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In vitro and *in vivo* pharmacological characterization of SSD114, a novel GABAB positive allosteric modulator

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ABSTRACT

Positive allosteric modulators (PAMs) of the $GABA_B$ receptor have emerged as a novel approach to the pharmacological manipulation of the $GABA_B$ receptor, enhancing the effects of receptor agonists with few side effects. Here, we identified N-cyclohexyl-4-methoxy-6-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine (SSD114) as a new compound with activity as a $GABA_B$ PAM in *in vitro* and *in vivo* assays. SSD114 potentiated GABA-stimulated [^{35}S]GTPγS binding to native $GABA_B$ receptors, whereas it had no effect when used alone. Its effect on GTPγS stimulation was suppressed when GABA-induced activation was blocked with CGP54626, a competitive antagonist of the $GABA_B$ receptor. SSD114 failed to potentiate WIN55,212,2-, morphine- and quinpirole-induced [^{35}S]GTPγS binding to cortical and striatal membranes, respectively, indicating that it is a selective $GABA_B$ PAM. Increasing SSD114 fixed concentrations induced a leftward shift of the GABA concentration-response curve, enhancing the potency of GABA rather than its efficacy. SSD114 concentration-response curves in the presence of fixed concentrations of GABA (1, 10, and 20 μM) revealed a potentiating effect on GABA-stimulated binding of [^{35}S]GTPγS to rat cortical membranes, with EC_{50} values in the low micromolar range. Bioluminescence resonance energy transfer (BRET) experiments in Chinese Hamster Ovary (CHO)-cells expressing $GABA_B$ receptors showed that SSD114 potentiates the GABA inhibition of adenylyl-cyclase mediated by $GABA_B$ receptors. Our compound is also effective *in vivo* potentiating baclofen-induced sedation/hypnosis in mice, with no effect when tested alone. These findings indicate that SSD114, a molecule with a different chemical structure compared to known $GABA_B$ PAMs, is a novel $GABA_B$ PAM with potential usefulness in the $GABA_B$-receptor research field.

1. Introduction

The identification of $GABA_B$ allosteric modulators constitutes a novel approach for pharmacologically manipulate this receptor; these molecules with little or no intrinsic agonistic activity modulate $GABA_B$ receptor activation by exploiting the presence of endogenous GABA, which may lead to a lower potential for side effects.

Positive allosteric modulators (PAMs) of $GABA_B$ receptor, including 3,5-bis(1,1-dimethylethyl)-4-hydroxy-β,β-dimethyl-benzenepropanol (CGP7930) and *N,N′*-dicyclopentyl-2-(methylthio)-5-nitro-4,6-pyrimidinediamine (GS39783), were firstly developed by Novartis. *In vitro* experiments showed that they increase potency and maximal efficacy of GABA at the $GABA_B$ receptor (Urwyler et al., 2001, 2003). However, the most potent compound, GS39783, has genotoxic effects likely associated with the nitro-group on the aromatic ring. These findings led Novartis group to study less toxic analogs of GS39783 containing a pyrimidine scaffold, such as *N*-[(1*R*,2*R*,4*S*)-bicyclo[2.2.1]hept-2-yl]-2-methyl-5-[4-(trifluoromethyl)phenyl]-4-pyrimidinamine-(NVP-BHF177), which exhibited PAM activity both *in vitro* and *in vivo* experiments (Guery et al., 2007). Then, Hoffmann–La Roche produced a novel $GABA_B$ PAM, namely (R,S)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one (rac-BHFF), which increases both potency and efficacy of GABA in [^{35}S]GTPγS binding assay and in electrophysiological experiments in hippocampal slices (Malherbe et al., 2008).

$GABA_B$ PAMs (*e.g.* CGP7930, rac-BHF, BHF177) *in vivo* potentiated the sedative/hypnotic effects of baclofen in mice (Carai et al., 2004; Koek et al., 2010), decreased alcohol intake and oral self-administration in alcohol-preferring rats (Agabio and

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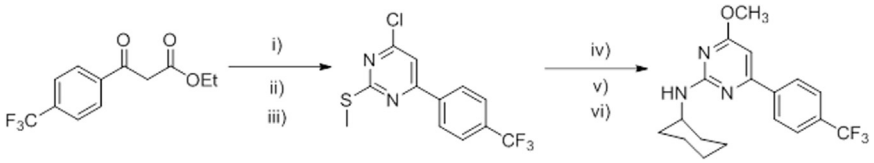

Scheme 1. Chemistry: Synthesis of *N*-cyclohexyl-4-methoxy-6-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine (SSD114). i) $(NH_2)_2CS$, NaOEt, EtOH; ii) CH_3I, NaOH, EtOH; iii) $POCl_3$; iv) CH_3ONa, CH_3OH; v) Oxone, CH_3OH; vi) cyclohexylamine, 1,4-dioxane.

Colombo, 2014), reduced intravenous self-administration of nicotine (Paterson et al., 2008) and blocked the rewarding properties of nicotine in rats (Filip et al., 2015). Furthermore, they showed anxiolytic- and antidepressant-like properties in mice and rats (Cryan et al., 2004; Jacobson and Cryan, 2008; Li et al., 2013).

Recently, using a virtual screening protocol, we identified 2-(acylamino)thiophene derivatives as a new class of $GABA_B$ PAMs; in particular, methyl 2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628) potentiated GABA–stimulated [^{35}S]GTPγS binding, leading to a decrease in the EC_{50} for GABA and a slight increase in Emax. In *in vivo* experiments, both compounds increased the sedative/hypnotic effect of a sub-threshold dose of baclofen in DBA mice (Castelli et al., 2012).

These results led us to investigate different 2-(acylamino) thiophene derivatives as potential $GABA_B$ PAMs; three new compounds, namely methyl 2-(4-trifluoromethylbenzamido)-4-ethyl-5-methylthiophene-3-carboxylate, 2-(4-chlorobenzamido)-4-ethyl-5-methylthiophene-3-carboxylate, and 2-(4-methylbenzamido)-4-ethyl-5-methylthiophene-3-carboxylate, potentiated [^{35}S]GTPγS binding displaying a potency comparable to that of reference compounds (GS39783 and CGP7930). They decreased the EC_{50} of GABA but slightly increased maximal GABA stimulation, affecting the potency of GABA rather than its efficacy (Mugnaini et al., 2013).

Here, we focused on design and synthesis of a series of trisubstituted pyrimidines based on a hybridization strategy. The structural overlays of the $GABA_B$ PAMs GS39783 and NVP-BHF177 were used as a starting point to synthesize a series of 2,4,6-trisubstituted pyrimidines. Among these new compounds, N-cyclohexyl-4-methoxy-6-(4-(trifluoromethyl)phenyl)pyrimidin-2-amine (SSD114) emerged as a novel $GABA_B$ PAM.

The pharmacological characterization of SSD114 was performed by *in vitro* experiments using [^{3}H]3-N-[1-(S)-3,4dichlorophenylethylaminol]-2-(S)hydroxypropylcy-clo-hexylmethyl phosphinic acid ([^{3}H]CGP54626) binding and 5′-O-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding to $GABA_B$ native receptors. In addition, a BRET approach was used to evaluate the effects on adenylate-cyclase activity in CHO cells stably expressing $GABA_B$ receptors. Finally, the *in vivo* potential of the putative $GABA_B$ PAM was evaluated using the baclofen-induced sedation/hypnosis test in mice.

2. Materials and methods

2.1. Reagents

[^{35}S]GTPγS was purchased from PerkinElmer Life and Analytical Science (Walthman, MA, USA); GABA, GDP, and GTPγS, were obtained from Sigma/RBI (Natick, MA, USA); CGP54626 and (*R*)-Baclofen were from Tocris Bioscience (Ellisville, MO, USA). [^{35}S]GTPγS (125 Ci/mM) and [^{3}H]Baclofen (49.7 Ci/mM) were obtained from PerkinElmer and [^{3}H]CGP54626 (85 Ci/mM)from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). All solvents and reagents used in the chemical synthesis were commercially available and used without further purification. ^{1}H NMR and ^{13}C NMR were recorded at 400 and 100 MHz on a Varian Mercury instrument. Elemental analyses (C, H, and N) were performed on a Perkin-Elmer PE 2004 elemental analyzer.

2.2. Synthesis of N-cyclohexyl-4-methoxy-6-(4-(trifluoromethyl) phenyl)pyrimidin-2-amine (SSD114)

The target molecule was obtained by a 6–step synthesis starting from the commercially available ethyl (4-trifluoromethylbenzoyl) acetate (Scheme 1). In the first step, the β-ketoester was reacted with thiourea and sodium ethoxide in refluxing ethanol to provide the substituted thiouracilat a yield of 50% (Botta et al., 1999). Subsequent methylation at the sulfur atom by iodomethane in a basic solution of H_2O/EtOH led to the corresponding *S*-methylated compound in almost quantitative yield after crystallization from acetone (Qin et al., 2010). The substituted 4-pyrimidinone was quantitatively converted by reaction with phosphorus oxychloride at 100 °C into the corresponding 4-chloro-2-(methylthio)-6-(4-(trifluoromethyl)phenyl)pyrimidine. The chlorine atom was displaced by nucleophilic substitution with CH_3ONa in dry methanol leading to the corresponding 4-methoxypyrimidine derivate. This last compound was then treated with potassium monopersulfate to oxidize the thioether to a sulfone in 80% yield (Radi et al., 2005). Finally, displacement of the sulfone with cyclohexylamine in refluxing 1,4-dioxane produced SSD114 as a colorless oil with a 71% isolated yield after chromatographic purification. ^{1}H NMR (400.1 MHz, $CDCl_3$): δ 8.07 (d, J=8.0 Hz, 2H, ArH), 7.70 (d, J=8.2 Hz, 2H, ArH), 6.43 (s, 1H, ArH), 5.07 (bs, 1H, NH), 3.94 (s, 3H, OCH_3) overlapped with (m, 1H, CH), 2.10 (m, 2H, CH_2), 1.78 (m, 2H, CH_2), 1.67 (m, 1H, CH_2), 1.44 (m, 2H, CH_2), 1.27 (m, 3H, CH_2), ppm. ^{13}C{1H} NMR (100.6 MHz, CDCl3): δ 171.4, 164.0, 161.9, 141.4, 131.6 (q, ^2J[CF]=32 Hz), 127.2 (2C), 125.4, (2C, q, ^3J[CF]=4 Hz), 124.1 (q, ^1J[CF]=272 Hz), 93.1, 53.3, 49.9, 33.2 (2C), 25.8 (2C), 24.9 ppm. Anal. calcd for $C_{18}H_{20}F_3N_3O$: C, 61.53; H, 5.74; N, 11.96; Found: C, 61.65; H, 5.80; N, 11.89%.

2.3. Animals

Male Sprague-Dawley rats and DBA mice (Charles River Laboratories, Calco, Italy), weighing 200–250 and 20–25 g, respectively, were used. Rats and mice were housed 4 and 8/cage, respectively, in standard plastic cages with wood chip bedding, under a 12:12-h artificial light/dark cycle (lights on at 7:00 a.m.) at a constant temperature of 22 °C and relative humidity of approximately 60%. Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the home cage.

2.4. In vitro experiments

2.4.1. Membrane preparation for binding assays

Rats (250 g) were killed; brains were rapidly removed and cerebral cortices and striatal were dissected on ice. Cortical tissues were homogenized using a glass-teflon homogenizer (Glass-Col, Terre Haute, IN, USA) in 15 volumes (v/w) of ice-cold 0.32 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was collected and re-