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The pharmacological properties of a novel MCH_1 receptor antagonist isolated from combinatorial libraries

Hiroshi Nagasaki[1,*], **Shinjae Chung**[2,3,*], **Colette T. Dooley**[4], **Zhiwei Wang**[2], **Chunying Li**[2], **Yumiko Saito**[5], **Stewart D Clark**[2], **Richard A. Houghten**[6], and **Olivier Civelli**[2,3]

1 *Dept. of Metabolic Medicine, School of Medicine, Nagoya University, Nagoya, Japan*

2 *Department of Pharmacology, University of California Irvine, Irvine, CA*

3 *Department of Developmental and Cell Biology, University of California Irvine, Irvine, CA*

4 *Torrey Pine Institute for Molecular Studies, Fort Pierce, FL*

5 *Laboratory for Behavioral Neuroscience, Graduate School of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan*

6 *Torrey Pine Institute for Molecular Studies, San Diego, CA*

Abstract

Melanin-concentrating hormone (MCH) is a neuropeptide that exhibits potent orexigenic activity. In rodents, it exerts its actions by interacting with one receptor, MCH_1 receptor which is expressed in many parts of the central nervous system (CNS). To study the physiological implications of the MCH system, we need to be able to block it locally and acutely. This necessitates the use of MCH_1 receptor antagonists. While MCH_1 receptor antagonists have been previously reported, they are mainly not accessible to academic research. We apply here a strategy that leads to the isolation of a high affinity and selective MCH_1 receptor antagonist amenable to in vivo analyses without further chemical modifications. This antagonist, TPI 1361-17, was identified through the screening of multiple non-peptide positional scanning synthetic combinatorial libraries (PS-SCL) totaling more than eight hundred thousand compounds in conditions that allow for the identification of only high-affinity compounds. TPI 1361-17 exhibited an IC_{50} value of 6.1 nM for inhibition of 1 nM MCH-induced Ca^{2+} mobilization and completely displaced the binding of [^{125}I] MCH to rat MCH_1 receptor. TPI 1361-17 was found specific, having no affinity for a variety of other G-protein coupled receptors and channels. TPI 1361-17 was found active in vivo since it blocked MCH-induced food intake by 75 %. Our results indicate that TPI 1361-17 is a novel and selective MCH_1 receptor antagonist and is an effective tool to study the physiological functions of the MCH system. These results also illustrate the successful application of combinatorial library screening to identify specific surrogate antagonists in an academic setting.

Index words

MCH_1 receptor; combinatorial libraries; antagonist; G-protein coupled receptors; TPI 1361-17

Corresponding Author: Olivier Civelli, Department of Pharmacology, University of California, Irvine, 369 Med Surge II, Irvine CA, 92612, Phone; 949-824-2522, Fax; 949-854-4106, civelli@uci.edu.
*These authors contributed equally

1. Introduction

Melanin-concentrating hormone (MCH) is a cyclic, 19-amino-acid peptide isolated from salmon pituitary as a melanophore concentrating factor (Kawauchi et al., 1983), that is also present in rat and human (Mouri et al., 1993; Vaughan et al., 1989). In the mammalian brain, MCH is predominantly expressed in the perikarya of the lateral hypothalamic area (LHA), and the zona incerta (ZI) and projects widely throughout the central nervous system (Bittencourt et al., 1992; Vaughan et al., 1989). MCH is known to interact with two receptors, MCH_1 receptor (Saito et al., 1999) and MCH_2 receptor (Mori et al., 2001; Sailer et al., 2001). MCH_1 receptor is expressed in the brain regions where MCH fibers were identified, in particular in centers modulating feeding behavior (Bittencourt et al., 1992; Saito et al., 2001a; Saito et al., 1999), and in some peripheral tissues (Bradley et al., 2000; Pissios et al., 2006; Saito et al., 2000; Saito et al., 2001b; Tadayyon et al., 2000; Verlaet et al., 2002). MCH_2 receptor, identified on the basis of its sequence similarity to MCH_1 receptor, is species-specific, most notably it is absent in rodents (Tan et al., 2002), a fact that has impaired study of its function.

In mammals, MCH has been mostly implicated in the regulation of food consumption and energy metabolism. Central administration of MCH has been shown to promote feeding (Qu et al., 1996), while MCH_1 receptor mRNA levels rise as a result of starvation and leptin deficiency (Kokkotou et al., 2005) and MCH circulatory levels are high in obese Zucker rats (Stricker-Krongrad et al., 2001). MCH also stimulates insulin and leptin release in insulinoma cell lines and 3T3-L1 adipose cells, respectively (Bradley et al., 2000; Tadayyon et al., 2000) and regulates pituitary hormones (Kennedy et al., 2003; Kennedy et al., 2001; Murray et al., 2000; Tsukamura et al., 2000). The importance of MCH system in energy metabolism was confirmed by genetic interventions. Mice devoid of MCH are lean and hypophagic (Shimada et al., 1998), while mice over-expressing MCH are obese and hyperphagic (Ludwig et al., 2001). Genetic disruption of MCH_1 receptor on the other hand results in mice that are surprisingly hyperphagic, but also hypermetabolic, and obesity-resistant (Chen et al., 2002; Marsh et al., 2002). The reasons for this discrepancy are unknown.

MCH_1 receptor antagonists have been shown to be effective at modulating feeding and diet induced obesity (Borowsky et al., 2002; Kowalski et al., 2004; Kowalski et al., 2006; Mashiko et al., 2005; Shearman et al., 2003); reviewed in Handlon and Zhou, 2006). Consistent with the wide distribution of MCH_1 receptor in the brain, the MCH system is expected to be involved in various physiological functions and indeed a MCH_1 receptor antagonist has been shown to have antidepressant and anxiolytic activity (Borowsky et al., 2002; Chaki et al., 2005; David et al., 2007; Georgescu et al., 2005; Shimazaki et al., 2006; Smith et al., 2006). MCH_1 receptor antagonists have also been reported to improve social memory in a social recognition test (Millan et al., 2008). On the other hand, MCH_1 receptor knockout mice exhibit cognitive deficits in inhibitory passive avoidance test (Adamantidis et al., 2005).

To fully understand physiological roles of the MCH system, we need to be able to study the behavioral effects resulting from acute blockade of the MCH system. This necessitates the isolation of an antagonist. A MCH antagonist presents the following advantages: 1) its effects can be monitored after both acute or chronic administration; 2) it is not plagued by the developmental complications that may be associated with genetic manipulations; 3) it can be injected locally allowing to differentiate peripheral and central effects for example; 4) it is not restricted to one animal species; 5) it allows to set up parameters for testing the system in humans. In order to isolate a specific MCH_1 receptor antagonist, we chose to screen mixture based combinatorial libraries. Combinatorial chemistry has emerged as a powerful tool for the discovery and optimization of new leads in the pharmaceutical industry (Pinilla et al., 2003).

This has been largely due to advances in high-throughput screening as well as more efficient parallel synthesis and purification techniques. The most commonly used mixture libraries and deconvolution methods include iterative, positional scanning and the sequencing of resin-bound peptides or tags from one-bead, one compound library. Furthermore, provided that access to a semi high-throughput technology is available, screening for a specific antagonist in the academic setting is possible. In this study, we screen heterocyclic positional scanning synthetic combinatorial libraries (PS-SCL) to isolate a specific MCH_1 receptor antagonist which will be useful to study physiological functions of the MCH system.

2. Materials and Methods

2.1. Drugs

Rat / human MCH, rat / human NPY, rat urotensin II, Dynorphin and angiotensin II were purchased from Bachem California (Torrance, CA). Human Orexin B was purchased from Anaspec (San Jose, CA). [^{125}I]-MCH was purchased from AP Biotech (Piscataway, NJ). Phosphoramidon and phenylmethylsulfonylfuloride (PMSF) were purchased from Sigma (St. Louis, MO).

2.2. Non-peptide positional scanning combinatorial library

We used several heterocyclic and peptidomimetic positional scanning combinatorial libraries (PS-SCL) for screening. Each library was prepared by using simultaneous multiple synthesis technology (Houghten, 1985) as described elsewhere (Ostresh, 1998). List of the libraries and a representation of an N-benzyl aminocyclic thiourea are presented (Table 1). The identity and purity of each individual compound were analyzed by mass spectral analysis interfaced with a liquid-chromatography system (Finnigan LCQ) and/or analytical reverse-phase high performance liquid chromatography (RP-HPLC) using a Vydac C18 column and a Beckman system Gold HPLC. The compounds were purified by using a Waters Milliprep 3400 preparative HPLC with a preparative Foxy fraction collector.

2.3. Cell culture and transfection

MCH_1 receptor was mixed with LipofectAMINE transfection reagents (Life Technologies, MD), and the mixture was diluted with Opti-MEM and added to 60–70 % confluent HEK 293T cells plated on 100-mm dishes. The transfected cells were cultured in DMEM containing 10 % FBS. For Calcium mobilization assay and radioligand binding assay, CHO cells or HEK-293T cells stably expressing rat MCH_1 receptor were used (Saito et al., 1999).

2.4. Measurement of calcium influx

Transfected HEK293T cells seeded on black-walled 96-well plates (Sigma, St Louis USA) were loaded for 1 h at 37°C with Ca^{2+}-sensitive fluorescent dye, fluo-4 (Molecular Device, CA, USA) in Hanks' balanced salt solution containing 20 mM HEPES (pH 7.4). The level of $[Ca^{2+}]_i$ was then monitored using a FLIPR system (Fluorometric Imaging Plate Reader; Molecular Devices, San Francisco, CA). For antagonists screening, each mixture from the compound library was first incubated with the cell for 10 minutes, before the addition of MCH. Data were expressed as fluorescence (arbitrary units) versus time.

2.5. MCH_1 receptor-binding experiments

HEK293T cells expressing the rat MCH_1 receptor were scraped with ice-cold PBS and centrifuged at 1000 × g for 5 min. Cell pellet was homogenized with ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, and ultra-centrifuged twice at 48,000 × g for 20 min at 4°C. The pellets were then suspended in 50 mM Tris-HCl (pH 7.4) buffer containing 5 mM EDTA and used as membrane fractions. The membrane fraction (30 μg protein for each assay)

dissolved in 500 μl assay buffer containing 50 mM Tris-HCl buffer (pH 7.4), 1 μM phosphoramidon, 0.5 mM phenylmethylsulfonylfluoride and 0.2 % BSA with 0.1 nM [^{125}I] (Phe^{13}, Tyr^{19}) MCH and various concentrations of cold MCH and TPI1361-17 at room temperature for 2 h. Nonspecific binding was determined by including 1 μM MCH in the binding reaction. The binding reaction was terminated by rapid filtration through GF/C filter plates pre-soaked in 0.2 % polyethylenimine, followed by washing three times with 3 ml of PBS. The radioactivity retained in the filters was determined with a γ-counter. IC_{50} values calculated as described above are expressed as the mean ± S.E.M. for three independent determinations.

2.6. Selectivity screening of TPI 1361-17

Binding analyses were carried out by MDS Pharma services (Taipei, Taiwan) and assay details and literature references are available online at http://discovery.mdsps.com/catalog/. hERG channel assay was carried out by AVIVA biosciences (San Diego, CA). Other activity analyses were carried out by using the FLIPR assay format as described above. Activity implies that activation of the listed receptors was tested in the presence of TPI 1361-17 by changes in intracellular calcium levels as described above.

2.7. Animals

Adult male Sprague-Dawley rats weighing 200 – 300 g were obtained from Charles River Laboratories (Wilmington, MA), bearing a 23-gauge stainless steel cannula placed into the right lateral ventricle. Rats were housed individually and maintained on a 12 h light-dark cycle (6:00 am–6:00 pm light) with free access to tap water and rat chaw (PROLAB RMH 2500, containing 23.0% protein, 4.5% fat, gross energy 4.5 kcal/g. PMI nutrition international, LLC. Brentwood, MO). Prior to all studies, rats were handled and habituated for 7 days, and 10 μl of artificial cerebrospinal fluid (aCSF: NaCl 124, KCl 5, $CaCl_2$ 2.4 $MgSO_4$ 2, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10, in mM) was injected through the cannula to minimize stress effects at the time of the experiment. Cannula placement was confirmed in all animals by evaluating the response to 50 ng of angiotensin II. Only animals drinking more than 5 ml of water in 1h were used in feeding studies. This study design was approved by the Institutional Animal Care and Use Committee, University of California, Irvine.

2.8. Pharmacokinetics

Pharmacokinetic studies in rats were carried out by Ricerca Biosciences (Concord, OH). Assay details are available at http://www.ricerca.com/pages/RC3/metavivo2.html.

2.9. Spontaneous locomotor activity

Ambulatory movements were recorded using a Hamilton-Kinder Activity Monitor (with the infrared beams in a 4 × 8 configured frame). Locomotion was quantified in transparent polycarbonate cages (32 × 26 × 20 cm) placed within the activity frames. Animal position is detected by two dimensional beam breaks. The ambulation is tabulated as a movement that results in the change of location. All other beam breaks are categorized as non-ambulatory, fine movement. Animals were acclimatized to the novel home cage-environment for 60 minutes before injection, then were removed, injected and immediately replaced in the same cage, and monitoring began. Locomotion was measured for 60 minutes after the i.c.v. administration of either vehicle or 5 nmole TPI 1361-17.

2.10. Conditioned taste aversion test

Rats had free access to food in their home cage, but water was restricted to 20 ml per day. Rats were accustomed to a 1hr tap water drinking session (per day) for 5 days in a separate cage where the amount of liquid consumed is monitored in an automated feeding/drinking recording

system (TSE, Germany). On day 6, the conditioning day, rats were exposed to 0.1% saccharin solution instead of water and the volume consumed was recorded. 15 min after saccharin bottle removal, rats were injected either with vehicle, LiCl (300 mg/kg, i.p.) or TPI 1361-17 (10 nmole, i.c.v.). On day 7, rats were offered both water and saccharin in separate bottles and the drinking volume was monitored for 1 hr. The volume of water and saccharin solution consumed was used to quantify the conditioned taste aversion (CTA). Lithium chloride (LiCl), a classic reagent to induce conditioned taste aversion was used as a positive control to induce aversion after saccharin consumption on conditioning day (day 6), and therefore influence rats to drink water instead of saccharin on the test day (day 7).

2.11. Measurement of MCH - induced food intake

To determine the effects of TPI 1361-17 on MCH-induced food intake, rats were randomly divided into three groups; 1) TPI 1361-17 and 2 nmole of MCH; 2) MCH 2 nmole, 3) aCSF. Each mixture contained a DMSO equivalent concentration to that of the antagonist solution (up to 5 % (v/v)). Ten μl of each solution was administered into lateral ventricle for 2 minutes. The injections were done between 7:00 – 8:00 a.m., and food intake was monitored for 4 h after the injection.

2.12. Data analysis

Prism software (GraphPad, San Diego, CA) was used for statistical analysis. Results were analyzed by t test or ANOVA followed by the appropriate post hoc comparisons, and $P<0.05$ was considered statistically significant.

3. Results

3.1. Screening of the positional scanning synthetic combinatorial libraries (PS-SCL)

A total of twelve heterocyclic or peptidomimetics PS-SCLs were tested (Table 1). Most libraries were generated in a PS format by using 40 ~ 50 amino acids at each of the R1 and R2 positions, and 40 ~ 80 carboxylic acids at the R3 position. A sample set of PS-SCLs was screened initially, sub-libraries with defined R3 positions and a mixture of building blocks at each of the other two positions were prepared (all compounds in the library are represented). Each sub-library was composed of 40 ~ 80 mixtures containing an average of 1300 individual compounds. A total of 618 separate mixtures were screened for inhibition of 30 nM MCH-induced Ca^{2+} mobilization in CHO cells expressing MCH_1 receptor. Mixtures showing IC_{50} values lower than 2.5 μg/ml were considered bioactive. At this concentration, an individual compound should have a nanomolar affinity for MCH_1 receptor. The N-benzyl aminocyclic thiourea PS-SCL exhibited 26 hits out of 80 sub-libraries, while the N-methyl aminocyclic thiourea PS-SCL had one out of 80. There were no agonistic or MCH_1 receptor antagonistic activities in the other libraries.

3.2. Deconvolution of the N-benzyl aminocyclic thiourea PS-SCL

The N-benzyl aminocyclic thiourea PS-SCL had been generated in a PS format by using 40 amino acids at R1 Position, 37 amino acids at the R2 position, and 80 carboxylic acids at the R3 position for a total of 118,400 (40 × 37 × 80) individual compounds (Fig. 1A). The building blocks are described in Table 2. The complete PS-SCL was composed of three sub-libraries, each of which had a single defined building block at one position and a mixture of building blocks at each of the other two positions (Fig. 1B). Each sub-library contained the same number of compounds (118,400). Pooling of each sub-library varied based on the number of building blocks included at the defined position of that sub-library. The PS-SCL was composed of 157 separate mixtures (i.e., 40 + 37 + 80 = 157 samples to be assayed), each mixture contained 1,480 (40 × 37) to 3,200 (40 × 80) individual compounds, depending on the location of the

defined position. The structure(s) of the active individual compound(s) present in the library can be directly determined from the screening of these 157 mixtures, since each individual compound is present in a single mixture in each of the three sub-libraries.

3.3. Screening of the N-benzyl aminocyclic thiourea PS-SCL

Each mixture was assayed in duplicate in three separate assays at three concentrations (1, 0.1 and 0.01 μg/ml) (Fig. 1B). Inhibition rates were calculated as the percent decrease in intracellular Ca^{2+} mobilization in MCH_1 receptor expressing CHO cells induced by 30 nM MCH in the presence or absence of the mixture. None of the 157 mixtures elicited increases in Ca^{2+} mobilization alone. Mixtures showing highest reproducible antagonism and dose-dependency were selected for deconvolution. A set of 84 individual N- benzyl aminocyclic thioureas was then generated to confirm the connectivity between the selected building blocks (i.e., if the activities of the selected mixtures are due to the same individual compounds), as well as to determine the relative activities of the individual compounds. These 84 selected compounds were generated containing 4, 3 and 7 different building blocks at position R1, R2 and R3 respectively (Fig. 2A). Each compound was assayed at five different concentrations derived from serial ten-fold dilutions starting at 10 μM (Fig. 2A). The lowest IC_{50} values were in the nM range, found with the combination of L-Alanine at R1 position, L-Arginine at R2, and phenylacetic acids at R3. The compound with the highest affinity, TPI 1361-17 (*N*-(3-{(4*S*)-1-[(1*S*)-2-(benzylamino)-1-methylethyl]-3-[2-(3-fluorophenyl)ethyl]-2-thioxoimidazolidin-4-yl}propyl)guanidine) (Fig. 2B) was selected for further pharmacological analyses. By mass spectrometry, the molecular weight of purified TPI 1361-17 was 470.26, which matched exactly the one expected.

3.4. TPI 1361-17 antagonism at MCH_1 receptor

MCH stimulated the Ca^{2+} mobilization in HEK 293T cell expressing rat MCH_1 receptor with an EC_{50} value of 0.9 nM. TPI 1361-17 showed IC_{50} value of 6.1 nM on 1 nM MCH-induced Ca^{2+} mobilization (Fig. 3B). TPI 1361-17 did not change the basal Ca^{2+} level by itself, but significantly inhibited the MCH-induced Ca^{2+} mobilization. The addition of increasing concentrations of TPI 1361-17 caused a progressive shift of the curve to the right (Fig. 3A). The Schild regression estimated a pA2 of 9.43 with a slope of 1.085 ± 0.010 ($r^2 = 0.975$), which predicts a Kb of 0.37 nM (Fig 3C). This compound was more than 1,000 fold selective for MCH_1 receptor when compared to the human MCH_2 receptor, as well as other G-protein coupled receptors including the neuropeptide Y2, orexin 1R, prolactin releasing peptide receptor, and kappa opioid receptors. TPI 1361-17 did also not show activity in a hERG channel assay. Its binding was also tested on a battery of receptors, channels and enzymes and showed no activity (Table 3). TPI 1361-17 exhibited a high affinity for rat MCH_1 receptor in binding assays as well. TPI 1361-17 completely displaced the binding of [^{125}I] MCH to rat MCH_1 receptor, with an IC_{50} value of 31.8 ± 8.66 nM (mean ± S.E.M.) (Fig. 3D).

3.5. Pharmacokinetics of TPI 1361-17

After intravenous administration of TPI 1361-17 (5 mg/kg), peak plasma concentrations were attained at 0.083 hr, the first sampling point postdose. TPI 1361-17 concentrations then declined, and values for plasma elimination half-life ranged from 1.7 to 4.2 hr (mean value of 2.7 hr). $AUC_{0-\infty}$ values ranged from 1964.6 to 2684.1 hr*ng/mL (mean value of 2438.6 hr*ng/mL).

3.6. Inhibitory effects of TPI 1361-17 on MCH-induced food intake

Rats were habituated to a wire bottom cage for more than 3 weeks prior to the experiment. Experiments were carried out from 7 a.m. to 8 a.m. when rats were satiated. Intracerebroventricular injection of MCH (2 nmole) induced rapid and robust feeding in

satiated rats. Addition of TPI 1361-17 (1 or 5 nmole) significantly suppressed MCH-induced food consumption in a dose dependent manner. One nmole TPI 1361-17 decreased MCH-induced food intake for 1h and 2h but this decrease was reversed over the next two hours. On the other hand, five nmole of TPI 1361-17 suppressed cumulative food intake by 75 % even at 4h (Fig. 4).

3.7. Effects of TPI 1361-17 on locomotor activity and taste aversion test

Intracerebroventricular cannulated rats were acclimatized to the testing situation. Five or one nmole of TPI 1361-17 administered i.c.v. did not exhibit any detectable changes in movement. Ambulatory movements and non-ambulatory movements were monitored for 60 minutes upon administration of five nmole TPI 1361-17 or vehicle (Fig. 5A). Both animal groups showed highest movements just after the injection, which gradually decreased and stabilizes. There were no significant differences at any time points, as there was no difference in the total amounts of non-ambulatory or ambulatory movements (Fig. 5A). TPI 1361-17 also did not result in any aversive effect in taste aversion test. Neither TPI 1361-17 (10 nmole, i.c.v.) nor vehicle changed the preference to drink a saccharin solution, whereas LiCl elicited a robust taste aversion effect (** $P<0.01$ vs. vehicle group) (Fig. 5B).

4. Discussion

The use of orphan G-protein coupled receptors as targets to identify novel transmitters has led to the discoveries of several new neuropeptides as well as to the matching of known peptides to their specific G-protein coupled receptors. Discovery of a new neuropeptide system ultimately leads to the question of its function. This question can be addressed either by genetic or chemical approaches. The development of specific antagonists allows evaluation of blockade of a system and of this effect on the organism in a timely fashion. It also provides putative candidates for therapeutic research. Our approach was to screen large numbers of randomly synthesized compounds in pools of an average 1300 compounds. By choosing appropriate pool concentrations, we calculated that any positive pool would include a compound that should inhibit MCH-induced intracellular calcium release with a low nanomolar affinity that reflected high specificity and could be of use *in vivo* without further chemical modification.

We describe the identification and characterization of a specific MCH_1 receptor antagonist, TPI 1361-17, isolated from a N-benzyl aminocyclic thiourea PS-SCL. The technology of mixture-based combinatorial library enables us to assay thousands of compounds at a time, thus decreasing the time and costs of screening and as shown here is suitable for an academic environment. So far, this strategy has proven useful for analysis of T-cell specificity (Zhao et al., 2001), to characterize enzymes (Nazif and Bogyo, 2001) and to discover ion channel blockers (Tai et al., 2001) and both agonists and antagonists of G-protein coupled receptors (Dooley et al., 1998). Since we had little information about structure-function relationship of MCH_1 receptor (Macdonald et al., 2000), we chose to test as many PS-SCL libraries as possible. Among the twelve non-peptide libraries tested, the N-benzyl aminocyclic thiourea library was found to have high affinity to the MCH_1 receptor. Since our high-throughput receptor assay system is able to detect both agonistic and antagonistic activities, we have also identified a few mixtures showing agonistic activities (data not shown). Upon deconvolution of the libraries, we identified one compound, TPI 1361-17, which most effectively inhibited MCH-induced Ca^{2+} mobilization in CHO or HEK 293T cells expressing the rat MCH_1 receptor. TPI 1361-17 exhibited an IC_{50} value of 6.1 nM at 1 nM MCH and completely displaced [125I] MCH binding to rat MCH_1 receptor. TPI 1361-17 was found specific for MCH_1 receptor since it displayed no affinity to an array of G-protein coupled receptors and channels. In particular, no activity

was detected on hERG channel assay which has been shown to plague many MCH_1 receptor antagonists (Mendez-Andino and Wos, 2007).

To prove its efficacy in vivo, TPI 1361-17 was tested for its ability to block MCH-induced food intake. TPI 1361-17 inhibited MCH-induced food intake up to 75% in dose dependent manner at the beginning of the light phase, when MCH shows maximal effect (Rossi et al., 1997). This effect lasted for over 4h, in agreement with the half-life that we found for the compound in plasma. TPI 1361-17 did not affect locomotion or fine movements and did not induce any taste aversion, indicating that its administration does not induce aversive effects.

Numerous MCH_1 receptor antagonists have been reported (Luthin, 2007). They have been developed by the pharmaceutical industry and show different pharmacological properties. TPI 1361-17 differs in that it has been isolated using an approach that aimed at identifying high affinity antagonists that did not require further chemical modifications for activity. TPI 1361-17 exhibits pharmacological characteristics that allow it to be used as a tool for studying the responses associated with the activation of the MCH system. MCH has been implicated in a variety of physiological functions, most notably the regulation of energy homeostasis. Centrally administered MCH stimulates feeding (Qu et al., 1996), while MCH-deficient mice are hypophagic, lean, and hypermetabolic (Shimada et al., 1998). Targeted disruption of MCH_1 receptor causes hyperphagia, hypermetabolism, and diet-resistant obesity (Chen et al., 2002; Marsh et al., 2002). This accumulating evidence suggests that MCH_1 receptor regulates energy metabolism. While there exist two MCH receptors in human, only MCH_1 receptor is expressed in rodents, blockade of MCH_1 receptor should therefore equal total blockade of the MCH system in rodents. Also, MCH_1 receptor antagonists have been reported to exhibit antidepressant effects when administered orally or centrally (Borowsky et al., 2002; Georgescu et al., 2005). Finally, a MCH_1 receptor antagonist has been shown to improve social memory suggesting that the blockade of the MCH system using an antagonist affects cognition (Millan et al., 2008).

In conclusion, we have screened PS-SCL libraries under conditions that would allow to identify high affinity antagonists. We have succeeded in isolating a MCH_1 receptor antagonist, TPI 1361-17 that exhibits high-affinity and is selective for the MCH_1 receptor. It does not induce aversive effects when administered centrally and thus can be used to explore the functions of the MCH_1 receptor system.

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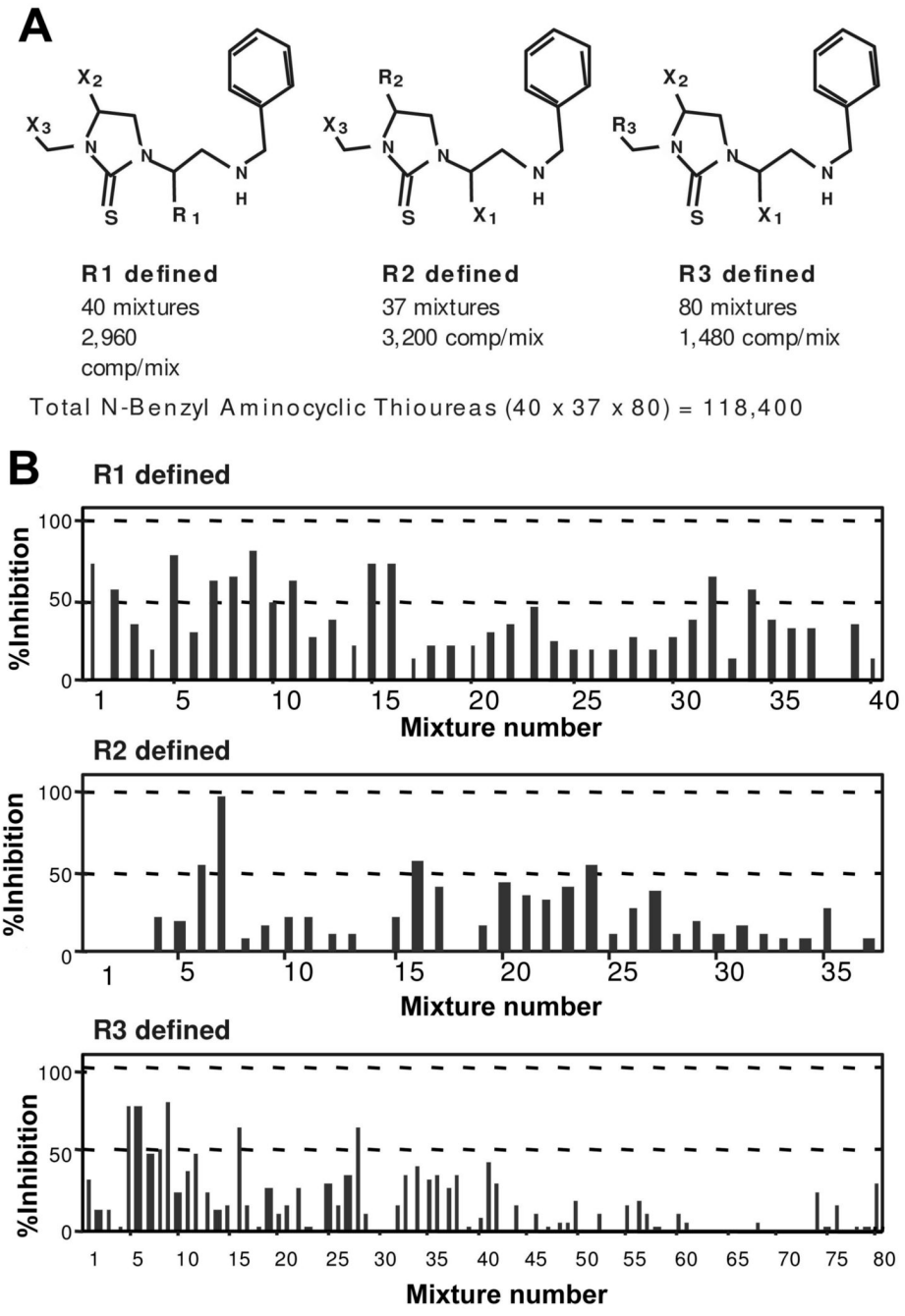

Figure 1.

A, Representation of the N-benzyl aminocyclic thiourea PS-SCL. R is defined as a single block; X is a mixture of building blocks. **B,** Profile of the inhibitory activity of the PS-SCL on 30 nM MCH-induced Ca^{2+} mobilization on MCH_1 receptor expressing CHO cells. Each graph represents a building block with one-position defined mixtures. The bars show the % inhibition by a single mixture at 0.5 μg / ml. A representative data set from three individual assays is shown. See Table 2 for the details of each mixture.

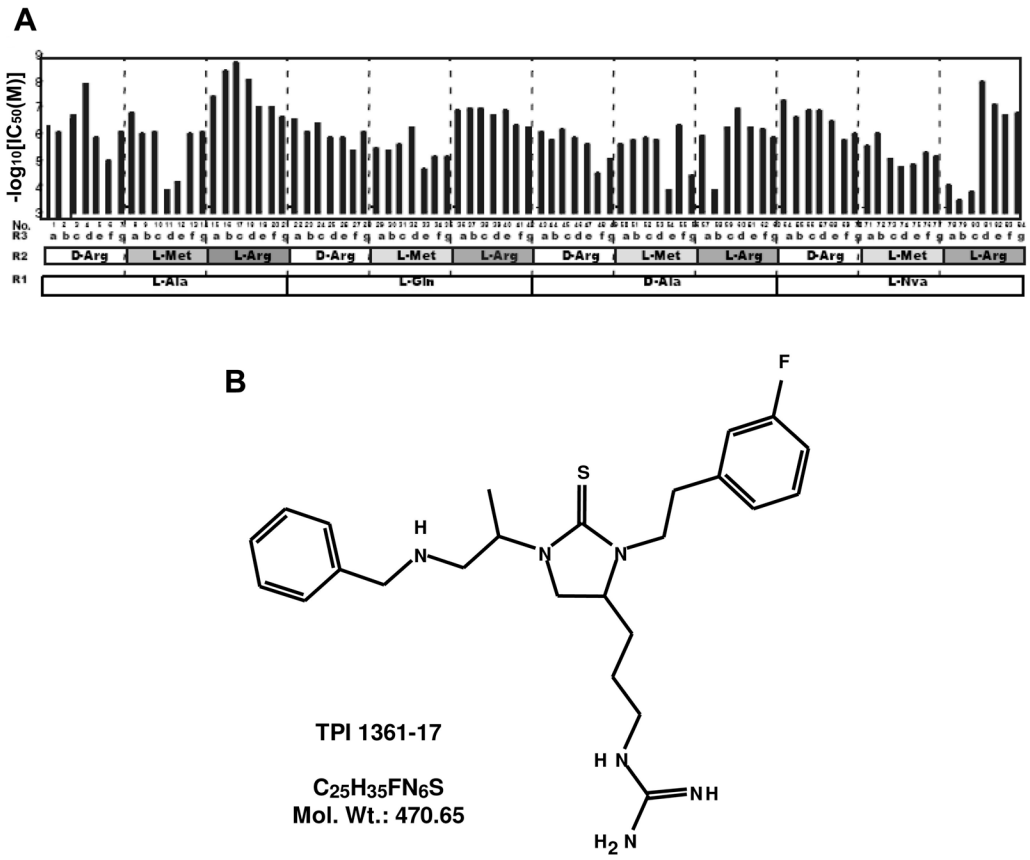

Figure 2.

A, Inhibitory activity of a series of 84 compounds individually synthesized based on the blocking profile of the library screening. IC_{50} values of each compound inhibiting 30 nM MCH-induced Ca^{2+} mobilization in MCH_1 receptor expressing CHO cells. Details of the each compound are shown in Table 2. Functionalities at the three diversity positions (R1-R3) for each compound is illustrated. For R3 position, each letter represents organic acids as follows; a; 3-Bromophenylacetic Acid, b; Phenylacetic Acid, c; 3-Fluorophenylacetic Acid, d; 4-Fluorophenylacetic Acid, e; 3,5 Bis (Trifluoromethyl) Phenylacetic acid, f; 4-Methylacetic Acid, and g; p-Tolylacetic acid. **B,** Chemical structure of TPI 1361-17.

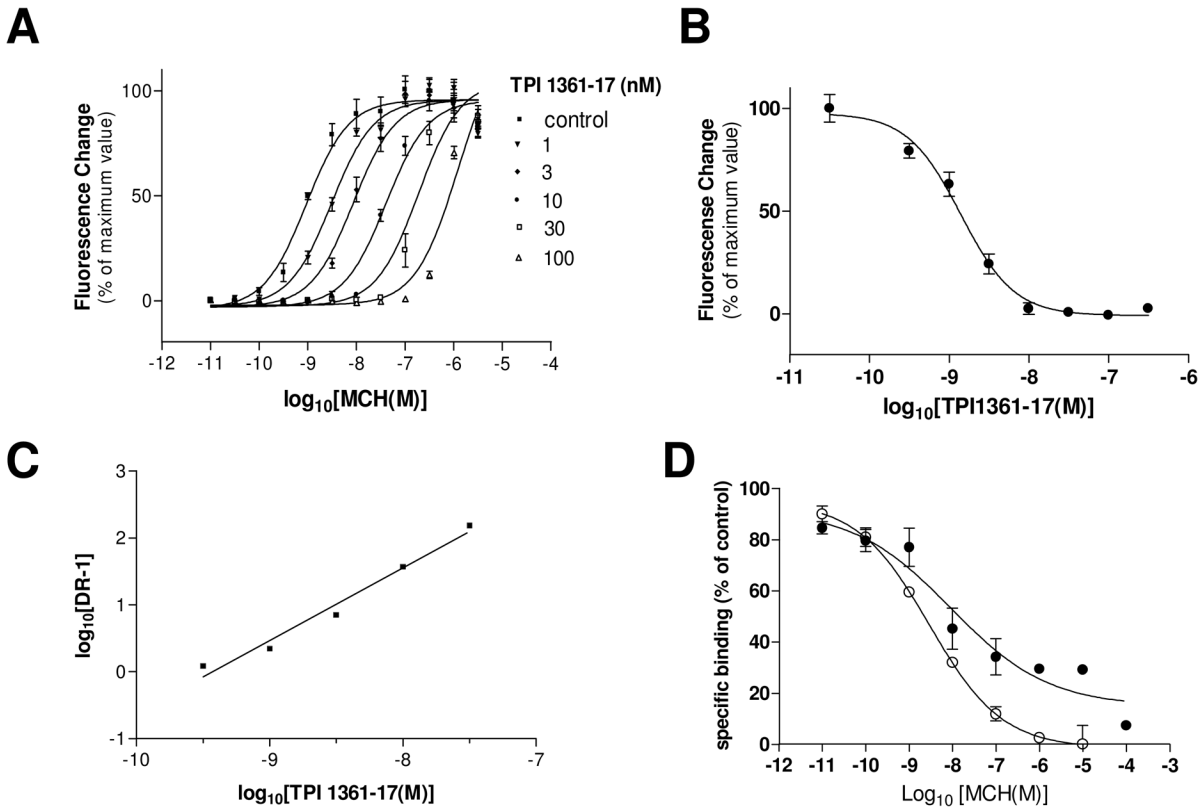

Figure 3.

In vitro pharmacology of TPI 1361-17. **A,** Dose response curves obtained for inhibition of MCH-induced fluorescence in MCH_1 receptor expressing HEK-293T cells by increasing concentrations of TPI 1361-17. **B,** IC_{50} value for TPI 1361-17 on 1 nM MCH-induced Ca^{2+} mobilization. **C,** Schild plot of the TPI 1361-17 antagonism. Data are mean ± S.E.M., n = 3. **D,** Displacement of [^{125}I] MCH binding to membrane fractions of MCH_1 receptor expressing HEK-293T, by MCH (●) and TPI 1361-17 (○). The data representative of three separate experiments with similar results are shown. Data are mean ± S.E.M.

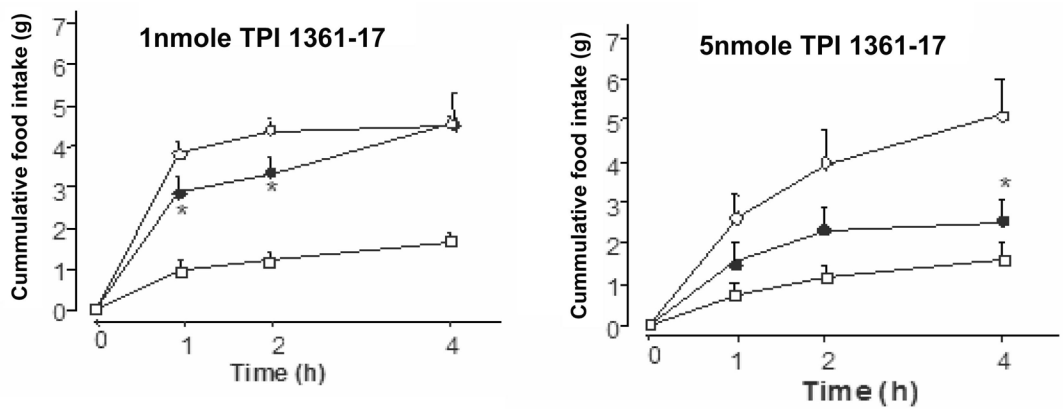

Figure 4.

Effect of i.c.v. administration of TPI 1361-17 on MCH-induced food intake. Rats were administered vehicle (□, n = 10), or 2 nmole MCH (○, n = 9), or 2 nmole MCH and TPI 1361-17 (●, n= 9). TPI 1361-17 was tested at two doses: 1 nmole and 5 nmole. Experiments were performed one to two hours into the light cycle. Ten minutes after the administration, rats were given access to regular low fat-diet, food intake was monitored and cumulative food intake was calculated (Each data point expresses mean value with S.E.M. * $P< 0.05$; ANOVA/ Bonferoni multiple comparison test for the effect of TPI 1361-17 + MCH versus MCH alone).

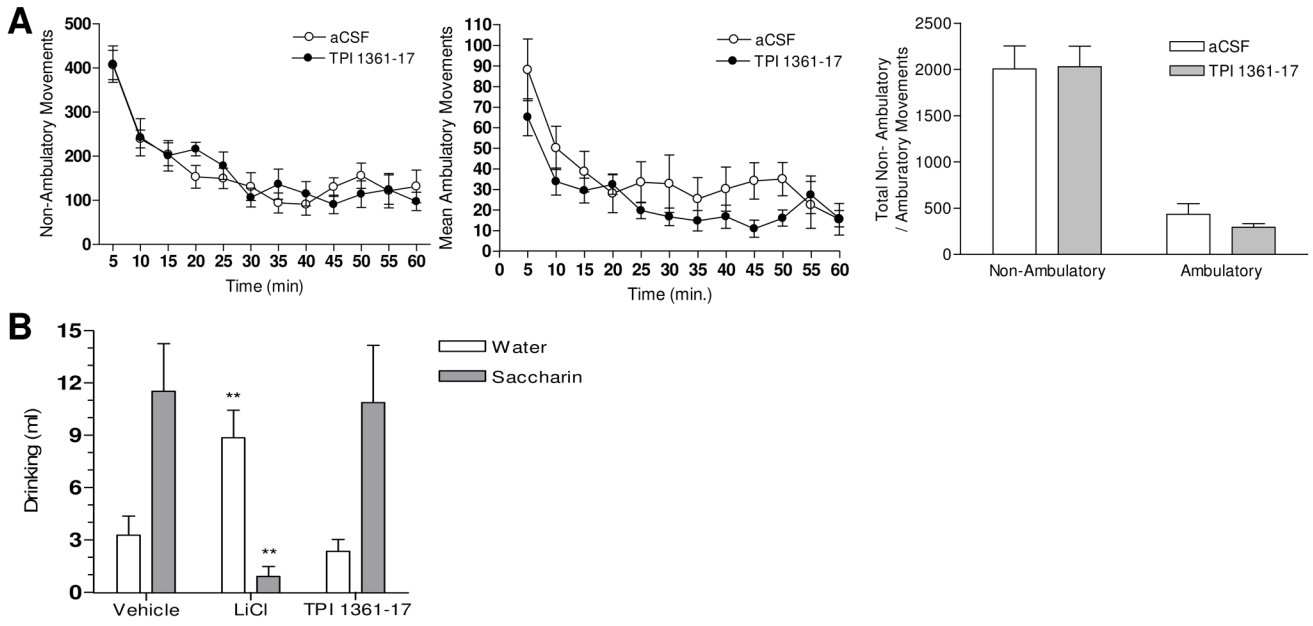

Figure 5.

A, Effect of i.c.v. administration of TPI 1361-17 on non-ambulatory and ambulatory movements. Both movements were monitored for one hour after the administration of vehicle (○) or 5 nmole TPI 1361-17 (●); Sums of non-ambulatory and ambulatory movements (Each data point represents the value with the S.E.M.; n=10). **B,** Effect of TPI 1361-17 (10 nmole, i.c.v.) on conditioned taste aversion (□ water; ■ saccharin) (** $P<0.01$ vs. vehicle group, ANOVA/Dunnett's multiple comparison test, n=6-8). Data expressed as mean ± SEM.

Table 1
PS-SCLs tested for MCH_1 receptor antagonism

Templates	Diversity (# of total cpds.)	# of active mixture / # of total mixture
Heterocycles		
N-benzyl aminocyclic thioureas	118,400	26 / 80
N-methyl amynocyclic thioureas	118,400	1 / 80
N-methyl diketopiperazines	31,320	0 / 40
N-benzyl diketopiperazines	31,320	0 / 40
N-methyl piperazines	31,320	0 / 40
N-benzyl piperadines	31,320	0 / 40
C-6-acylamino bicyclic guanidines	72,283	0 / 40
bicyclic guanidines	102,459	0 / 88
bis-diketopiperazines	45,864	0 / 40
Peptidemimetics		
triphenylureas	85,248	0 / 40
N-acyl triamines	125,000	0 / 50
N-methyltriamines	31,320	0 / 40

Table 2

Building blocks used in the N-benzyl thiourea PS-SCL

	Building block		
No.	**R1**	**R2**	**R3**
1	L-Ala	L-Ala	1-Phenyl-1-cyclopropanecarboxylic acid
2	L-Phe	L-Phe	2-Phenylbutyric acid
3	L-Gly	Gly	3-Phenylbutyric acid
4	L-Ile	L-Ile	m-Tolylacetic acid
5	L-Lys(Boc)	L-Leu	3-Fluorophenylacetic acid
6	L-Leu	L-Met(O)	3-Bromophenylacetic acid
7	L-Met(O)	L-Arg(Pmc)	(α-α-α-Trifluoro-m-tolyl)acetic acid
8	L-Asn	L-Ser(tBu)	p-Tolylacetic acid
9	L-Gln	L-Thr(tBu)	4-Fluorophenylacetic acid
10	L-Arg(Pmc)	L-Val	3-Methoxyphenylacetic acid
11	L-Ser(tBu)	L-Trp(Boc)	4-Bromophenylacetic acid
12	L-Thr(tBu)	L-Tyr(BrZ)	4-Methoxyphenylacetic acid
13	L-Val	L-Tyr(tBu)	4-Ethoxyphenylacetic acid
14	L-Trp	D-Ala	4-Isobutyl-α-methylphenylacetic acid
15	L-Tyr(BrZ)	D-Phe	3,4-Dichlorophenylacetic acid
16	L-Tyr(tBu)	D-Ile	3,5-bis-(Trifluoromethyl)phenylacetic acid
17	D-Ala	D-Leu	3-(3,4-Dimethoxyphenyl)propionic acid
18	D-Phe	D-Ser	4-Biphenylacetic acid
19	D-Ile	D-Thr(tBu)	α-Methylcinnamic acid
20	D-Lys(Boc)	D-Val	2-(Trifluoromethyl)cinnamic acid
21	D-Leu	D-Trp(Boc)	(3,4-Dimethoxyphenyl)acetic acid
22	D-Asn	D-Tyr(tBu)	3,4-(Methylenedioxy)phenylacetic acid
23	D-Gln	D-Arg(Pmc)	2-Methoxycinnamic acid
24	D-Ser	L-Nle	Benzoic acid
25	D-Thr(tBu)	D-Nle	4-Chlorocinnamic acid
26	D-Val	L-Nva	trans-Cinnamic acid
27	D-Trp	D-Nva	m-Toluic acid
28	D-Tyr(tBu)	L-NapAla	Phenylacetic acid
29	D-Arg(Pmc)	D-NapAla	Hydrocinnamic acid
30	L-Nle	L-Phg	4-Phenylbutyric acid
31	D-Nle	L-Glu(tBu)	3,5-bis-(Trifluoromethyl)benzoic acid
32	L-Nva	D-Glu(tBu)	Butyric acid
33	D-Nva	β–Ala	Heptanoic acid
34	L-NapAla	L-ChAla	Isobutyric acid
35	D-NapAla	D-ChAla	(+/−)-2-Methylbutyric acid
36	L-Phg	L-His(Trt)	Isovaleric acid
37	L-ChAla	D-His(Trt)	3-Methylvaleric acid
38	D-ChAla		4-Methylvaleric acid
39	L-His(Trt)		Crotonic acid
40	D-His(Trt)		Vinylacetic acid

	Building block		
No.	**R1**	**R2**	**R3**
41			p-Toluic acid
42			Trimethylacetic acid
43			tert-Butylacetic acid
44			Cyclohexanecarboxylic acid
45			Cyclohexylacetic acid
46			Cyclohexanebutyric acid
47			Cycloheptanecarboxylic acid
48			Acetic acid
49			2-Methylcyclopropanecarboxylic acid
50			Cyclobutanecarboxylic acid
51			Cyclopentanecarboxylic acid
52			3-Cyclopentylpropionic acid
53			Cyclohexanepropionic acid
54			4-Methyl-1-cyclohexanecarboxylic acid
55			4-tert-Butyl-cyclohexanecarboxylic acid
56			4-Methylcyclohexaneacetic acid
57			Tiglic acid
58			1-Adamantaneacetic acid
59			Niflumic acid
60			4-Nitrophenylacetic acid
61			4-(Nitrophenyl)-butyric acid
62			4-Nitrocinnamic acid
63			2-Nitrobenzoic acid
64			2,4-Dinitrophenyl acetic acid
65			4-Biphenylacetic acid
66			2-Chloro-5-nitrobenzoic acid
67			(4-Pyridylthio)acetic acid
68			3-3 Diphenylpropionic acid
69			2-Chloro-4-nitrobenzoic acid
70			4-Dimethylaminobenzoic acid
71			4-Nitrobenzoic acid
72			3-Dimethylaminobenzoic acid
73			Abietic acid
74			2-Methyl-4-nitro-1-imidizole-propionic acid
75			trans-Styrylacetic acid

Table 3

Selectivity of TPI 1361-17

Activity implies that activations of the listed receptors were tested in the FLIPR assay in the presence of increasing concentrations of TPI 1361-17 as described in Methods. hERG channel assay was carried out by using in vitro hERG electrophysiology assay at AVIVA biosciences. Binding implies that the following receptors and channels were tested for displacement of specific radioactive ligands by TPI 1361-17 (done by MDS Pharma Services).

ACTIVITY		BINDING	
MCH2R	>10uM	Adenosine A_1	>1uM
NPY1R	>10uM	Adenosine A_{2A}	>1uM
NPY2R	>10uM	Adrenergic $\alpha_{1A}/\alpha_{1B}/\alpha_{2A}$	>1uM
NPY5R	>10uM	Adrenergic β_1/β_2	>1uM
GPR7	>10uM	Dopamine D_{2s}	>1uM
GPR10	>10uM	Dopamine D_1	>1uM
GPH14	>10uM	GPR103	>1uM
Dopannine D_1	>10uM	Histamine H_1	>1uM
DopannineD_2	>10uM	Imidazoline I_2, Central	>1uM
Opiate κ (KOR)	>10uM	Muscarinic M_2	>1uM
Orexin 1R	>10uM	Muscarinic M_3	>1uM
hERG	>10uM	Prostanoid EP4	>1uM
		Opiate μ (OP3, MOP)	>1uM
		Phorobol Ester	>1uM
		Potassium Channel [K_{ATP}]	>1uM
		Rolipram	>1uM
		Nicotinic Acetylcholine	>1uM
		Nicotinic Acetylcholine α1, Bungarotoxin	>1uM
		$GABA_A$, Agonist site	>1uM
		$GABA_A$, Benzodiazepine, Central, Flunitrazepam	>1uM
		Glutarmate, NMDA, Phencyclidine	>1uM
		Calcium channel L-type	>1uM
		Transporter, Norepinephrine (NET)	>1uM