Neuropharmacology 86 (2014) 259–272

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Mapping the binding pocket of a novel, high-affinity, slow dissociating tachykinin NK_3 receptor antagonist: Biochemical and electrophysiological characterization

Pari Malherbe [a, *], Frédéric Knoflach [a], Anne Marcuz [a], Claudia Bohnert [a], Michael Weber [a], Henner Knust [b], Hasane Ratni [b], Will Spooren [a], Theresa M. Ballard [a], Caterina Bissantz [b]

[a] Discovery Neuroscience, F. Hoffmann-La Roche AG, pRED, Pharma Research & Early Development, Grenzacherstrasse 124, CH4070, Basel, Switzerland
[b] Discovery Chemistry, F. Hoffmann-La Roche AG, pRED, Pharma Research & Early Development, Grenzacherstrasse 124, CH4070, Basel, Switzerland

ARTICLE INFO

Article history:
Received 1 April 2014
Received in revised form
21 July 2014
Accepted 23 July 2014
Available online 5 August 2014

Keywords:
RO5328673
Dual NK2/NK3 antagonist
Inositol phosphate accumulation
Firing frequency
Locomotor activity
NK_3 modeling

ABSTRACT

The NK_3 receptor is a GPCR that is prominently expressed in limbic areas of the brain, many of which have been implicated in schizophrenia. Phase II clinical trials in schizophrenia with two selective NK_3 antagonists (osanetant and talnetant) have demonstrated significant improvement in positive symptoms. The objective of this study was to characterize the properties of a novel dual NK_2/NK_3 antagonist, RO5328673. [^{3}H]RO5328673 bound to a single saturable site on hNK_2, hNK_3 and $gpNK_3$ with high-affinity. RO5328673 acted as an insurmountable antagonist at both human and guinea-pig NK_3 receptors in the [^{3}H]IP accumulation assay. In binding kinetic analyses, [^{3}H]RO5328673 had fast association and dissociation rates at hNK_2 while it had a fast association rate and a remarkably slow dissociation rate at gp and hNK_3. In electrophysiological recordings of gp SNpc, RO5328673 inhibited the senktide-induced potentiation of spontaneous activity of dopaminergic neurons with an insurmountable mechanism of action. RO5328673 exhibited *in-vivo* activity in gerbils, robustly reversing the senktide-induced locomotor activity. The TM2 residue $gpNK_3$-A114$^{2.58}$ (threonine in all other species) was identified as the critical residue for the RO5328673's slower dissociation kinetics and stronger insurmountable mode of antagonism in the guinea-pig as compared to hNK_3-T139$^{2.58}$. Using site-directed mutagenesis, [^{3}H]RO5328673 binding and rhodopsin-based modeling, the important molecular determinants of the RO5328673-binding pocket of hNK_3 were determined. A comparison of the RO5328673-binding pocket with that of osanetant showed that two antagonists have similar contact sides on hNK_3 binding crevice except for three mutations V95L$^{1.42}$, Y247W$^{5.38}$, V255I$^{5.46}$, which behaved differently between interacting modes of two antagonists in hNK_3.

1. Introduction

The tachykinins are a family of neuropeptides comprised mainly of substance P (SP: RPKPQQFFGLM-NH_2), neurokinin A (NKA: HKTDSFVGLM-NH_2) and neurokinin B (NKB: DMHDFFVGLM-NH_2). Tachykinin peptides, which act as neurotransmitters/neuromodulators, exert their effects through three distinct receptors called NK_1, NK_2 and NK_3 receptors. Tachykinin receptors belong to the class A family (rhodopsin-like) of G-protein-coupled receptors (GPCRs) that are coupled via $G_{q/11}$ to the activation of the phospholipase C-IP3/DAG signaling pathway leading to an elevation of intracellular Ca^{2+} levels (Almeida et al., 2004; Severini et al., 2002). Tachykinin receptors are widely distributed throughout the CNS and in peripheral tissues including gut, lung, bladder and bone marrow. They are involved in numerous physiological functions including nociception, neuroimmunomodulation and reproduction. Dysfunction of tachykinin receptors has been implicated in diseases such as emesis, bronchial asthma, gastrointestinal disorders, inflammatory bowel syndrome and overactive bladder as well as in the pathology of psychiatric diseases such as depression,

Abbreviations: SP, substance P; NKA, neurokinin A; NKB, neurokinin B; GPCRs, G-protein coupled receptors; IP, inositol phosphates; DA, dopamine; NA, noradrenaline; D_2 receptor, dopamine 2 receptor; SNpc, substantia nigra pars compacta; gp, guinea-pig; h, human; 7TMD, seven-transmembrane domain; WT, wild-type; 3D, three-dimensional; OPSD, bovine rhodopsin; β_2AR, β_2-adrenergic receptor; A_{2A}, A_{2A} adenosine receptor.

* Corresponding author. pRED Neuroscience, Building 69, Room 332, F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland.
E-mail address: pari.malherbe@bluewin.ch (P. Malherbe).

http://dx.doi.org/10.1016/j.neuropharm.2014.07.017
0028-3908/

anxiety, schizophrenia (Albert, 2004; Lecci and Maggi, 2003; Quartara et al., 2009).

The expression of NK_3 receptor (by in situ hybridization and NKB/senktide autoradiography) was mostly detected in brain regions including cortex (frontal, parietal and cingulate cortex), various nuclei of the amygdala, the hippocampus, and midbrain structures (Langlois et al., 2001; Rigby et al., 2005; Stoessl, 1994). Among tachykinin receptors, NK_3 is a novel target under investigation to treat schizophrenia. It is prominently expressed in limbic areas of the brain, many of which have been implicated in schizophrenia (Harrison, 1999). Importantly, the NK_3 receptor is also located on dopaminergic neurons in the midbrain regions such as substantia nigra (A9), ventral tegmental area and nucleus raphe linealis (A10) (Chen et al., 1998; Langlois et al., 2001; Stoessl, 1994). Activation of the midbrain NK_3 receptor with the endogenous ligand NKB or the highly specific agonist succinyl-DFMe-FGLM-NH2 (senktide) increased the firing frequency of dopaminergic neurons and as a consequence the dopamine (DA) release in the nucleus accumbens (Alonso et al., 1996; Marco et al., 1998; Nalivaiko et al., 1997). This effect of NK_3 receptor stimulation on the dopaminergic neurons and dopamine release implies that NK_3 receptor may provide a means for modulating the dopaminergic system without the strong adverse side effects produced by D_2 receptor antagonists (Leucht et al., 2003). Data from two double blind placebo controlled (short term) clinical trials studying the effects of two selective NK_3 antagonists from two distinct chemical classes, osanetant (SR 142801) and talnetant (SB 223412), have demonstrated significant improvement in positive symptoms (Evangelista, 2005; Meltzer and Prus, 2006; Meltzer et al., 2004; Spooren et al., 2005). Both NK_3 antagonists were better tolerated than their respective controls, e.g. haloperidol and risperidone, and efficacy was established in the absence of the typical side-effects of first and second generation antipsychotics e.g. weight gain, diabetes and extrapyramidal symptoms (EPS) (Leucht et al., 2003). Preclinical studies investigating the effects of talnetant on prefrontal DA and noradrenaline (NA) release have shown that both DA and NA release were significantly increased following treatment with talnetant. The effect was comparable to that observed with the atypical anti-psychotic clozapine (Dawson et al., 2008). These studies suggest that NK_3 antagonists may hold potential for treating cognitive deficits in schizophrenia. Interestingly, clinical investigation of talnetant in healthy volunteers has shown that it slightly improves visuomotor coordination and vigilance (Liem-Moolenaar et al., 2010). Moreover, recent findings have demonstrated that four out of nine multiplex families affected by hypogonadotropic hypogonadism carry loss-of-function mutations in NKB or the hNK_3 receptor, hence indicating that NKB/NK_3 signaling also plays a key role in the hypothalamic regulation of reproduction in humans (Silveira et al., 2010; Topaloglu et al., 2009).

Although NK_2 receptors are mainly found in the peripheral nervous system on end-organs, such as airway, bladder, and gastrointestinal (GI) tract smooth muscles (Evangelista et al., 2003), highly localized expression of NK_2 receptors were also detected in brain areas related to emotional networks, anxiety and depression including PFC (infralimbic cortex), cingulate cortex, hippocampus, nucleus accumbens, amygdala, mediodorsal thalamus, and lateral septum (Bensaid et al., 2001; Nagano et al., 2011; Saffroy et al., 2003). Preclinical investigations with an NK_2 antagonist, saredutant (SR48968) have shown anxiolytic and antidepressant-like effects in various rodent models (Louis et al., 2008; Micale et al., 2008; Steinberg et al., 2001). Saredutant has been investigated in major depressive disorders (MDD) patients in randomized double-blind placebo controlled phase II clinical trials, with positive results (Ebner et al., 2009; Quartara et al., 2009), and has made it recently into clinical phase III trials (Hopkins, 2010). Based on this evidence, it can be hypothesized that the combination of NK_2 and NK_3 antagonism may provide a therapeutic benefit for both positive and negative symptoms associated with schizophrenia; and an improved side effect profile over existing antipsychotics.

The objective of the current study was to provide a comprehensive *in-vitro* characterization of a novel NK_2/NK_3 antagonist, RO5328673. The RO5328673's mode of antagonism was determined at human and guinea-pig NK_3 wild-type and mutated receptors by binding kinetics and functional analysis using [MePhe^7] NKB-induced formation of [^{3}H]IP assay. Our data show that RO5328673 is a potent dual NK_3/NK_2 antagonist with an insurmountable (also referred as apparent non-competitive or pseudo-irreversible (Vauquelin et al., 2002)) mode of antagonism at both human and guinea-pig NK_3 receptors in a recombinant and native preparation (electrophysiological recordings in guinea-pig midbrain slices). RO5328673 inhibits NK_3-mediated *in-vivo* effect (gerbil locomotor activity) induced by selective NK_3 agonist. Furthermore, by carrying out [^{3}H]RO5328673 binding on wild-type and mutated hNK_3, the critical determinants for high-affinity binding site of RO5328673 were identified. These data together with rhodopsin-based 3D modeling allowed developing a model that suggests a likely docking mode of RO5328673 to hNK_3 receptor and its comparison with osanetant−hNK_3 binding pocket.

2. Materials and methods

2.1. Materials

Aprepitant (Emend or MK-869, 2-(R)-(1-(R)-3,5-Bis(triflurometyhl)phenyl-ethoxy)-3-(S)-(4-fluoro)phenyl-4-(3-oxo-1,2,4-triazol-5yl)methylmorphine), CP-96,345 ((2S, 3S)-cis-2-(Diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine), MDL 105,212 (1-(2-[(R)-3-(3,4-Dichloro-phenyl)-1-(3,4,5-trimethoxy-benzoyl)-pyrrolidin-3-yl]-ethyl)-4-phenyl-piperidine-4-carboxylic acid amide), osanetant (SR 142801, (S)-(+)-N-((3-[1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl]prop-1-yl)-4-phenylpiperidin-4-yl}-N-methylacetamine), RO5328673 (((3S,4R)-4-(3,4-Dichloro-phenyl)-1-[1-(1-methyl-cyclopropanecarbonyl)-piperidine-4-5 carbonyl]-pyrrolidin-3-yl)-methyl-carbamic acid 4-fluoro-phenyl ester), saredutant (SR 48968, N-[(S)-4-(4-Acetylamino-4-phenyl-piperidin-1-yl)-2-(3,4-dichloro-phenyl)-butyl]-N-methyl-benzamide), SB222200 ((*S*)-(−)-*N*-(α-ethylbenzyl)-3-methyl-2-phenylquinoline-4-carboxamide), talnetant (SB223412, (*S*)-(−)-*N*-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide) were synthesized within the Chemistry department of F. Hoffmann-La Roche. [^{3}H]RO5328673 (specific activity: 21.3 Ci/mmol) was synthesized at the Roche chemical and isotope laboratories. [^{3}H]osanetant ([^{3}H]SR 142801, specific activity: 74.0 Ci/mmol), [^{3}H]SP ([^{3}H]Substance P, specific activity: 40.0 Ci/mmol), [^{3}H]SR 48968 (specific activity: 27.0 Ci/mmol), [*myo*-1,2−^{3}H]inositol with PT6-271 (specific activity: 16.0 Ci/mmol) and yttrium silicate (Ysi) RNA binding beads were purchased from GE Healthcare UK limited (Chalfont St. Giles, UK). [MePhe^7]Neurokinin B (H-Asp-Met-His-Asp-Phe-Phe-NMe-Phe-Gly-Leu-Met-NH_2) was purchased from NeoMPS SA (Strasbourg, France). Senktide (succinyl-Asp-Phe-Me-Phe-Gly-Leu-Met-NH2) were obtained from Tocris Biosciences (Bristol, UK).

2.2. Construction of point-mutated NK_3 receptors

cDNAs encoding the human NK_1 (hNK_1, accession no.: P25103), human NK_2 (hNK_2, accession no: P21452), human NK_3 (hNK_3, accession no.: P29371), and guinea-pig NK_3 receptors, ($gpNK_3$, accession no.: P30098) were subcloned into pCI-Neo expression vectors (Promega Corporation, Madison, WI). All point-mutants were constructed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions and using pCI-Neo-NK as a DNA template. Complementary oligonucleotide primers (sense and antisense) containing the single site or double sites of mutations were synthesized by Microsynth AG (Balgach, Switzerland). The following PCR conditions were used for repeated extensions of the plasmid template: 95 °C for 1 min and 20 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 8 min using 50 ng plasmid DNA, 100 ng each of primers and 2.5 units Pfu Turbo DNA polymerase (Stratagene). The entire coding regions of all positive point-mutants were sequenced from both strands using an automated cycle sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Cell culture, transient transfection, and membrane preparation

Human embryonic kidney (HEK)293 cells were transfected as previously described (Malherbe et al., 2008). After forty-eight hours posttransfection, the cells were harvested and washed three times with ice-cold PBS and frozen at −80 °C. The pellet was suspended in ice-cold 50 mM Tris−HCl pH 7.4 buffer containing 10 mM EDTA (10× volume) and homogenized with a polytron (Kinematica AG, Basel,