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# Preclinical profile of a novel metabotropic glutamate receptor 5 positive allosteric modulator

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ABSTRACT

Recent reports have indicated that patients with schizophrenia have a profound hypo-functionality of glutamatergic signaling pathways. Positive allosteric modulation of $mGlu_5$ receptor has been postulated to augment NMDA function and thereby alleviate the glutamatergic hypo-function observed in schizophrenic patients. Here we report the in vitro and in vivo characterization of CPPZ (1-(4-(2-chloro-4-fluorophenyl) piperazin-1-yl)-2-(pyridin-4-ylmethoxy)ethanone), a structurally novel positive allosteric modulator selective for $mGlu_5$ receptor. In HEK293 cells stably over-expressing human $mGlu_5$ receptor, CPPZ potentiates the intracellular calcium response elicited by a suboptimal concentration of the endogenous agonist glutamate. CPPZ does not have any intrinsic agonist activity and behaves functionally as a positive allosteric modulator. This is further supported by binding data, which demonstrate that CPPZ is able to displace the negative allosteric modulator MPEP but does not compete with the orthosteric ligand quisqualic acid. Instead, CPPZ enhances the binding of the orthosteric ligand. In native preparations, CPPZ potentiates calcium flux in rat cortical neurons stimulated with the group I agonist dihydroxyphenylglycine (DHPG). In addition, CPPZ modulates long-term potentiation in rat hippocampal slices, a process known to be NMDA dependent. In vivo, CPPZ reverses hyper locomotion triggered by the NMDA open channel blocker MK801 in CD1 mice. CPPZ was also able to reduce rat conditioned avoidance responding to electric shock. Both in vitro and in vivo data demonstrate that this novel compound acts as an $mGlu_5$ receptor positive allosteric modulator, which modulates NMDA dependent responses and suggests that the enhancement of $mGlu_5$ receptor activity may prove useful in the treatment of schizophrenia.


## 1. Introduction

Current therapies for schizophrenia are successful mainly in treating positive symptoms and do not significantly affect negative and cognitive symptoms. Through pharmacological and genetic research, investigators have identified possible deficiencies in glutamatergic dependent pathways and thereby identified novel potential targets (Olney et al., 1999). The glutamatergic hypothesis is supported by the clinical observation that phencyclidine (PCP) and ketamine, two use-dependent N-methyl-D-aspartate (NMDA) receptor antagonists, are able to induce psychotic symptoms in normal volunteers as well as exacerbate psychotic symptoms in schizophrenic patients (Javitt and Zukin, 1991). According to this hypothesis one might alleviate symptoms of schizophrenia by augmenting NMDA receptor signaling. Several different mechanisms are currently under evaluation among them is activation of $mGlu_5$ receptors (Fitzjohn et al., 1996). The $mGlu_5$ receptor is coupled to $G_{\alpha q/11}$ and glutamate binding results in the activation of phospholipase C, increase in inositol phosphate hydrolysis and the release of intracellular $Ca^{++}$ stores (Hermans and Challiss, 2001; Pin and Duvoisin, 1995). This leads to the phosphorylation of NMDA receptor and enhancement of its function (Benquet et al., 2002; Salter, 1998). Many reports have shown that the activation of $mGlu_5$ receptors can regulate NMDA receptor function in neuronal circuits located in the frontal cortex and hippocampus which are thought to be affected in schizophrenic patients (Anikstejn et al., 1991; Awad et al., 2000; Mannaioni et al., 2001). The orthosteric binding site of $mGlu_5$ receptor is an unfavorable target because of the difficulty to obtain subtype specific agonists. Therefore, much effort has been placed in identifying compounds that bind to an allosteric site which is located in the 7-transmembrane domain and positively modulate the receptor response. These allosteric sites are more heterogeneous than the orthosteric site which should allow for greater subtype selectivity (Raddatz et al., 2007). In addition to selectivity, positive allosteric modulators would have the

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advantage that receptor activation will be controlled by endogenous glutamate release and native neural circuit activity thereby selectively enhancing $mGlu_5$ receptor-related neurotransmission and reducing possible adverse affects of tonic agonist activation. Several research groups have attempted to discover $mGlu_5$ receptor positive allosteric modulators and these efforts have resulted in the identification of several novel chemical entities with allosteric modulator activity (de Paulis et al., 2006; Kinney et al., 2005; O'Brien et al., 2003). All of these compounds bind to an allosteric site that is shared with the negative allosteric modulator MPEP (Kuhn et al., 2002). In addition, several compounds have been tested in various in vivo animal models for antipsychotic activity and were active in these assays (Kinney et al., 2005; Liu et al., 2008). Here we describe the identification of a structurally novel $mGlu_5$ receptor positive allosteric modulator, which was able to enhance $mGlu_5$ receptor activity, modulate NMDA receptor dependent responses and demonstrate antipsychotic activity.

## 2. Materials and Methods

### 2.1. Calcium Mobilization Assay in HEK293 Cell Line

A $Ca^{++}$ influx assay similar to that described by Andreas Ritzén et al. (Ritzen et al., 2009) was used to detect $mGlu_5$ receptor positive allosteric modulator activity in an HEK293 cell line stably expressing the d-isoform of human $mGlu_5$ receptor. Positive allosteric modulator activity was assessed by measuring potentiation of the $EC_{20}$ response to L-glutamate in the presence of a test compound. The measurements were made using the fluorometric imaging plate reader (FLIPR) 384 (Molecular Devices, Sunnyvale, CA). The day before the experiment 25,000 cells/well were plated in DMEM containing 10% dialyzed FBS (Hyclone, Logan, UT) in 384 well Poly-D-Lysine coated plates (Becton Dickinson, Franklin Lakes, NJ). After the removal of the plating medium the following day, the cells were labeled for 1 h at 37 °C in 4.3 μM Fluo-4 AM (Invitrogen, Carlsbad, CA) containing 10% Pluronic F-127 (TeffLabs, Austin, TX) in assay buffer (Hank's balanced salt solution (HBSS) (Mediatech, Manassas, VA), 20 mM HEPES, 1 mM Probenecid (Sigma, St. Louis, MO), at pH 7.4). The cells were washed at room temperature to remove excess dye prior to the addition of test compounds serially diluted (11-point concentration response curve) from 10 mM in 100% DMSO into assay buffer. Compounds were assayed for any underlying agonist activity by addition of test compounds to the cells on the FLIPR instrument (first addition: 13 μl test compound to 25 μl assay buffer/well) and the response was recorded for 1 min. After the 1st addition (15 min), positive modulator activity was assayed by challenge with $EC_{20}$ (200–300 nM) L-glutamate (2nd addition, 14 μl) and the response was recorded for 1 min. Positive modulator activity was calculated from the fluorescence max–min data normalized to yield responses for no modulation ($EC_{20}$ response) and full stimulation (10 μM L-glutamate) as 0% and 100% modulation, respectively. Inhibition by the silent binder 5MPEP used an $EC_{80}$ of glutamate (~1 μM). Concentration–response data were fitted to a four-parameter logistic equation to estimate compound potency ($EC_{50}$) and efficacy ($E_{max}$) using Prism software (Graphpad). The selectivity of CPPZ was determined by Calcium Mobilization Assay in HEK cell lines stably transfected with respective mGlu receptors ($mGlu_{1, 2, 3, 4, 6, 7}$ and $_8$) using the methods described above for $mGlu_5$ receptor. CPPZ was evaluated for selectivity not only as positive modulator but also for any agonist or antagonist activity using twelve point concentration curves. To determine the antagonist activity, the cells were first incubated for 15 min with CPPZ followed by an $EC_{20}$ challenge with glutamate. Agonist activity was recorded immediately after adding CPPZ to the cells using FLIPR.

### 2.2. Calcium Mobilization Assay in Rat Cortical Neurons

Time pregnant Sprague–Dawley rats (Charles River, Malvern, PA) were euthanized by $CO_2$ inhalation. The abdominal cavity was opened and the uterine horns containing E17–18 rat embryos were removed and placed into ice-cold HBSS without calcium or magnesium. The embryos were removed from the uteruses, decapitated and the brains were extracted from the skulls. Meninges were carefully removed and the cortical sheets were collected. The cortices were digested for 15 min at 37 °C in 10 ml of a trypsin/EDTA solution (Invitrogen, Carlsbad, CA). The trypsin/EDTA solution was inactivated by the addition of 10% fetal bovine serum in Neurobasal media. Tissue pieces were dissociated by passing through heat-sterilized Pasteur pipettes of decreasing diameters. Then the cell suspension was pelleted by low speed centrifugation twice to remove all trypsin. The cell pellet was then resuspended in a serum free chemically defined medium (Neurobasal, B27, Penicillin/streptomycin and Glutamax I (Invitrogen, Carlsbad, CA)) and passed through a 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove cell debris and undissociated tissue pieces. The cells were counted and seeded at $1 \times 10^5$ cells/well in 96-well black, Poly-D-Lysine plates (Corning, Lowell, MA). The plates were incubated at 37 °C, 95% humidity and 5% $CO_2$. The plates were examined before each assay. Only plates with confluent monolayers of neurons were tested.

Neurons were tested in calcium assays on days 4–6 of culture. The media were aspirated from the plates. The cells were washed once in HBSS/HEPES (20 mM, pH 7.3). Calcium-4 dye (Molecular Devices, Sunnyvale, CA) diluted in HBSS/HEPES (20 mM, pH 7.3) was added to the plates. The dye-loaded plates were incubated for 1 h at 37 °C. After the one hour incubation, the $mGlu_5$ receptor positive modulator, CPPZ was added to the plates and the plates were incubated for 10 min at room temperature. The plates were then loaded into a Flexstation II Microplate Reader (Molecular Devices, Sunnyvale, CA). The potent agonist of group I metabotropic glutamate receptors, DHPG (Tocris, Ellisville, MO) was added to each well and the instrument measured calcium mobilization at 485 nm excitation with emission at 525 nm. Dose response curves were calculated using Prism software (Graphpad) using a 4-parameter open fit with no constraints.

### 2.3. Radioligand Binding Assay in Membranes Prepared from Rat Cortical Tissue

The membranes used in the binding experiments were prepared from male Sprague–Dawley rat (Taconic Charles River) cortical tissue. [$^3$H]MPEP (American Radiolabeled Chemicals, Inc., St. Louis, MO) was used as the ligand and non-specific binding was determined in the presence of 1 μM ABP-688 (AstraZeneca). CPPZ was prepared as a 10 mM stock in DMSO and serially diluted in DMSO. The membranes were diluted in assay buffer which contained 50 mM HEPES, 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, at pH 7.4. The compounds and membranes were mixed in a 96-well plate and then the assay was initiated by adding [$^3$H]MPEP at a final concentration of 2.0 nM. The total volume was 200 μl with 10 μg/well membranes and a final concentration of 1% DMSO. The serially diluted compounds were present at concentrations starting at 100 μM. The competitive binding assays were done three times in duplicate. All assays were terminated after 1 h incubation at room temperature by filtering the assay mixture through GFB filter plates (PerkinElmer, Waltham, MA) pre-treated with 0.1% polyethylene imine Sigma, St. Louis, MO. The plates were then washed 5 times (total of 4 ml/well) with ice cold wash buffer containing 0.05 M Tris–HCl and 0.003 M $MgCl_2$, at pH 7.0. After drying the plates, 40 μl of Microscint40 were added and the plates were read on a TopCount NXT microplate plate reader (PerkinElmer, Waltham, MA). Binding of an orthosteric ligand, [$^3$H]quisqualic acid (PerkinElmer, Waltham, MA), was also determined to see if CPPZ had any affinity for the orthosteric site of $mGlu_5$ receptor. The membranes used in these binding experiments were prepared from HEK293 cells expressing human $mGlu_5$ receptor isoform b. The assay buffer described for [$^3$H]MPEP binding was used in these experiments. Saturation binding of