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Discovery Of An Orexin Receptor Positive Potentiator

Jiyong Lee^{a,c}, M. Muralidhar Reddy, and Thomas Kodadek^b

Thomas Kodadek: Kodadek@scripps.edu

^aDivision of Translational Research, Departments of Internal Medicine and Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd. Dallas, TX 75390 (USA)

^bDepartments of Chemistry & Cancer Biology, The Scripps Research Institute, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458 (USA). Tel: 561-228-2461

Abstract

The orexin neurohormones control a variety of important physiological processes by signaling through two related G protein-coupled receptors, including appetite and feeding, wakefulness and energy homeostasis. Pharmacological manipulation of orexin signaling is an important goal. Here we describe the isolation of orexin receptor ligands from a library of microarray-displayed peptoids via a novel two-color, cell-based screen. Functional analysis of derivatives of these "hits" resulted in the development of moderate potency, low molecular weight receptor antagonists. Moreover, further optimization efforts resulted in the fortuitous discovery of a compound that positively potentiates the activity of the receptor. This compound is the first small molecule reported to up-regulate orexin signaling.

Introduction

The neurohormones orexin-A and orexin-B are produced in the hypothalamus in response to a drop in blood glucose levels. By signaling through two related G protein-coupled receptors, OX1 and OX2, the orexins regulate a number of important behaviors, including feeding^{1, 2} wakefulness^{3, 4}, anxiety⁵ reward seeking and addiction⁶. Very recently, chronic stimulation of orexin signaling, mostly via OX2, has been demonstrated to strongly resist high fat diet-induced obesity, hyperglycemia and hyperinsulinemia⁷. This was shown to be due to increased leptin sensitization, increased energy expenditure and reduced food intake.

Not surprisingly, there has been considerable interest in the development of compounds with which to manipulate orexin signaling. Non-peptide antagonists of OXRs have been reported by a number of groups (for a review, see ref⁸) and show promise in the treatment of insomnia⁹. They may also be useful for the treatment of panic anxiety attacks⁵ and drug addiction¹⁰, though no clinical trials for these indications have been carried out. On the other hand, activation of orexin signaling will be required to treat narcolepsy. The majority of human narcoleptics lack orexin-producing neurons, possibly due to an autoimmune attack on these cells¹¹, so in this case a full agonist will be required as a therapeutic lead¹². Based on the recent findings of Funato, et al.⁷ an OX2 receptor agonist might be useful in treating diet-induced obesity and diabetes. Since diabetics presumably produce normal

physiological levels of orexin, a positive allosteric potentiator of receptor signaling might be interesting as well for this indication since it would hyper-activate orexin signaling in response to the native hormone.

To the best of our knowledge there have been no published reports of non-peptidic small molecules capable of stimulating orexin signaling. In this paper, we describe the discovery of the first positive allosteric potentiator of the orexin receptors. This molecule resulted from the analysis of derivatives of modest potency orexin receptor antagonists that were isolated from a novel microarray-based screen.

Results and discussion

Array-based screening identifies a selective orexin receptor ligand

To identify orexin receptor ligands, we employed an assay that is an elaboration of previously reported cell-based receptor-binding assays^{13–15}. As shown in Fig. 1, our plan was to display thousands of peptoids (N-substituted glycine oligomers)¹⁶ on a microarray platform, expose them to cells expressing the orexin receptor and identify peptoids that bind these cells with high selectivity. To encourage the isolation of selective ligands, we employed a two-color assay in which cells that do or do not express OXR1, but are otherwise identical, are exposed to the array and compounds that exhibit a strong preference for retention of the OXR1-expressing receptors are then identified.

To effect this strategy, we first examined if microarray-displayed peptoids¹⁷ are capable of binding to cells stably expressing human OXR1 (HEK293/hOXR1)¹⁸. The cells were incubated with a peptoid array (Fig. S1) for one hour after pre-blocking the array with 3% BSA in DMEM to inhibit non-specific binding. The slide was then washed with PBS, and the binding of the cells to the array was examined under a microscope. As shown in Fig. 1A, circular patterns of cell monolayers were observed at certain points on the array and the diameters of the monolayers were similar to those of the printed peptoid spots (200 – 300 μm), suggesting binding of cells to peptoids spotted at these positions. This experiment suggested that the hybridization conditions were appropriate to assay for peptoid-receptor interactions.

HEK293 cells, which do not detectably express orexin receptors (as determined by Western blotting and quantitative RT-PCR; data not shown), were stained with SYTO85 (green), and HEK293/hOXR1 cells were stained with SYTO60 (red). After mixing the cells in an approximately 1:1 ratio, they were applied to a peptoid microarray displaying 5760 different 9-mers and incubated for 1 hour at 37°C. After washing followed by fixation, the slide was scanned at 635 nm to visualize HEK293/hOXR1 binding and 532 nm to visualize HEK293 binding. The two images were then superimposed (Fig. 1B). We hypothesized that spots showing a high ratio of red over green fluorescence display peptoids that bind to OXR1 specifically. After measuring the level of fluorescence in each channel (532 nm and 635 nm, respectively) using a standard microarray scanner, we identified 99 spots displaying above background fluorescence in one or both channels (Fig. S2). Some representative images of these spots are shown in Fig. 1C. Calculated ratios of the fluorescence intensities in the red and green channels are illustrated in Fig. 1D. The mean fluorescence ratio (635/532) of the 99 spots was 5.9. The fact that this ratio was not 1.0 was due to the optics of the scanner. We measured the fluorescence emissions ratio with 635- and 532- nm excitation wavelengths of a known 1:1 mixture of the differentially labeled cells and measured a ratio of 6.1. Thus, we concluded that the ratio of 5.9 reflects equal binding of the two cells on most of the features of the microarray. The 12 peptoid features displaying the highest red/green ratio above the mean were chosen as possible OXR1 ligands. Tandem MALDI mass spectrometry (using compound from the solution used to spot the microarray) provided unambiguous

structures for five of these peptoids, which we named orexin receptor binding peptoids (OBPs; see Fig. S3).

Characterization and improvement of the orexin receptor ligands

These five OBPs were re-synthesized, purified by HPLC and employed in functional assays. OXR1 receptor activation by orexin-A up-regulates adenylyl cyclase, which subsequently increases cAMP production in OXR1-expressing cells¹⁹. To quantify orexin-A induced cAMP elevation in HEK293/hOXR1 cells, we employed a reporter gene (3×CRE-Luc) assay in which the promoter of the reporter gene contains a cAMP responsive element upstream of the firefly luciferase gene. As shown in Fig. S4A, orexin-A stimulates cAMP elevation in a dose-dependent fashion ($EC_{50} = 43$ nM). This stimulation was blocked by the commercially available OXR1-selective antagonist SB408124 (Fig. S4B) with an IC_{50} of 180 nM, which is similar to the reported value²⁰, validating that this assay properly measures orexin-triggered receptor activity.

As shown in Fig. S5A, none of the OBPs showed agonist activity, but some weak antagonist activity was observed (Fig. S5B). For example, OBP1 showed an approximately 30% inhibition of orexin A-induced cAMP elevation at 300 μ M. The activity of OBP2 was not quantified since it was cytotoxic to both HEK293 and HEK293/hOXR1 cell lines. The weak activity of OBP1 as an antagonist shows that the microarray-based binding assay is capable of registering even modest affinity receptor-binding molecules, probably because of avidity effects.

To further address the specificity of OBP1 binding to OXR1, we utilized a different assay. OBP1 on Tentagel beads (TG-OBP1, Fig. S6) were pre-incubated with 3% BSA in DMEM and then incubated with Syto60 stained-HEK293/hOXR1 cells or Syto60 stained-HEK293 cells, washed, and visualized by fluorescence microscopy. As shown in Fig. S7, HEK293/hOXR1 cells showed binding to TG-OBP1 while HEK293 cells did not. This indicates that the peptoid does not bind stably to any other molecule on the surface of the HEK293 cells.

To determine if the peptoid recognizes the hormone-binding site of the receptor or some other surface of the extracellular region of the protein, we carried out a competition experiment. Whereas binding of HEK293/hOXR1 cells to TG-OBP1 was abolished by free OBP1, the addition of excess orexin A did not compete binding of the cells to the immobilized peptoid (Fig. S7). This suggests that OBP1 binds to a site distinct from that recognized by orexin itself and that it acts via an allosteric mechanism.

The low potency of OBP1 clearly must be improved for it to be a useful reagent. As a first step towards this goal, we sought to identify the minimal pharmacophore in the peptoid. Derivatives of OBP1 were synthesized in which each side chain (R) was replaced, in turn, with a methyl group (Fig. 2A and Fig. S8)²¹. As shown in Fig. 2B, placement of the methyl group at the fifth and sixth positions of the peptoid (compounds OBP1-5 and OBP1-6) resulted in a complete loss of antagonist activity. Likewise, Tentagel beads displaying a peptoid in which both the Nmba and Npip residues of OBP1 were replaced with methyl groups did not show any binding to HEK293/hOXR1 cells (Fig. S7). Finally, a truncated form of OBP1 (OBPt) containing only these two residue was found to exhibit antagonist activity equal to or even slightly better than the parent OBP1 peptoid (Fig. 2C). These results argue that the Nmba and Npip residues in the OBP1 hit comprise the minimal pharmacophore.

We noticed some structural similarity between OBPt and ACT-078573 (also known as Almorexant; see Fig. 3A), an orexin receptor antagonist developed by Actelion Pharmaceuticals⁹. Based on this purported similarity, we hypothesized that appending a

hydrophobic unit to the N-terminus of OBPt would place this group in approximately the same region of space as the CF₃-substituted aryl ring in ACT-078573 and improve binding. The N-terminal secondary amine of OBPt was benzylated or benzoylated to afford OBPt-1 and OBPt-2, respectively (Fig. 3A). OBPt-1 was found to block orexin-A-induced cAMP elevation much more efficiently (IC₅₀ = 20 μM; Fig. 3B) than OBPt. OBPt-2 showed a weaker, but still improved, activity (IC₅₀ = 55 μM). Similar results were obtained by monitoring ERK phosphorylation, a known downstream mediator of OX_R1 (Fig. 3C)¹⁹.

To identify even better compounds, a small library of additional derivatives was synthesized in which positions R1 through R4 (Fig. 4A) were varied. Detailed information about library construction and determination of the structure activity relationships will be reported elsewhere. We found that OBPt-3 and OBPt-4 were more potent OX_R1 antagonists than OBPt-1 with IC₅₀s of 4.5 μM and 15.1 μM, respectively (Fig. S10). We then synthesized a derivative combining the two substitutions in OBPt-3 and OBPt-4 that distinguished them from OBPt-1 to afford OBPt-5. As shown in Fig. 4B, OBPt-5 showed increased potency (IC₅₀ = 1.7 μM). The potency of the enantiomer OBPt-6 was almost identical (IC₅₀ = 2.9 μM). Finally, we found that OBPt-5 also antagonized OX_R2, which is 64% identical to OX_R1 (Fig. 4C). OBPt-5 did not directly interfere with forskolin (adenyl cyclase activator)-induced cAMP production (Fig. S12), which does not depend on orexin signaling. This argues that the antagonist effect of OBPt-5 was not due to some receptor-independent activity.

Discovery of a positive potentiator or orexin receptor-mediated signaling

While testing various OBPt-1 derivatives we fortuitously identified three compounds (Fig. 5A) that did not antagonize the response of OX_R1 to orexin A, but rather appeared to slightly enhance OX_R1-mediated signaling in an orexin-responsive luciferase reporter gene assay (data not shown). This was somewhat surprising, given the very modest structural alterations in these compounds relative to OBPt-1. These original experiments employed concentrations of the orexin A peptide near the EC₁₀₀. Therefore, to determine if these compounds are indeed OX_R1 potentiators, we carried out experiments at 16 nM orexin A, which drives OX_R1-mediated reporter gene expression in this assay at only about 20% of the maximum possible level (i.e., EC₂₀ ≈ 16 nM). As shown in Fig. 5B, all of the compounds tested stimulated OX_R1 activity under these hormone-limiting conditions. The most potent, OBPt-9, was chosen for further characterization. As shown in Fig. 5C, the maximal potentiation at this concentration of orexin A was about 2.5-fold, with an EC₅₀ value for potentiation of approximately 120 nM. Note that a control peptoid (CON) did not show any activity. Importantly, OBPt-9 alone caused no activation of OX_R1 (Fig. S13).

We next determined the effect of OBPt-9 on the potency and efficacy of orexin A. Cells were pre-incubated with OBPt-9 or DMSO (vehicle) and subsequently stimulated with increasing concentrations of orexin A. As shown in Fig. 5D, OBPt-9 induced a leftward and upward shift of the orexin A response curve. The EC₅₀ value for orexin A in the presence of vehicle was 41 nM, whereas, the EC₅₀ values were 27 and 12 nM in the presence of 0.1 μM and 5 μM of OBPt-9, respectively. Moreover, the maximal response to orexin A was about 2-fold higher in the presence of OBPt-9 (5 μM) than with vehicle alone.

Next, we examined if OBPt-9 can also potentiate the response of OX_R2 to orexin A. After transient expression of OX_R2, HEK293 cells also carrying a receptor-driven reporter gene were treated with OBPt-9 and then with increasing concentrations of orexin A. As depicted in Fig. S14, OBPt-9 showed a similar potentiation pattern as was observed with the OX_R1-containing cells. The Orexin A EC₅₀ was 60 nM in the presence of vehicle and 22 nM in the presence of OBPt-9. Moreover, the maximum level of reporter gene activation in the presence of OBPt-9 was almost twice that observed in the presence of vehicle. We also

found that OBPT-9 did not affect ATP-dependent, endogenous P2 receptor-mediated ERK phosphorylation in HEK293 (Fig. S15). This suggests the orexin receptor potentiation activity of OBPT-9 is not due to some non-specific cell surface receptor activation.

Conclusion

In summary, we have discovered new orexin receptor regulators using a microarray-based, two-color, cell-binding screen. An important feature of the screen is that it examines binding of prospective receptor ligands to differentially-labeled cells that do and do not display the target receptor but are otherwise identical. This allows the identification of compounds that display considerable selectivity for the target receptor, in contrast to typical screening methods that provide only information on potency, leaving selectivity issue to be worked out later. We also demonstrated a rapid and efficient route to pharmacophore identification and a preliminary optimization of the hit, which resulted in an antagonist with approximately 180-fold improved potency.

Recently, we reported a similar two-color, cell-based screen carried out on Tentagel beads that allows up to several million peptoids to be screened simultaneously¹⁵. While the microarray platform is limited to a few thousand compounds, it has the advantage of allowing facile and quantitative comparisons of the binding properties of all of the compounds on the array²³. Thus, while we demonstrated here the utility of microarrays for screening primary, naïve libraries, its most useful application may be in evaluating libraries of derivatives of primary hits in the search for improved ligands. Experiments along these lines are underway.

Importantly, we have discovered the first small-molecule allosteric potentiator of the orexin receptor. Allosteric potentiators bind to a site on the receptor distinct from that of the native ligand and accentuate the response of the receptor to that ligand, but cannot stimulate receptor function independently. It has been suggested that allosteric potentiators might have advantages over classical orthosteric agonists from the therapeutic point of view. For example, allosteric potentiators would not drive chronic receptor activation, but rather accentuate natural cycles of activation of the receptor^{24, 25}. Animal experiments to test the utility of these compounds in vivo are underway.

Experimental

General remarks

All chemicals and solvents were purchased from commercial suppliers and used without further purification. Mass spectra were obtained with a Voyager-DETM PRO (Applied Biosystems) for MALDI-TOF with α -cyano-4-hydroxycinnamic acid matrix. MS/MS spectra were obtained on a 4700 Proteomics Analyser (Applied Biosystems).

Peptoid microarrays

Peptoid microarrays featuring 5760 different 9-mer peptoids were prepared as described before¹⁷ using the peptoid library shown in Fig. S1.

Cell culture

HEK293 cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Invitrogen) supplemented with 110 mg/L sodium pyruvate, 2mM L-glutamine and 10% (v/v) fetal calf serum at 37°C in a 5% CO₂ environment. For HEK293/hOXR1 cells, G418 (500 μ g/mL, Gibco) was included in the medium.

A microarray-based, two-color, cell-binding screen and ratio-metric image analysis

Cells were grown in culture plates to 95% confluency. The medium was replaced with a Syto dye staining solution (5 μ M in PBS, pH = 7.4) and incubated for 10 min at 37°C. Syto85 was used for HEK293 and Syto60 was used for HEK293/hOXR1. Cells were washed with PBS three times and re-suspended with an incubation medium (3% BSA in DMEM). The two differentially stained cells were mixed at a 1:1 ratio in incubation media (10 mL), using 1×10^6 cells from each cell type. The cell suspension was added onto a microarray slide which had been washed with PBS and equilibrated with an incubation medium for 1hr. The Super PAP Pen (The Binding Sites, Inc.) was used to make a boundary along the edge of the microarray to hold cell suspension. After incubation for 1hr at 37°C, the cell suspension was removed by suction and the microarray slide was placed on a Petri dish. PBS was added to cover the slide and the plate was gently shaken to wash off cells that bind nonspecifically to the microarray surface (30 sec $\times$ 5 times). The microarray slide was then fixed (3% formaldehyde in PBS) for 10 min at room temperature and washed with PBS (10 sec $\times$ 2 times). After brief washing with ddH₂O, excess water was decanted and the microarray slide was dried with reduced exposure to light. The microarray slide was scanned with a ScanArray ExpressHT microarray scanner (PerkinElmer) using 532-nm (Syto 85) and 635-nm (Syto 60) lasers at 100% power and 300 PMT with pixel size of 5. All the scanned images were analyzed by using the GENEPIX PRO 5.0 software (Axon Instruments, Union City, CA). Fluorescence intensities (F) of each spot at 635-nm or 532-nm after subtraction of mean local background intensity (B) were used for ratio-metric analysis in which ratio = (F635-B635) / (F532-B532).

Peptoid synthesis

All peptoids were synthesized by on Rink Amide AM resin (NovaBiochem) or TentaGel Macrobeads NH₂ resin (OBPt-3, OBPt-5, OBPt-6, PBpt-7, and OBPt-8. RAPP Polymere) using the sub-monomer approach by a microwave-assisted protocol²⁶ and purified by preparative RP-HPLC (Waters) and confirmed with MALDI-TOF/MS. For the preparation of sarcosine derivatives (OBP1-1 to OBP1-9), methyl amine (2.0 M in THF) was used. For the preparation of CON, 2-methoxyethylamine was used. For OBPt-1, after addition of second amine, N-terminal secondary amine was benzylated under reductive alkylation condition using benzaldehyde (10 eq.) and BAP (10 eq). For OBPt-2, N-terminal secondary amine was benzoylated with BzOH (3 eq.), DIC (3 eq.), and HOAt(3 eq). All peptoids prepared on Rink Amind AM resin were cleaved from resins with cleavage cocktail (95% TFA, 2.5% water, 2.5 % TIS) and purified by preparative RP-HPLC. See Table S1 for characterization of synthesized peptoids.

Preparation of OBPt-3, OBPt-5, OBPt-6, OBPt-7, and OBPt-8

TentaGel Macrobeads NH₂ resin (RAPP Polymere) was reacted with 4-(bromomethyl)-3-nitrobenzoic acid (5 eq) in the presence of DIC (5 eq) and HOBt (5 eq) for 3 hr. After washing with DMF, the resin was reacted with (R)-, (S)- α -methylbenzylamine, or benzylamine in DMF (2 M) for 3 hr. The resin was washed with DMF and then reacted with DIC (2 M) and bromoacetic acid (2 M) for 3 hr. After washing with DMF, the resin was reacted with corresponding second amine for 3 hr to afford secondary amine. The resin was washed with DMF and then reacted with corresponding benzaldehyde (10 eq) and BAP (10 eq) for reductive alkylation. After washing, peptoids were cleaved from the resin by exposing to UV (365 nm, 6W) for 12 hr in 2% v/v TFA/MeOH and purified by preparative RP-HPLC. See Table S1 for characterization of synthesized peptoids.

Bead-binding assay

Peptoids were synthesized with TentaGel Macrobeads NH₂ resin (RAPP Polymere) as described previously¹⁵ and suspended in PBS. About 200 beads were dispensed into each well in 96-well plates. After incubation with 3% BSA in DMEM (100 μ L) for 30 min at 37°C, DMEM (100 μ L) containing HEK293/hOXR1 or HEK293 cells (20,000 cells) which were stained with Syto60 as described above were added. After 1hr incubation at 37°C, the beads were washed with PBS (200 μ L) five times before visualization under light/fluorescence microscopy. Cy-5 channel was used to detect Syto60 emission.

Fluorescence measurement with plate reader

HEK293/hOXR1 and HEK293 cells were stained as described above with Syto60 and Syto85 and mixed at 1:1 ratio in PBS. Total 5000 cells in PBS were added into 96-well plate and fluorescence emission at 670 nm and 575 were measured with excitation wavelengths of 635 nm and 532 nm, respectively. Three independent experiments were done to calculate the mean ratio of emissions at two wavelengths.

cAMP production assay

HEK293/hOXR1 Cells were grown to ~60% confluency in 48-well plates and transfected with the luciferase reporter plasmid, pGL3-3 $\times$ CRE-TATA (100 ng/well)²⁷. 24 hr after transfection, the cells were serum-starved for 4hr and treated with the indicated concentrations of orexins. In the case of evaluating antagonists or allosteric potentiators, cells were pre-treated with indicated concentrations of peptoids for 20 min. 6 hr after orexin addition, cells were lysed with passive lysis buffer (Promega) for 10 min at room temperature. To test agonist activity of peptoids, cells were treated with peptoids in the absence of orexin. The luciferase activity of the cell lysate was measured using the dual-luciferase assay kit (Promga) following the manufacture's protocol and normalized to Renilla luciferase activity from the co-transfected Renilla luciferase expression plasmid (pRLuc, 2 ng/well). Fold increase values of the normalized luciferase activities were presented in data. When HEK293 cells were used, indicated concentration of forskolin was used instead of orexin. To examine the effect of peptoids on OXR2 expressing cells, HEK293 cells were transfected with phOXR2 (OriGene Technology Inc., 50 ng/well), pGL3-3 $\times$ CRE-TATA (50 ng/well), and pRLuc (1 ng/well) for 24 hr before starvation.

ERK phosphorylation assay

HEK293/hOXR1 cells were grown (~75% confluency) in 6-well plates and serum-starved overnight prior to stimulation with orexin A for 12 min. For the antagonist evaluation, cells were treated with antagonists (SB408124 or peptoids) 20 min before the addition of orexin A. Cells were lysed with reporter lysis buffer (Promega) for 15 min at 4°C. The collected lysates were mixed with 2 $\times$ SDS sample buffer and heated for 5 min at 95°C. The samples were separated by SDS-PAGE and transferred to PDVF membranes (Immobilon, Millipore). The membranes were probed with anti-phospho ERK(1/2) or anti-ERK(1/2) primary antibodies (Santa Cruz) and subsequently developed with appropriate HRP-conjugated secondary antibody (BioRad) followed by chemiluminescence detection using SuperSignal[®] West Pico substrate (PIERCE). Quantifications of blot bands were performed using "Image J" software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A microarray-based, two-color, cell binding screen to isolate ligands of human OXR1. (A) Light microscopic images of cells binding to a peptoid microarray. Scale bar = 250 μm (B) Schematic illustration of the screening procedure. (C) Representative superimposed images (red and green) of cells on a microarray after washing. (D) Ratiometric analysis of microarray images. The ratio of red to green fluorescence on 99 spots that displayed above background signal is shown. The unfilled bar at the far right of the graph represents the mean of the 99 spots. Bars marked with the asterisk represent peptoids that were subjected to sequence analysis by tandem mass spectrometry. Unambiguous sequences were obtained for peptoids marked OBP1-OBP5 (see Electronic Supplementary Information). F635 and F532 are mean fluorescence emission intensities of spots with excitation wavelengths of 635 nm and 532 nm, respectively. B635 and B532 are background fluorescence emission intensities with 635 nm and 532 nm, respectively

Fig. 2. Pharmacophore identification via “sarcosine scanning”. (A) Each side chain (R) of the Nth residue was replaced, in turn, with a methyl group to afford OBP1-N (where N=1–9), sarcosine containing peptoids. (B) Effects of sarcosine replacements on the antagonist activity of OBP1. The y axis shows the measured increase in cAMP concentrations in the cells relative to cells not treated with orexin (i.e., treatment with 0.5 μM orexin results in a four-fold stimulation of cAMP production). (C) Chemical structure of truncated OBP1 (OBPt) and its antagonist activity. Error bars represent the standard deviation of the mean of triplicate experiments.

Fig 3. Effect of the introduction of hydrophobic groups at the N-terminus of OBPt on activity (A) Comparison of the structures of ACT-098573, a potent antagonist of OX_R1 and OX_R2, and OBPt. The structures are oriented to illustrate the hypothesis that addition of an aryl substituent on the N-terminal nitrogen of the peptoid (marked with a filled arrow) would fill space that is occupied by the trifluoromethylphenyl-containing side chain in ACT-098573. i) benzaldehyde, BAP ii) Benzoic acid, DIC, HOAt (B) Antagonist activities of OBPt-1 and OBPt-2. Increasing concentrations (2, 20, 30, 50, and 75 μM) of peptoids were used. CON is a N-benzylated control peptoid (see Fig. S9). Error bars represent the standard deviation of the mean from triplicate experiments. (C) Inhibition of orexin-mediated ERK phosphorylation by OBPt-1. A representative figure from three different experiments is shown.

Fig. 4. Attempted optimization of OBPt-1 (A) Chemical structures of some of the OBPt-1 derivatives examined. (B) Antagonist activities of OBPt-5 and OBPt-6. (C) Effect of OBPt-5 on orexin A- or orexin B-induced OX2 activation of HEK293 cells expressing human OX2. SB408124 is an OX1 selective antagonist²⁰. Proline bis-amide is an OX1/2 dual antagonist²² (Fig. S11).

Fig. 5.

Discovery of a positive allosteric potentiator of the orexin receptors (A) Chemical structures of tested compounds (OBPt-7, OBPt-8, and OBPt-9). (B) Effects of the compounds on the response (cAMP elevation) of OX1R-expressing cell to an EC₂₀ concentration of orexin A. The level of cAMP elevation by 0.3 μM orexin A (the EC₁₀₀ concentration) is also shown for comparison. (C) Concentration-responsive curves of OBPt-9 and CON on cAMP elevation of OX1R expressing cells in the presence of EC₂₀ concentration of orexin A. (D) Concentration-response curves of orexin A on cAMP elevation of OX1R expressing cells in the presence or absence of OBPt-9.