Acta Pharmacol Sin 2008 Jun; 29 (6): 752–758

Full-length article

High-throughput screening of novel antagonists on melanin-concentrating hormone receptor-1[1]

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Key words

melanin-concentrating hormone; melanin-concentrating hormone receptor-1; antagonist

[1]Project supported in part by the Shanghai Municipality Science and Technology Development Fund (No 06DZ22907 and 07DZ22920), the Ministry of Science and Technology (No 2004CB518902), and Servier Beijing Pharmaceutical Research and Development Co, Ltd.
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Received 2008-01-22
Accepted 2008-03-13

doi: 10.1111/j.1745-7254.2008.00800.x

Abstract

Aim: To find new antagonists on human melanin-concentrating hormone receptor-1 (MCHR-1) through high-throughput screening (HTS) of a diverse compound library. **Methods:** MCHR-1, [^{3}H]SNAP7941, and FlashBlue G-protein-coupled receptor beads were used to measure the receptor-binding activities of various compounds based on scintillation proximity assay (SPA) technology. The guanosine 5' (γ-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) binding assay was subsequently applied to functionally characterize the "hits" identified by the HTS campaign. **Results:** Of the 48 240 compounds screened with the SPA method, 12 hits were confirmed to possess MCHR-1 binding activities, 8 were functionally studied subsequently with the [^{35}S]GTPγS binding assay, and only 1 compound (NC127816) displayed moderate human MCHR-1 binding affinity (K_i=115.7 nmol/L) and relatively potent antagonism (K_B=23.8 nmol/L). This compound shares a novel scaffold (1-ethoxy-2*H*-2-aza-1-phospha-naphthalene 1-oxide) with 3 other analogs in the group. **Conclusion:** Considering the marked difference in molecular shape and electrostatic status between NC127816 and the structures reported elsewhere, we anticipate that its derivatives may represent a new class of potent MCHR-1 modulators.

Introduction

Melanin-concentrating hormone (MCH) is a 17-amino acid cyclic peptide originally isolated and sequenced from the salmon pituitary in 1983. It was regarded as a regulator of the pigmentary changes in background adaptation[1]. Rat MCH, purified from the hypothalamus, is identical to that of humans, and is 19 amino acids in length, 2 amino acids longer than its salmon counterpart[2]. In mammalian species, MCH is predominately synthesized in 2 brain centers, namely the perikarya of the lateral hypothalamus and zona incerta, and becomes widely distributed throughout the central nervous system (CNS). The extensive terminal distribution suggests that MCH may serve as a neurotransmitter or modulator in regulating brain functions[3]. Previous studies have revealed that the biological effects of MCH are complex, including feeding and energy balance[4-6], sexual behavior[7], stress responses[8], neuroendocrine functions[9-11], anxiety[7,12], seizure[13], memory and/or learning[14,15], grooming and locomotor activities[16], and arousal regulation[17].

In 1999, 5 independent groups almost simultaneously reported the identification of a MCH receptor [MCHR; MCHR-1, MCH-1R, MCH1, MCH_1, or SLC-1 (orphan somatostatin-like receptor 1)] through a reverse pharmacology approach[18-22]. A second MCH receptor (MCHR-2, MCH-2R, MCH2, MCH_2, or SLT) was identified subsequently based on the sequence homology to MCHR-1[23-27]. However, the functions of MCHR-2 remain unclear at present due to its species-specific expression and the lack of non-rodent *in vivo* models for behavioral studies[28,29]. Therefore, the focus of the current research is directed towards MCHR-1.

The stimulation of MCHR-1 leads to the elevation of the intracellular Ca^{2+} level, inhibition of forskolin-stimulated cyclic adenosine monophosphate (cAMP) production, and activation of the mitogen-activated protein kinase cascade, indicating that MCHR-1 is a G-protein-coupled receptor (GPCR) linked to both $G_{i/o}$ and G_q proteins[30]. MCHR-1 is different from the limited expression of MCH in the lateral hypothalamus and zona incerta, as it is widely distributed throughout the CNS, including the cerebral cortex, caudate-putamen, hippocampal formation, subiculum, the shell of the nucleus accumbens, amygdala, hypothalamus and thalamus, the locus coeruleus of the brainstem, various nuclei of the mesencephalon and rhombencephalon, as well as most anatomical areas implicated in the control of olfaction, with the exception of the main olfactory bulb[31,32]. Based upon a series of studies using MCHR-1 antagonists and knock-out mice lacking MCHR-1, it has been established that MCHR-1 mediates feeding behavior and energy expenditure[33-35]. Experimental evidence also demonstrates that MCHR-1 plays an important role in the regulation of mood and stress responses. Furthermore, the pharmacological properties of MCHR-1 antagonists in rodent models of stress-related disorders (such as depression and anxiety) have been elucidated[35,36].

The discovery of the MCH receptors has prompted pharmacological scientists and behavioral biologists to evaluate their potential therapeutic applications. Both peptidic and non-peptidic MCHR-1 ligands have been reported, and the effects of more than 50 MCH analogs have been investigated to understand their structure–activity relationships (SAR)[37]. Intracerebroventricular injection of these MCH analogs led to a rapid and significant increase in food intake, the efficacy of which was strongly correlated with their potency at MCHR-1[38]. The results of this study clearly suggest that MCHR-1 mediates the orexigenic effects of MCH[38]. Some non-peptidic MCHR-1-selective antagonists have been identified, such as T-226296 and SNAP7941, both of which have anorectic effects[35,39].

The scintillation proximity assay (SPA) uses homogeneous and radioisotopic technology that does not involve post-reaction liquid handling steps, and is well-suited to automation and high-throughput screening (HTS)[40]. In the SPA system, membranes that express a particular receptor are attached to a microbead coated with wheat germ agglutinin. An isotope (e.g. [^{3}H]) is brought very close to the scintillant-impregnated microbead by binding to its surface. Because the emitted β particles can only travel short distances in the bulk solution, the microbead preferentially captures electrons from the bound radiolabeled ligand. The amount of light emitted from the scintillant in the microbead is thus directly proportional to the amount of bound radiolabeled ligands. In this study, we describe a SPA-based HTS campaign involving a diverse library of 48 240 synthetic and natural compounds. Using the SPA technology, human MCHR-1 (hMCHR-1) binding affinities were determined with [^{3}H] SNAP7941 competitive displacement. The functionality (agonist or antagonist activities) was subsequently assessed by a guanosine 5' (γ-[^{35}S]thio) triphosphate ([^{35}S]GTPγS) binding assay[41]. As a result, a novel hMCHR-1 antagonist was discovered together with a series of ligands previously unreported for this receptor.

Materials and methods

Reagents The radioligand [^{3}H]SNAP7941 (specific activity: 2.26 TBq/mmol) and [^{35}S]GTPγS (specific activity: 37 TBq/mmol) were purchased from Amersham (Buckinghamshire, UK). FlashBlue GPCR beads were obtained from Perkin–Elmer (Boston, MA, USA). Guanosine diphosphate (GDP), GTPγS, saponin, HEPES, glutamine, bovine serum albumin (BSA), and MCH are the products of Sigma–Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was bought from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose was the product of GIBCO BRL (Grand island, NY, USA). SNAP7941 was provided by Servier (Neuilly-sur-Seine, France).

Membrane preparation Human embryonic kidney cells (HEK293) stably expressing the hMCHR-1 receptor were cultured in DMEM with high glucose supplemented with 10% FBS, 2 mmol/L glutamine, 1×10^5 IU/L penicillin, 100 mg/L streptomycin, and 400 mg/L G418. The cells were grown at confluence, harvested in phosphate-buffered saline, and centrifuged at 1000×*g* for 5 min (4 °C). The resulting pellet was suspended in isotonic buffer (5 mmol/L Tris/HCl, 0.2 mmol/L $MgCl_2$, and 0.25 mol/L sucrose, pH 7.4) and homogenized using the BioNeb Cell Disruption System (Terre Haute, IN, USA). The homogenate was then centrifuged (20 000×*g* for 30 min at 4 °C), and the resulting pellet was resuspended in the binding buffer 1 (50 mmol/L Tris/HCl, 120 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, pH 7.4). Protein content was determined using the Bradford assay[42]. Aliquots of membrane preparations were stored at –80 °C until use.

Homogeneous hMCHR-1 binding assay The membranes were incubated overnight in binding buffer 1 containing 2.5 nmol/L [^{3}H]SNAP7941, 0.75 g/L FlashBlue GPCR beads, various titrations of SNAP7941 from a stock solution of 40 μmol/L, and the library compounds with an average concentration of 6.7 μmol/L (final volume: 100 μL). Non-specific binding was defined with 1 μmol/L SNAP7941. Data were analyzed with GraphPad PRISM (GraphPad Software, San Diego, CA, USA).

The competitive inhibition constant (K_i) was calculated according to the Cheng–Prussof equation: $K_i=IC_{50}/[1+(L/K_d)]$, where IC_{50} is the concentration that produced 50% inhibition, and L is the concentration of radiolabeled ligands used[43].

[^{35}S]GTPγS binding assay The membranes and test compounds were diluted in the binding buffer 2 (50 mmol/L HEPES, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 3 μmol/L GDP, 0.1% BSA, pH 7.4) in the presence of 10 mg/L saponin, in order to enhance the agonist-induced stimulation level[37,44], and then premixed with 1 g/L FlashBlue GPCR beads. For the agonist test, incubation was started by the addition of 0.2 nmol/L [^{35}S]GTPγS to the membranes and test compounds, and carried on for 1 h at room temperature in a final volume of 100 μL. To study the antagonist activity, the membranes were pre-incubated for 2 h with MCH (200 nmol/L) in conjunction with a given concentration of a test compound. Reaction was begun with the addition of 0.2 nmol/L [^{35}S] GTPγS followed by 1 h incubation at room temperature. Non-specific binding was assessed using non-radiolabeled GTPγS (10 μmol/L). Data were analyzed with GraphPad PRISM to calculate the 50% effective concentration (EC_{50}), and the maximal effect (E_{max}) was expressed as a percentage of that observed with 1 μmol/L MCH (100%) for agonists. K_B was used to describe antagonist potency: $K_B=IC_{50}/(1+[agonist]/EC_{50})$, where IC_{50} is the antagonist concentration that gives 50% inhibition of [^{35}S]GTPγS binding in the presence of a fixed concentration of an agonist, and EC_{50} is the 50% effective concentration of an agonist when tested alone[43]. The maximal inhibitory effect (I_{max}) was expressed as a percentage of that observed with 200 nmol/L MCH on hMCHR-1.

Results

HTS campaign In the validation process, various assay parameters were studied. Different concentrations of [^{3}H] SNAP7941 were tested, and it was found that low concentrations of this radioligand resulted in poor signal detection. When the concentration was increased to 2.5 nmol/L, a satisfactory signal (~100-fold over the instrument noise) and signal/background ratio were achieved. Under this experimental condition, the optimal concentrations of receptor protein extract and microbeads were determined to be 1.3 mg/L and 0.75 g/L, respectively (Figure 1). The control compound, SNAP7941, was then tested on hMCHR-1 to validate the assay system. As shown in Figure 2, the K_i value of SNAP7941 was calculated to be 0.16 nmol/L, similar to that reported in the literature[35,45]. In order to apply the assay to a HTS format, the Z' value and signal/background (S/B) ratio (i.e. specific *vs* non-specific binding) were studied. As shown in Figure 3, the Z' value of this SPA method was 0.77, with an S/B ratio of 6.59 and a coefficient of variation (CV) value of 4.7%. These characteristics suggest that the assay system is of high quality and suitable for HTS[46]. The scatter plots of the HTS campaign directed towards hMCHR-1 are shown in Figure 4. Of the 48 240 samples screened, 24 initial hits were identified (≥50% inhibition of SNAP7941 binding to hMCHR-1). Among them, 12 hits were subsequently confirmed and their IC_{50} values determined.

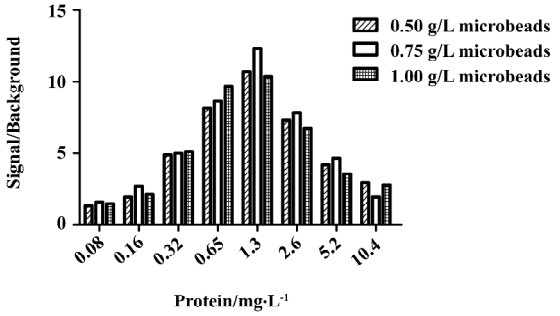


Figure 1. Optimization of assay parameters. When the concentration of [^{3}H]SNAP7941 was increased to 2.5 nmol/L, satisfactory signal and S/B ratio were achieved. Under this condition, various concentrations of receptor protein extract and microbeads were studied, and 1.3 mg/L and 0.75 g/L were chosen for the experimentation, respectively.

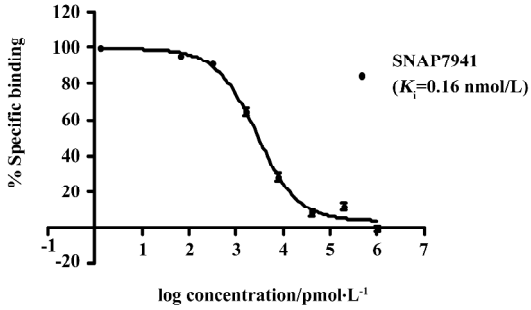


Figure 2. Displacement of [^{3}H]SNAP7941 binding to hMCHR-1-expressing HEK293 cell membrane preparation by SNAP7941. Data shown are mean±SD of triplicate measurements and representative of at least 3 independent experiments.

Functionality characterization To examine the pharmacological properties (agonist or antagonist activities) of these hit compounds on hMCHR-1, [^{35}S]GTPγS binding assays were performed. The antagonist activity of SNAP7941 is shown in Figure 5. Based on the potency displayed in the receptor-binding assay, we selected 8 confirmed hits identified from the HTS campaign for simultaneous functionality studies. Two compounds exhibited an antagonist profile and none demonstrated agonist activity (Table 1). Four of

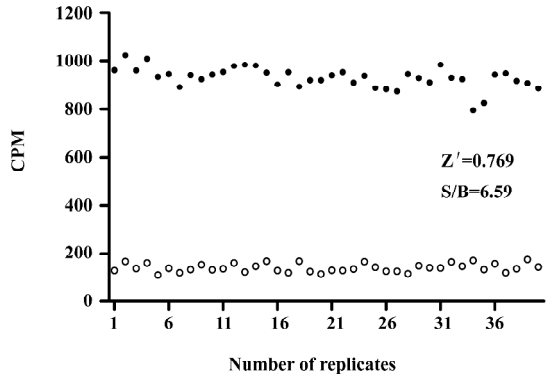


Figure 3. Z' value and S/B ratio determination. Homogenous hMCHR-1 binding assay was performed at optimized conditions with hMCHR-1-expressing HEK293 cells, [^{3}H]SNAP7941 (2.5 nmol/L), and SNAP7941 (1 μmol/L). Background is the radioactivity counts in 2.5% DMSO solution where SNAP7941 is dissolved. Forty replicates of signal and background readouts were studied.

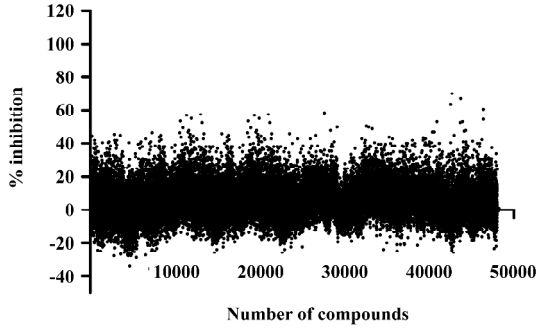


Figure 4. HTS of 48 240 compounds using hMCHR-1 receptor binding assay. Results are expressed as the percentage inhibition of [^{3}H]SNAP7941 binding to the receptor by each sample.

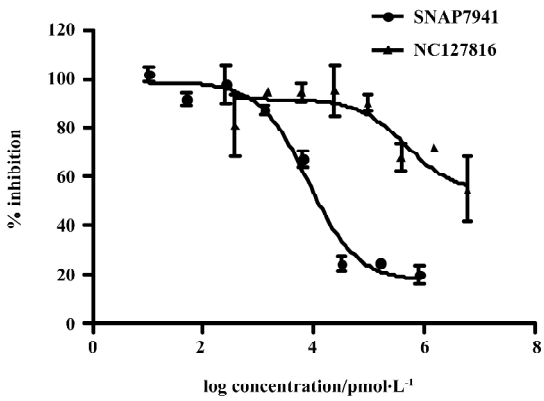


Figure 5. Dose–response curves of SNAP7941 and NC127816 on hMCHR-1 measured by the GTPγS assay from which IC_{50} values were calculated. Data shown are mean±SD of triplicate measurements and representative of at least 3 independent experiments.

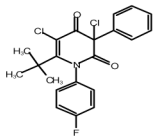
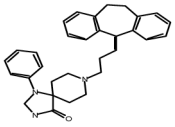
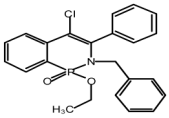
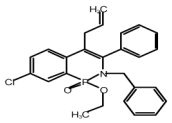
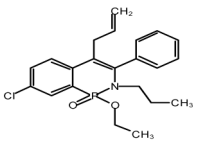
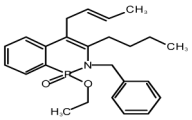
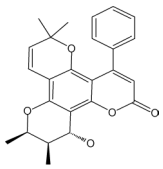
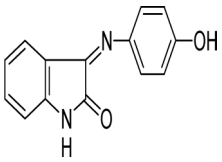
the 8 compounds (NC127816, NC127844, NC127847, and NC127853) share a general chemical skeleton of 1-ethoxy-2*H*-2-aza-1-phospha-naphthalene 1-oxide (Figure 6), including 1 hMCHR-1 antagonist (NC127816; Figure 5), as determined by the GTPγS assay. Compound NC127398 with antagonist activity and a different chemical scaffold had a lower hMCHR-1 binding affinity and was less potent compared to NC127816 (Table 1). The other 3 confirmed hits, namely, NC091703, NC093198, and NC127858, with different scaffolds, bound to the receptor at submicromolar range, without apparent functional properties (Table 1).

Discussion

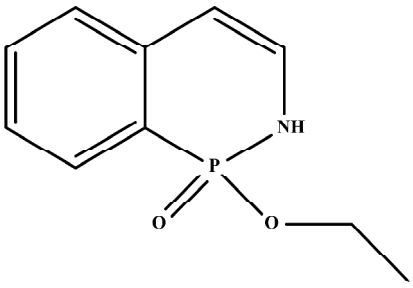
The increasing demand for better therapeutic agents to control the worldwide obesity epidemic has led to some major efforts in this highlighted research area. As reported previously, transgenic mice overexpressing the MCH gene are susceptible to insulin resistance and obesity, while mice lacking the gene encoding MCH or MCHR-1 are lean, resistant to diet-induced obesity, and maintain elevated metabolic rates[47]. There exists compelling evidence indicating that the antagonism of the MCHR-1 system could be an effective means of treating obesity. This understanding has generated much interest among pharmaceutical/biotechnology companies in the discovery of MCHR-1 antagonists. As a result, many MCHR-1 antagonists with diverse structures have been identified, including aminoquinoline, indazole, biphenyl carboxamide, and thienopyrimidinone scaffolds. However, significant development hurdles concerning safety, selectivity, and metabolic stability remain to be overcome before they can be declared as drug candidates[48]. In addition, the broad distribution of MCH and MCHR-1 within the CNS bring about a number of diverse functions, which may cause adverse effects in chronic treatment of obesity. Thus novel scaffolds are needed to achieve the ultimate goal of discovering safer and more selective MCHR-1 antagonists.

In the present study, we describe an HTS campaign and subsequent identification of a novel antagonist to MCHR-1 and a series of new ligands with chemical skeletons not reported previously. The primary screening assay, that is, the SPA technology-based competitive receptor binding assay, was fully validated to possess an optimal Z' factor, S/B (or signal to noise) ratio, and CV value for HTS. Of the 48 240 synthetic and natural compounds screened, 24 hits that showed ≥50% inhibition in competition with the control compound, SNAP7941, were initially identified (initial hit rate =0.05%). Among them, 12 hits (final hit rate =0.025%) were subsequently confirmed. [^{35}S]GTPγS binding methods in both agonist and antagonist modes were used as secondary functionality screening. Among the 8 confirmed hits selected, only 1 compound (NC127816) displayed moderate hMCHR-1

Table 1. Binding properties and functionality characteristics of 8 confirmed hits on hMCHR-1.

Compound	Structure	Molecular formula	Molecular weight	K_i (nmol/L)	I_{max} in GTPγS assay	K_B in GTPγS assay (nmol/L)
NC091703		$C_{21}H_{18}NO_2FCl_2$	406.3	938.4	<20%	NA
NC093198		$C_{31}H_{33}N_3O$	463.6	729.9	<20%	NA
NC127816		$C_{23}H_{21}NO_2PCl$	409.8	115.7	45%	23.8
NC127844		$C_{26}H_{25}NO_2PCl$	449.9	381.5	54%	NA
NC127847		$C_{22}H_{25}NO_2PCl$	401.9	332.2	<20%	NA
NC127853		$C_{25}H_{32}NO_2P$	409.5	502.7	<20%	NA
NC127858		$C_{25}H_{24}O_5$	404.5	426.9	41%	NA
NC127398		$C_{14}H_{10}N_2O_2$	238.2	1775.2	79%	54.4

NA, not active, that is,, unable to calculate the K_B value.



1-Ethoxy-2*H*-2-aza-1-phospha-naphthalene 1-oxide

Figure 6. Skeleton of 4 newly discovered MCHR-1 ligands.

binding affinity (K_i=115.7 nmol/L) and relatively potent antagonism (K_B=23.8 nmol/L). Compared to the long chain structures of MCHR-1 modulators documented in the literature, NC127816 and its 3 analogs have an obviously simpler structure (Figure 6) and smaller molecular size. Based on this skeleton, we made some slight modifications according to electronic properties, steric effects, and bioisosteric replacement principles. To our surprise, most of the newly synthesized analogs bound to MCHR-1 with different affinities (data not shown), indicating the potential of using 1-ethoxy-2*H*-2-aza-1-phospha-naphthalene 1-oxide as a scaffold for further SAR analyses.

It is known that three dimensional (3-D) shape and electrostatic similarity play important roles in defining MCHR-1 antagonists[49]. Considering the marked difference in molecular shape and electrostatic status between NC127816 and the structures reported elsewhere, we anticipate that the derivatives of 1-ethoxy-2*H*-2-aza-1-phospha-naphthalene 1-oxide may represent a new class of potent MCHR-1 modulators, which could lead to not only new therapeutics for obesity, but also further comprehension of MCH/MCHR-1-related biology.

Acknowledgements

We are indebted to Mr Xin HUI, Ms Hong YU, Dr Ming-kai XU, and Mr Qiang SHEN for their technical assistance, and to Dr Dale E Mais for valuable discussion.

Author contribution

Ming-wei WANG designed the research; Jian-hua YAN, Qun-yi LI performed the research;Jean A BOUTIN, M Pierre RENARD, Yi-xiang DING, Xiao-jiang HAO, Wei-min ZHAO contributed new reagents or analytic tools; Ming-wei WANG and Jian-hua YAN analyzed data; Ming-wei WANG, Jian-hua YAN wrote the paper.

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