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A novel glycine transporter-1 (GlyT1) inhibitor, ASP2535 (4-[3-isopropyl-5-(6-phenyl-3-pyridyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole), improves cognition in animal models of cognitive impairment in schizophrenia and Alzheimer's disease

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ABSTRACT

Hypofunction of brain N-methyl-D-aspartate (NMDA) receptors has been implicated in psychiatric disorders such as schizophrenia and Alzheimer's disease. Inhibition of glycine transporter-1 (GlyT1) is expected to increase glycine, a co-agonist of the NMDA receptor and, consequently, to facilitate NMDA receptor function. We have identified ASP2535 (4-[3-isopropyl-5-(6-phenyl-3-pyridyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole) as a novel GlyT1 inhibitor, and here describe our *in vitro* and *in vivo* characterization of this compound. ASP2535 potently inhibited rat GlyT1 (IC_{50} = 92 nM) with 50-fold selectivity over rat glycine transporter-2 (GlyT2). It showed minimal affinity for many other receptors except for μ-opioid receptors (IC_{50} = 1.83 μM). Oral administration of ASP2535 dose-dependently inhibited *ex vivo* [^{3}H]-glycine uptake in mouse cortical homogenate, suggesting good brain permeability. This profile was confirmed by pharmacokinetic analysis. We then evaluated the effect of ASP2535 on animal models of cognitive impairment in schizophrenia and Alzheimer's disease. Working memory deficit in MK-801-treated mice and visual learning deficit in neonatally phencyclidine (PCP)-treated mice were both attenuated by ASP2535 (0.3–3 mg/kg, p.o. and 0.3–1 mg/kg, p.o., respectively). ASP2535 (1–3 mg/kg, p.o.) also improved the PCP-induced deficit in prepulse inhibition in rats. Moreover, the working memory deficit in scopolamine-treated mice and the spatial learning deficit in aged rats were both attenuated by ASP2535 (0.1–3 mg/kg, p.o. and 0.1 mg/kg, p.o., respectively). These studies provide compelling evidence that ASP2535 is a novel and centrally-active GlyT1 inhibitor that can improve cognitive impairment in animal models of schizophrenia and Alzheimer's disease, suggesting that ASP2535 may satisfy currently unmet medical needs for the treatment of these diseases.

1. Introduction

Dysfunction of N-methyl-D-aspartate (NMDA) receptors is implicated in several psychiatric disorders. NMDA receptors require agonists and co-agonists such as glycine for their activation (Kemp and Leeson, 1993). Although glycine is widely distributed in the brain, the concentration in the synaptic cleft is regulated by glycine transporters (GlyTs), of which two (GlyT1 and GlyT2) have been identified (Aragón and López-Corcuera, 2003). They are both expressed in the brain stem and spinal cord, but GlyT1 is also expressed in forebrain areas such as the hippocampus and cerebral cortex, where it is suggested to modulate NMDA receptor activity by regulating glycine concentrations (Bergeron et al., 1998).

Schizophrenia is a debilitating psychiatric disease involving three major symptom classes (positive, negative and cognitive). Although existing antipsychotics are effective for positive symptoms, they have limited efficacy for negative symptoms (Buchanan et al., 1998) and cognitive deficits (Gold, 2004). NMDA receptor antagonists such as phencyclidine (PCP) produce all three symptoms in normal humans and, in stabilized patients, can precipitate relapse (Javitt and Zukin, 1991; Krystal et al., 1994). Hypofunction of NMDA receptors has therefore been proposed as a potential contributory factor in schizophrenia. GlyT1 inhibitors are expected to increase glycine, and thus facilitate NMDA receptor function and reduce the symptoms of schizophrenia. Several GlyT1 inhibitors have shown efficacy in animal models of schizophrenia (Depoortere et al., 2005; Harsing et al., 2003; Kinney et al., 2003). Furthermore, a small-scale clinical study

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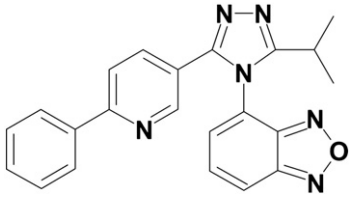

Fig. 1. Chemical structure of ASP2535, 4-[3-isopropyl-5-(6-phenyl-3-pyridyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole.

has demonstrated that sarcosine, a prototypic GlyT1 inhibitor, ameliorates the three symptoms in schizophrenia (Tsai et al., 2004). Although the clinical efficacy of small molecules that enhance NMDA receptor function is controversial (*e.g.*, Buchanan et al., 2007), a meta-analysis incorporating more than 20 studies found glycine, D-serine and sarcosine to have beneficial effects on multiple symptoms of schizophrenia (Tsai and Lin, 2010). Previous findings collectively suggest that GlyT1 might be a promising drug target for the treatment of schizophrenia, especially for cognitive impairment.

Alzheimer's disease is also characterized by cognitive impairment. Although several drugs are used for the treatment of the disease, their efficacy is unsatisfactory. NMDA receptors play a crucial role in learning and memory (Riedel et al., 2003): dysfunction of NMDA receptors leads to learning impairment (Morris, 1989), whereas their enhancement facilitates learning and memory (Myhrer et al., 1993). Considering that a decrease in NMDA receptors was observed in postmortem brains from Alzheimer's disease patients (Greenamyre et al., 1987), facilitation of NMDA receptors through GlyT1 inhibition could also be beneficial in treating cognitive impairment in Alzheimer's disease.

Based on these hypotheses, we have chemically optimized the functional properties of a series of compounds to design novel drugs for the treatment of cognitive impairment in neuropsychiatric diseases, and we have recently identified ASP2535, 4-[3-isopropyl-5-(6-phenyl-3-pyridyl)-4*H*-1,2,4-triazol-4-yl]-2,1,3-benzoxadiazole (Fig. 1). In the present study, we first evaluated the *in vitro* inhibitory effects of this compound on glycine transporters, its affinity for various receptors, *ex vivo* inhibitory effects on GlyT1, and its pharmacokinetics. We then evaluated the compound in animal models of cognitive impairment of schizophrenia and Alzheimer's disease.

2. Materials and methods

2.1. Drugs and drug treatment

[^{3}H]-Glycine was obtained from Perkin Elmer Inc. (Wellesley, MA). Rat glioma C6 cells and human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection (Manassas, VA). ASP2535 (Tobe et al., 2003), donepezil hydrochloride (used as donepezil) and PCP hydrochloride (used as PCP) were synthesized at Astellas Pharma Inc. (Tsukuba, Japan). Sarcosine, haloperidol, clozapine, (+)-MK-801 hydrogen maleate (used as MK-801) and (−)-scopolamine hydrobromide trihydrate (used as scopolamine) were purchased from Sigma Aldrich (St. Louis, MO). ASP2535 was suspended in 0.5% methyl cellulose. Donepezil was dissolved in distilled water, whereas PCP, sarcosine, MK-801 and scopolamine were dissolved in saline. Haloperidol and clozapine were dissolved in 0.3% tartaric acid. All compounds were administered at volumes of 10 ml/kg in mice and 1 ml/kg in rats. Drug doses were corrected for salt content (except PCP).

2.2. Inhibition of glycine transporters

2.2.1. Effect of ASP2535 on rat GlyT1

The inhibitory effect of ASP2535 on rat GlyT1 was examined as previously described (Gomeza et al., 1995) with some modifications. Rat glioma C6 cells, which endogenously express GlyT1, were plated at a density of 2.0×10^4 cells per well in 96-well white plates and incubated for two days in a CO_2 incubator (37 °C, 5% CO_2). After washing each well with HEPES buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 5 mM L-alanine and 10 mM HEPES, pH 7.4), the buffer was replaced with HEPES buffer containing ASP2535 (0.003–1 μM) and plates were incubated at 37 °C for 30 min. After incubation, [^{3}H]-glycine (final concentration: ~0.2 μM) was added and the plate was incubated at 37 °C for a further 10 min. The reaction was stopped by washing cells three times with ice-cold buffer. After solubilizing the cells with 0.1 N NaOH, MicroScint™ PS scintillation fluid (PerkinElmer Inc.; Waltham, MA) was added and the radioactivity counted using a TopCount™ instrument (Hewlett–Packard Company; Palo Alto, CA). Non-specific uptake was determined using 3 mM sarcosine. Sarcosine was also used as a reference compound.

2.2.2. Effect of ASP2535 on rat GlyT2

The inhibitory effect of ASP2535 on rat GlyT2 was examined using [^{3}H]-glycine uptake into primary cultured cells from rat brain stem. Pregnant female Wistar rats (17th gestational day, Japan SLC, Inc., Hamamatsu, Japan) were euthanized under ether anesthesia. Infants were recovered from the uterus and the brain stem was isolated. After digestion with papain, cells were plated at a density of 5.0×10^4 cells per well in poly-L-lysine-coated 96-well white plates. Cells were incubated for 14–21 days in a CO_2 incubator. After washing each well with HEPES buffer containing 3 mM sarcosine, the buffer was replaced with HEPES buffer containing 3 mM sarcosine and ASP2535 (0.1–30 μM) and plates were incubated at 37 °C for 30 min. After the incubation, [^{3}H]-glycine (final concentration: ~0.2 μM) was added and the plate was incubated at 37 °C for a further 10 min. The reaction was stopped and radioactivity was counted in the same way as rat GlyT1. Non-specific uptake was determined with 3 mM unlabeled glycine. Glycine was also used as a reference compound.

2.2.3. Effect of ASP2535 on human GlyT1

The inhibitory effect of ASP2535 on human GlyT1 was examined using human neuroblastoma SK-N-MC cells, which endogenously express GlyT1a (Depoortere et al., 2005). The cells were plated at a density of 5.0×10^4 cells per well in collagen-I-coated 96-well white plates and incubated for 24 h in a CO_2 incubator. After washing each well with HEPES buffer, fresh HEPES buffer containing ASP2535 (0.01–3 μM) was added and cells were incubated at 37 °C for 30 min. After incubation, [^{3}H]-glycine (final concentration: ~0.2 μM) was added and the plate was incubated at 37 °C for a further 10 min. The reaction was stopped by washing cells three times with ice-cold phosphate-buffered saline (PBS). Radioactivity was counted in the same way as for rat GlyT1. Non-specific uptake was determined using 3 mM sarcosine. Sarcosine was also used as a reference compound.

2.3. Affinity for multiple receptors, ion channels and transporters

To determine the affinity of ASP2535 for a wide range of receptors, transporters and ion channels, initial receptor binding screens (65 assays) were conducted with duplicate samples of 10 μM ASP2535 by Sekisui Medical Inc. (Tokyo, Japan) using proprietary assay formats. The subsequent dose–response study for μ-opioid receptors was also conducted by Sekisui Medical Inc. Experimental conditions for these assays are summarized in Supplementary Table 1 along with the results.

2.4. Animals

Animals were housed in groups of 10 (mice), 5 (young rats) or 3 (aged rats) in temperature- and humidity-controlled colony rooms (23 ± 1 °C and 55 ± 5%) under a 12-h light/dark cycle with water and