain and plasma decreased.

Fig. 4. Antidepressant-like activity of Met-1, the primary metabolite of AMN082, and correlating brain and plasma exposure levels in Swiss-Webster mice. A, acute administration of Met-1 (60-min pretreatment, intraperitoneally) produced antidepressant-like activity in the tail suspension test. *, $p < 0.05$ versus appropriate vehicle-treated control. B, at the correlating efficacy time point (60 min) Met-1 continued to increase in brain.

### Table 2

<table>
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<tr>
<th>Parameter</th>
<th>Brain (ng/g or ng/ml)</th>
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<td>$C_{max}$</td>
<td>339 ± 18</td>
<td>424 ± 42</td>
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<tr>
<td>$t_{max}$ (h)</td>
<td>0.5</td>
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<tr>
<td>AUC0-t (h/18528 ng/g or ng/ml)</td>
<td>569 ± 40</td>
<td>575 ± 51</td>
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test consistent with an antidepressant-like effect (Fig. 5A; \( F_{2,38} = 2.973; p = 0.0638 \)). Although the overall ANOVA was not statistically significant, planned post hoc analysis revealed a statistically significant 34% reduction in immobility time at the highest dose tested (10 mg/kg) relative to vehicle-treated controls (\( p < 0.05 \) relative to vehicle treatment).

**Pharmacokinetic Properties of Met-1 after Acute Administration of AMN082.** Brain and plasma exposure levels were calculated for the primary metabolite (Met-1) after a single 10 mg/kg i.p. injection of AMN082 (Fig. 5B). Met-1 levels continued to rise in brain with peak exposures of 897 ng/g recorded at 5 h post-treatment with AMN082. In plasma, peak exposure levels of 494 ng/ml for Met-1 were observed at 30 min post-treatment with AMN082 and were reduced to 54.9 ng/ml by 5 h post-treatment with AMN082. The brain/plasma ratio was 4.54 based on AUC\(_{0-4h}\) 3712 (h·ng/g) in brain and 818 (h·ng/g/ml) in plasma (Table 4).

**SERT Occupancy.** AMN082 produced a modest time-dependent inhibition of \([3H]DASB\) binding in hippocampus and striatum, indicating an interaction with SERT. One-hour pretreatment with 5.6 mg/kg AMN082 resulted in 1.75 and 2.25% receptor occupancy, respectively, in hippocampus and striatum. After 1-h pretreatment of 10 mg/kg AMN082, the percentage of receptor occupancy increased in both hippocampus and striatum to 6.25 and 3.75%, respectively. It is noteworthy that 4 h after administration of AMN082 (5.6 mg/kg) the percentage of receptor occupancy levels in hippocampus and striatum increased to 17.0 and 7.75%, respectively. Four hours after 10 mg/kg AMN082 administration the percentage of receptor occupancy was increased >2-fold relative to the levels achieved at the 1-h pretreatment time for hippocampus and striatum to 14.75 and 15.50%, respectively. These data are consistent with increasing brain concentrations of the primary metabolite of AMN082 over time and with increasing doses after administration of the parent compound (Fig. 5C).

**Discussion**

The primary purpose of these studies was to extend the characterization of the first reported mGluR7-specific allosteric modulator, AMN082, by evaluating its pharmacokinetic and metabolite profile. These data are the first to report that after peripheral administration this compound is rapidly metabolized from the parent compound to a major metabolite (Met-1) that demonstrates appreciable affinity at multiple monoamine transporters (SERT, DAT, and NET), which likely contribute to its antidepressant-like efficacy in preclinical behavioral models as well as some reports of non-specific behavioral effects (Palucha et al., 2007; Salling et al., 2008).

The initial pharmaceutical profiling of AMN082 was conducted in vitro in rat liver microsomes. AMN082 demonstrated rapid metabolism with a \( t_{1/2} \) of less than 1 min. From these studies we identified a major metabolite (Met-1). Based on the rapid metabolism observed with AMN082, albeit in vitro, we hypothesized that this primary metabolite may be contributing to the in vivo activity of its parent compound and synthesized it for further profiling for functional activity, selectivity, in vivo pharmacokinetics, and antidepressant-like efficacy.

Consistent with previous reports (Mitsukawa et al., 2005), AMN082 acts as a positive allosteric agonist of the mGluR7 receptor in vitro. Met-1 also demonstrated allosteric agonist activity of mGluR7 in vitro, but with approximately 100-fold less potency than the parent compound AMN082. Further profiling of Met-1 in a cross-screening panel consisting of a variety of 16 CNS receptors revealed that Met-1 demon-

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**Fig. 5.** Antidepressant-like efficacy of AMN082 after 4-h pretreatment in the tail suspension test and correlating exposure levels in brain and plasma of the primary metabolite Met-1 relative to percentage of receptor occupancy at SERT. A, 4-h pretreatment of AMN082 produced antidepressant-like activity in the tail suspension test in Swiss-Webster mice. *\( p < 0.05 \) versus appropriate vehicle-treated control. B, increasing exposure of Met-1 in brain after 4-h pretreatment of the parent compound AMN082 implicated a role for this metabolite in centrally mediating the behavioral effects of AMN082. C, the relationship of percentage of receptor occupancy at SERT relative to increasing brain concentrations of Met-1 after 1- and 4-h pretreatment with AMN082.

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**TABLE 3**

<table>
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<tr>
<th>Parameter</th>
<th>Brain (ng/g or ng/ml)</th>
<th>Plasma (ng/g or ng/ml)</th>
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<tr>
<td>( C_{\text{max}} )</td>
<td>9267 ± 1189</td>
<td>1055 ± 37</td>
<td>3.77</td>
</tr>
<tr>
<td>( t_{\text{max}} ) (h)</td>
<td>3.00</td>
<td>0.5</td>
<td>6.00</td>
</tr>
<tr>
<td>( AUC_{0-4h} ) (h·ng/g or ng/ml)</td>
<td>46715 ± 3404</td>
<td>2482 ± 116</td>
<td>18.80</td>
</tr>
</tbody>
</table>

**TABLE 4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Brain (ng/g or ng/ml)</th>
<th>Plasma (ng/g or ng/ml)</th>
<th>Brain/Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} )</td>
<td>897 ± 103</td>
<td>494 ± 67</td>
<td>1.80</td>
</tr>
<tr>
<td>( t_{\text{max}} ) (h)</td>
<td>5.00</td>
<td>0.5</td>
<td>10.00</td>
</tr>
<tr>
<td>( AUC_{0-4h} ) (h·ng/g or ng/ml)</td>
<td>3712 ± 203</td>
<td>818 ± 41</td>
<td>4.54</td>
</tr>
</tbody>
</table>
strained 300 to 3000 nM affinity at SERT, NET, and DAT. Unexpectedly, the parent compound AMN082 also demonstrated micromolar affinity at NET and α1-adrenergic receptors, which has not been previously reported to our knowledge. Irrespective of the modest affinity of AMN082 for receptors in addition to mGluR7, we hypothesized that the affinity of Met-1 for NET, DAT, and most particularly SERT, may contribute to the antidepressant-like activity previously reported for its parent compound AMN082 given that inhibitors of the serotonin transporter, either selectively (SSRIs) or in combination with inhibitors of the norepinephrine transporter (serotonin/norepinephrine reuptake inhibitors) and the dopamine transporter (triple reuptake inhibitors) have proven antidepressant efficacy. Therefore, the next set of experiments aimed to evaluate the antidepressant-like and pharmacokinetic profiles of Met-1 and the parent compound AMN082.

Consistent with previous literature reports, AMN082 demonstrated antidepressant-like activity in the tail suspension test in mice with efficacy comparable with the profile of the standard SSRI antidepressant fluoxetine in this assay. Analysis of brain and plasma levels after AMN082 administration demonstrated that AMN082 crosses the blood-brain barrier, peaks at approximately 30 min in both brain and plasma, and is reduced to modest exposure levels (<200 ng/g) by the 1-h efficacy time point. Acute administration of Met-1 demonstrated antidepressant-like activity in the tail suspension test as would be predicted by its binding profile and in particular its affinity for SERT. These data are in line with a report from Palucha-Poniewiera et al. (2010), who report that the antidepressant-like profile of AMN082 in rodents may be serotonin-dependent. It is noteworthy that at the corresponding efficacy time point Met-1 continued to increase in brain, exceeding 6000 ng/g.

Based on the impressive pharmacokinetic profile of Met-1 and its antidepressant-like activity in mice, the next set of experiments aimed to assess the antidepressant-like efficacy of AMN082 at a time point at which the parent compound AMN082 was reduced in brain while its primary metabolite Met-1 was present. Four-hour pretreatment of AMN082 to mice produced an antidepressant-like effect in the tail suspension test. The most important observation from this study was that at this time point AMN082 is significantly reduced in brain and plasma to <100 ng/g. However, analysis of Met-1 from brain and plasma of mice treated with AMN082 demonstrated that Met-1 is indeed present at brain levels in excess of 800 ng/g, suggesting that the primary metabolite of AMN082 may likely be responsible for the centrally mediated behavioral effects of AMN082. In addition, based on the exposure data indicating that Met-1 continues to increase in brain over time, it seems that this metabolite is likely formed in brain after peripheral administration of AMN082.

Given the reduced potency of AMN082 and Met-1 for SERT relative to fluoxetine, it is interesting to note that comparable reductions in immobility time were achieved in the tail suspension test. Although the tail suspension test is useful in predicting antidepressant-like effects in vivo and AMN082 demonstrated similar levels of reduced immobility time relative to fluoxetine, the assay does not provide the level of sophistication to predict clinical response nor can the test be used to determine whether compounds that have greater reduction in immobility time in mice would have improved efficacy in the clinic. It is noteworthy that 80% SERT occupancy has been reported to be required to achieve efficacy in vivo (Meyer et al., 2004), whereas in vivo in animal models used to predict antidepressant-like activity, such as the tail suspension test, subeffective doses of SSRIs achieve >80% receptor occupancy. However, it is well known that this assay is sensitive to SSRIs and other monoaminergic-based mechanisms of action; although novel nonmonoaminergic compounds have also been reported to demonstrate efficacy in this test. Therefore to demonstrate that the antidepressant-like activity of AMN082 is likely mediated by activity at SERT, we conducted ex vivo binding experiments after pretreatment with AMN082. Although the percentage of receptor occupancy levels reported in the present study were modest at best at the doses tested (<20%), the data demonstrate time-dependent increases in the percentage of receptor occupancy in brain regions relevant for antidepressant efficacy, thereby suggesting that there is indeed an interaction at SERT. The SERT occupancy measured in our ex vivo binding may underestimate the SERT occupancy in vivo because of moderate affinity of the Met-1 interaction. Because the off-rate of the primary metabolite of AMN082 is unknown, we used optimized experimental conditions for fluoxetine, a high-affinity SERT ligand, which achieved 95% receptor occupancy under these conditions (data not shown). It is likely that during the 1-h ex vivo binding incubation the metabolite may dissociate and under-represent the true receptor occupancy in vivo (Li et al., 2006). Nonetheless, these data demonstrate a positive relationship between the brain concentration of the primary metabolite of AMN082 and SERT occupancy and provide additional support for a role of SERT activity in the antidepressant-like effects of AMN082.

In summary, the present data indicate that peripheral administration of AMN082 results in rapid metabolism of parent compound to a primary metabolite with appreciable affinities for SERT, NET, and DAT. Although this may be a likely explanation for its antidepressant-like efficacy and is consistent with the report by Palucha-Poniewiera et al. (2010), which suggests a serotonin-dependent effect for the antidepressant-like activity of AMN082, other potential unknown mechanisms as well as contributory activity at mGluR7 cannot be fully discounted. Taken together, the present data suggest that the historical interpretation of the antidepressant-like properties of AMN082 being attributed specifically to mGluR7 modulatory activity may be confounded by additional contributing mechanisms of action of the compound and caution should be taken for interpreting the in vivo activity of AMN082.

Acknowledgments

We thank Dr. Mauricio Leal and the biometrics staff at Pfizer, Pearl River, NY for expert biostatistical guidance and helpful discussions of the pharmacokinetic data.

Authorship Contributions

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**Conducted experiments:** Sukoff Rizzo, Gilbert, Smith, M.-Y. Zhang, Di, Platt, Neal, Dwyer, Bender, J. Zhang, Lock, Kramer, and Randall.

**Contributed new reagents or analytic tools:** Dollings and Bender.

Wrote or contributed to the writing of the manuscript: Sukoff Rizzo, Leonard, Gilbert, Smith, Di, Tse, Ring, Hughes, and Dunlop.

References


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