

Identification and Characterization of a Small Molecule Antagonist of Human VPAC₂ Receptor

Alan Chu, Jeremy S. Caldwell, and Yu Alice Chen

GPCR Platform, Genomics Institute of the Novartis Research Foundation, San Diego, California

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ABSTRACT

The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) and their class II G protein-coupled receptors VPAC₁, VPAC₂, and PAC₁ play important roles in human physiology. No small molecule modulator has ever been reported for the VIP/PACAP receptors, and there is a lack of specific VPAC₂ antagonists. Via high-throughput screening of 1.67 million compounds, we discovered a single small molecule antagonist of human VPAC₂, compound 1. Compound 1 inhibits VPAC₂-mediated cAMP accumulation with an IC₅₀ of 3.8 μM and the ligand-activated β-arrestin2 binding with an IC₅₀ of 2.3 μM. Compound 1 acts noncompetitively in Schild analysis. It is a specific VPAC₂ antagonist with no detectable agonist or antagonist activities on VPAC₁ or PAC₁. Com-

ound 2, a close structural analog of compound 1, was also found to be weakly active. To our surprise, compound 1 is completely inactive on the closely related mouse VPAC₂. Chimera experiments indicate that compounds 1 and 2 bind to the seven transmembrane (7TM) region of the receptor as opposed to the N-terminal extracellular domain, where the natural ligand binds. Compound 1, being the first small molecular antagonist that is specific for VPAC₂, and the only VPAC₂ antagonist molecule known to date that allosterically interacts with the 7TM region, will be a valuable tool in further study of VPAC₂ and related receptors. This study also highlights the opportunities and challenges facing small molecule drug discovery for class II peptide G protein-coupled receptors.

The hormones VIP and PACAP belong to a nine-member peptide hormone family that includes glucagon, glucagon-like peptide (GLP)-1, GLP-2, glucose-dependent insulinotropic polypeptide, growth hormone-releasing hormone, peptide histidine-methionine, and secretin (Sherwood et al., 2000). Peptides in this PACAP/glucagon hormone superfamily are related by structure, distribution (abundant in the brain and gut), function (increasing intracellular cAMP concentration), and receptors (a homologous subset of class II G_s-coupled GPCRs). VIP exists as a 28-amino acid peptide, and PACAP exists as a 27- or 38-amino acid peptide. They have equal affinities for two shared GPCRs, VPAC₁ (also known as VIPR₁) and VPAC₂ (also known as VIPR₂). In addition, PACAP binds to PAC₁ (also known as ADCYAP1R1), a PACAP-specific receptor. Both VIP and PACAP, as well as their receptors, have a widespread distribution. A wide array of potential functions of VIP/PACAP system has been demonstrated, including regula-

tion of circadian rhythm (Harmar et al., 2002), neuronal survival (Rangon et al., 2005), tumor progression (Moody and Gozes, 2007; Valdehita et al., 2009), immune responses (Goetzl et al., 2001; Gonzalez-Rey et al., 2006), metabolic homeostasis (Tsutsumi et al., 2002), and megakaryocyte maturation (Freson et al., 2008). VPAC₂ knockout mice showed loss of electrical rhythmicity in suprachiasmatic neurons in the brain and a reduced behavioral circadian rhythm (Harmar et al., 2002). They also showed altered immune hypersensitivity (Goetzl et al., 2001) and an increased basal metabolic rate (Asnicar et al., 2002). Although the VIP/PACAP system has emerged as a potentially useful therapeutic target for inflammatory, metabolic, or circadian functions, VIP and PACAP peptides themselves are not practical as therapeutics because of their short half-lives and lack of oral bioavailability and brain penetration. Therefore, development of nonpeptide small-molecule modulators is of high interest.

VPAC₁, VPAC₂, and PAC₁ receptors belong to the class II (or class B) secretin family of GPCRs, rather than the larger class I (or class A) rhodopsin family of GPCRs. A distinct feature of class II GPCRs is that they have a large N-termi-

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ABBREVIATIONS: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; GPCR, G protein-coupled receptor; ECD, extracellular domain; PG-99-465; HTRF, homogeneous time-resolved fluorescence; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; GLP, glucagon-like peptide; RLU, relative luminescence unit; aa, amino acid(s); h, human; m, mouse.

nal extracellular domain (ECD) that has a conserved three-dimensional structural fold and is largely responsible for the peptide ligand binding. The NMR structures of mouse corticotropin-releasing factor receptor 2 ECD, both free (Grace et al., 2004) and in complex with a peptide antagonist (Grace et al., 2007), have been reported. In both forms, the ECD folds into a short consensus repeat or Sushi domain that contains two antiparallel β -sheets, three disulfide bonds, and a salt bridge between conserved residues aspartic acid 65 and arginine 101, sandwiched between tryptophans 71 and 109. The two subsequent reports of Sushi domain folds for the ECD of a PAC₁ (Sun et al., 2007) and of an incretin receptor (Parthier et al., 2007) provide strong support for the proposal of a general Sushi module in the ECDs of all class II receptors. The activation model of this class of receptor is thought to involve first the binding between ECD of the receptor and the central and C-terminal parts of the ligand, and this interaction then positions the N-terminal part of the ligand close to the receptor transmembrane core domain for activation and downstream signaling.

Over the years, there have been a number of tool agonist and antagonist compounds reported for VIP/PACAP receptors (for review, see Dickson and Finlayson, 2009). All of these modulators are peptides targeting orthosteric ligand binding sites; a small molecular modulator or an allosteric modulator has not been discovered for these receptors. Because it is often difficult to achieve selectivity among family members when targeting orthosteric sites, a truly specific VPAC₂ antagonist is still lacking. PG-99-465 (Moreno et al., 2000), a VIP analog that was initially reported to be a VPAC₂-specific antagonist with an IC₅₀ of 2 nM, seems to show complex pharmacology. It also is a partial agonist at VPAC₂ (EC₅₀ = 5 nM) and a full agonist at VPAC₁ and PAC₁ receptors with reported EC₅₀ values of 8 and 70 nM, respectively (Dickson et al., 2006).

We carried out a high-throughput screen on human VPAC₂ receptor using a cell-based cAMP assay. This functional

screening approach did not bias toward identifying compounds that compete for binding with the peptide ligand. It is noteworthy that a single confirmed antagonist hit was discovered from a 1.67 million-compound collection, highlighting the difficulty of small-molecule drug discovery for certain class II peptide GPCRs. This compound is the first specific antagonist reported for human VPAC₂ receptor.

Materials and Methods

Materials. PACAP6-38, PACAP1-38, and VIP were purchased from Tocris Bioscience (Ellisville, MO). PG-99-465 was a kind gift from the laboratory of Steve Kay at University of California San Diego. cAMP HighRange HTRF kits were purchased from Cisbio US (Bedford, MA). β -Arrestin assay technology was licensed from DiscoverX Corp. (Fremont, CA).

cAMP HTRF Assay. HEK293 cells were transfected with hVPAC₂ and hVPAC₁ in pCDNA3.1 vector, and selected with 800 μ g/ml G418. Clonal stable cell lines were generated by limited dilution to single cells before clonal expansion and test of their VIP-dependent cAMP responses. hVPAC₂-HEK stable clone 11 and hVPAC₁-HEK stable clone 12 were selected for all the later studies. On the day of assay, 3000 cells (4 μ l/well) or 15,000 cells (25 μ l/well) were seeded and incubated in cell incubator overnight in 1536-well or 384-well white solid plates, respectively (Greiner Bio-One, Longwood, FL). Next day, 50 nl (1536 format) or 500 nl (384 format) of antagonist compound was added by PinTool (GNF Systems), followed by addition of 1 to 5 μ l of agonist VIP in growth medium. Assay plates were returned to cell incubator for 30 min before addition of 2.5 μ l/well (1536 format) or 15 μ l/well (384 format) of cAMP conjugate and equal volume of anti-cAMP conjugate (Cisbio). After at least 1 h of room-temperature incubation, HTRF signal was read on Viewlux or EnVision (PerkinElmer Life and Analytical Sciences, Waltham, MA). Ratio of absorbance at 665 nm and 620 nm times 10,000 was calculated and plotted.

β -Arrestin Pathhunter Assay. GPCRs of interest were cloned into the ProLink vector (DiscoverX) for GPCR-ProLink fusion protein production. Parental HEK293 cells that stably express β -arrestin2- β -gal-EA fusion protein (DiscoverX) were detached and transiently transfected with the receptor of interest using

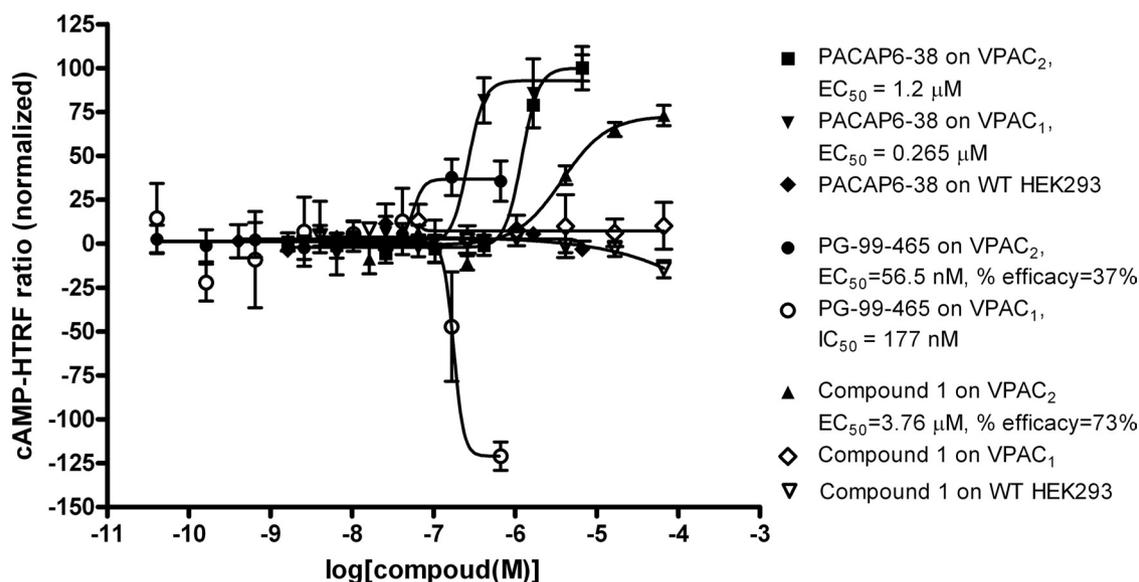


Fig. 1. Human VPAC₂ and VPAC₁ cAMP antagonist assays. Cellular cAMP responses were measured in a human VPAC₂-HEK293 stable cell line (clone 11), a human VPAC₁-HEK293 stable cell line (clone 12), and a wild-type (WT) HEK293 cell line as indicated. Varying amounts of the test compounds were added to the specific cell line before the following agonist was added: 1 nM VIP for hVPAC₂ cells, 5 nM VIP for hVPAC₁ cells, and 1 nM VIP for WT HEK293 cells. Thirty minutes later, the cells were lysed and cAMP concentrations were measured using a cAMP-HTRF dynamic kit. The ratio plotted is inversely proportional to the free cAMP concentrations in the cell. Thus, the higher the signal, the lower cellular [cAMP] is.

Fugene6 transfection reagent in suspension mode. Transfected cells in assay medium were plated into white solid 384-well plates at 15,000 cells/25 μ l/well. After overnight incubation, 500 nl of test molecules were transferred into the cell plates by PinTool (GNF Systems, San Diego, CA) followed by 2 h incubation at 37°C, 5% CO₂. Flash detection reagents were added at 12.5 μ l/well. After 5 min to 1 h of room-temperature incubation, the cell plates were read on CLIPR (PerkinElmer Life and Analytical Sciences) or Acquest (Molecular Devices, Sunnyvale, CA) for luminescence signal.

Data Analysis. EC₅₀ or IC₅₀ values were obtained by fitting the data with the sigmoidal dose-response curve-fitting tool of the Prism software (GraphPad Software, San Diego, CA). Eight or twelve different concentrations and three data points per concentration were usually used for curve fitting. Linear regression in Schild plot was also done using Prism software.

Results

Identification of Compound 1 as a Novel hVPAC₂ Receptor Antagonist. To establish an assay for high-throughput screening, clonal-derived HEK293 stable cell lines expressing human VPAC₂ and VPAC₁ were created. A number of stable cell lines were tested for VIP- or PACAP-stimulated cAMP response and for inhibition of VIP- or PACAP-stimulated cAMP responses by an antagonist. PACAP6-38 and PG-99-465 were used as control antagonist compounds. It is noteworthy that cell lines that gave robust agonist responses with low agonist EC₅₀ values showed little appreciable antagonist responses, whereas the cell lines that gave weaker agonist curves with high EC₅₀ values gave much better antagonist responses (data not shown). In addition, we found that phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) enhanced agonist assay sensitivity but reduced antagonist assay window and sensitivity (data not shown). After antagonist assay optimization with various parameters such as cell line, seeding cell number, IBMX and agonist concentrations, consistent cellular results with the control antagonists PACAP6-38 and PG-99-465 were obtained with a hVPAC₁-HEK293 and a hVPAC₂-HEK293 stable cell line, respectively. PG-99-465 demonstrated VPAC₁ agonist activity and partial VPAC₂ antagonist activity (Fig. 1). On human VPAC₂ receptor, its antagonist activity reached only 37% efficacy compared with PACAP6-38 (100%). In fact, PG-99-465 showed *agonist* activity in the presence of 1 mM IBMX and *agonist* activity in most of the other hVPAC₂-HEK293 stable cell lines with or without IBMX (data not shown). Therefore, PG-99-465 seems to be an agonist on hVPAC₁ and a partial agonist/antagonist on hVPAC₂. In comparison, PACAP6-38 showed antagonist activities on both VPAC₁ and VPAC₂ in these assays (Fig. 1).

A high-throughput screen was performed on the 1.67 million compounds of the GNF compound library in 1536 format using an automated high-throughput screening system. The assay was a homogeneous cAMP-HTRF assay using hVPAC₂-HEK293 stable cell line and 1 nM VIP as the agonist stimulation. After hit reconfirmation, only one true hit belonging to the hydroxyethylene class was confirmed – compound 1 (Fig. 2). Compound 1 had antagonist activity on hVPAC₂ receptor with an IC₅₀ of 3.8 μ M and no activity on parental HEK293 cells (Fig. 1). After a compound structural similarity search, an analog compound,

compound 2 (Fig. 2), was also found to be active. The activities of the two compounds were confirmed in an independent assay, the β -arrestin Pathhunter assay, in which β -arrestin2 binding to the activated receptor was measured (Yin et al., 2009). Compound 1 was the more potent inhibitor with an IC₅₀ of 2.3 μ M in the β -arrestin assay (Fig. 5, top left).

Compound 1 Noncompetitively Antagonizes hVPAC₂-Mediated cAMP Accumulation and β -Arrestin2 Binding. Inclusion of increasing concentrations of compound 1 dose dependently increased the VIP concentrations needed to trigger cAMP responses in hVPAC₂-HEK293 cells (Fig. 3A). In Schild regression analysis, if compound 1 were a competitive antagonist, a perfect linear line with a slope of 1 would be expected. However, the data, after Schild transformation (Fig. 3B), gave a slope of 0.73 ± 0.10 , which suggests a noncompetitive mechanism of action and a pA₂ of 5.63, predicting a binding affinity of ~ 2.3 μ M. A similar experiment with the β -arrestin Pathhunter assay was carried out. Here, compound 1 not only increased the apparent EC₅₀ values of VIP in triggering β -arrestin binding but also reduced the maximal level of β -arrestin binding (Fig. 3C). The decrease of the maximal level is inconsistent with the possibility that compound 1 is a competitive antagonist of VIP. In conclusion, compound 1 dose-dependently antagonized hVPAC₂-mediated cAMP accumulation and β -arrestin2 binding, but it seemed to act noncompetitively.

Compound 1 is Specific to Human VPAC₂ and Does Not Antagonize hVPAC₁ or hPAC₁. The specificities of compounds 1 and 2 for hVPAC₂, hVPAC₁, and hPAC₁, the three human VIP/PACAP receptors, were determined. Although PACAP6-38 antagonized VIP-triggered cAMP accumulation by hVPAC₁ in the cAMP assay, compound 1 showed no detectable activity (Fig. 1). In the hVPAC₁ transient β -arrestin Pathhunter assay (Fig. 4A), the control compound PACAP6-38 showed a very small amount of agonist activity (7% efficacy compared with VIP), and

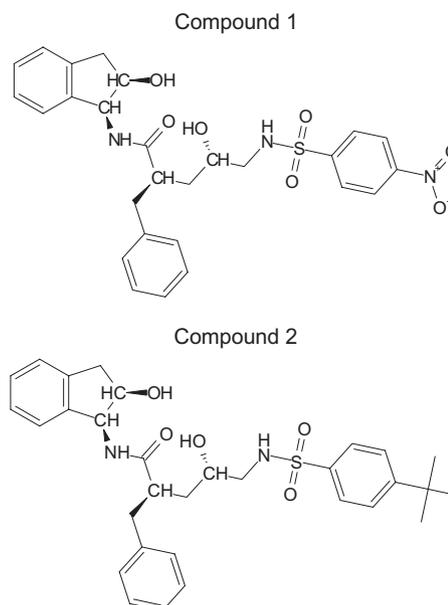


Fig. 2. Structures of VPAC₂ antagonist hits compounds 1 and 2. The full name of compound 1 is (2*R*,4*S*)-2-benzyl-4-hydroxy-*N*-((1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl)-5-(4-nitrophenylsulfonamido)pentanamide. The full name of compound 2 is (2*R*,4*S*)-2-benzyl-5-(4-*tert*-butylphenylsulfonamido)-4-hydroxy-*N*-((1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl)pentanamide.

small yet detectable inhibitory activity at micromolar concentrations. Compound 1 exhibited no appreciable agonist or antagonist activities, whereas compound 2 had some agonist activity at high micromolar concentrations. Likewise, in the hPAC₁ transient β -arrestin Pathhunter assay (Fig. 4B), compound 1 exhibited no appreciable agonist or antagonist activities, whereas compound 2 showed agonist activity with an EC₅₀ of 26.3 μ M and 51% efficacy (VIP = 100%) in the absence of a ligand. PACAP6-38, a PAC₁ antagonist, showed potent antagonist activity (IC₅₀ = 9.3 nM) and a small amount of agonist activity in the absence of an agonist (12% efficacy; Fig. 4B). In summary, compound 1 showed specific antagonist activity toward

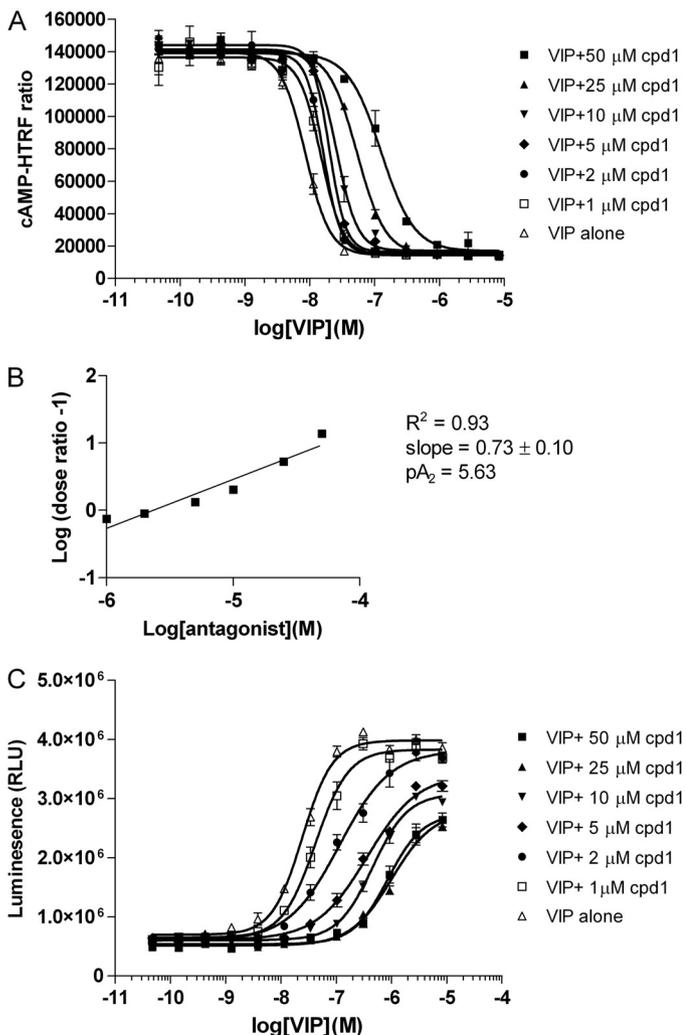


Fig. 3. Compound 1 noncompetitively antagonizes hVPAC₂-mediated cAMP accumulation and β -arrestin2 binding. **A**, cellular cAMP responses were measured in a human VPAC₂-HEK293 stable cell line after 30 min of compound stimulation (varying amounts of VIP and a fixed concentration of compound 1 as indicated) using a cAMP-HTRF kit. The ratio plotted on y-axis is inversely proportional to the free cAMP concentrations in the cell. **B**, Schild plot of the data in **A**. Dose ratio is the ratio of the apparent EC₅₀ of VIP in the presence of a given concentration of compound 1 over the EC₅₀ of VIP in the absence of compound 1. R^2 indicates the goodness of the linear fit. **C**, human VPAC₂ was transiently transfected into HEK293-arrestin2-EA parental cell line for β -arrestin assay. β -Arrestin binding to activated hVPAC₂, which is measured by the reconstituted β -gal activity, was detected after 2 h of stimulation with compounds (varying amounts of VIP and a fixed concentration of compound 1 as indicated). Relative luminescence unit, or RLU is plotted on the y-axis.

hVPAC₂ and no detectable activity toward hVPAC₁ and hPAC₁ receptors. Compound 2, on the other hand, albeit showing specific antagonist activity toward hVPAC₂, also showed a tendency toward receptor activation on VPAC₁ and PAC₁ at higher concentrations.

Compound 1 Interacts with the Transmembrane Core Region of Human VPAC₂. Compounds 1 and 2 were tested in a transient mouse VPAC₂ β -arrestin assay to check for cross-species activity. We were surprised to find that they completely lack inhibitory activity on the mouse receptor (Fig. 5), even though the human and mouse VPAC₂ receptors are 87% identical in sequence (Fig. 6). The differential activity of the compound gave us a unique opportunity to map the compound binding site on the human receptor. We first tested the possibility that compound 1 binds to the ECD of the receptor, where the N-terminally truncated VIP binds and inhibits the receptor activation. We made five single human-to-mouse mutations (Fig. 6, residues highlighted with • above) covering all the differences between human and mouse receptors in the putative ligand binding region in the ECD domain, and also a double mutant with both His⁴⁹→Gln and Asn⁶⁶→Asp mutations. No mutants showed any altered sensitivity to the inhibition by compound 1 (data not shown), indicating that the VIP binding region on the ECD is unlikely to be involved in binding to compound 1. We then made two receptor chimeras, one with mouse receptor ECD and human 7TM region (m-h-VPAC₂), and another one with human receptor ECD and mouse 7TM region (h-m-VPAC₂) (Fig. 6, domain switch position marked with a vertical line with arrows). Consistent with the mutagenesis results, h-m-VPAC₂ abolished the inhibitory activity of compounds 1 and 2 but not m-h-VPAC₂ (Fig. 5), suggesting

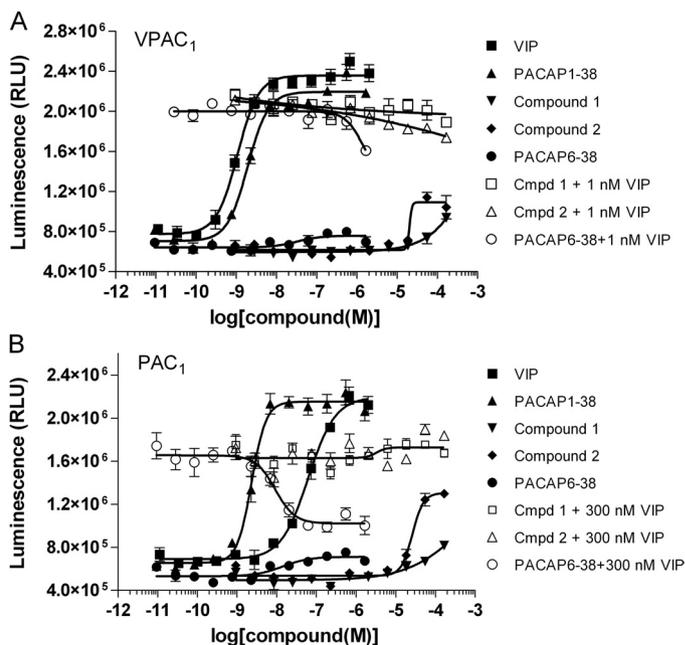


Fig. 4. hVPAC₁ and hPAC₁ transient β -arrestin Pathhunter assays. HEK293-arrestin2-EA parental cells were transiently transfected with human VPAC₁ (**A**) or human PAC₁ (**B**). After 16 to 24 h, test compounds were added to the cells at the indicated concentrations. Two hours later, the reaction was stopped by the addition of flash detection reagent, and β -gal activity was measured. RLU are plotted on the y-axis and the data are expressed as mean \pm S.E.

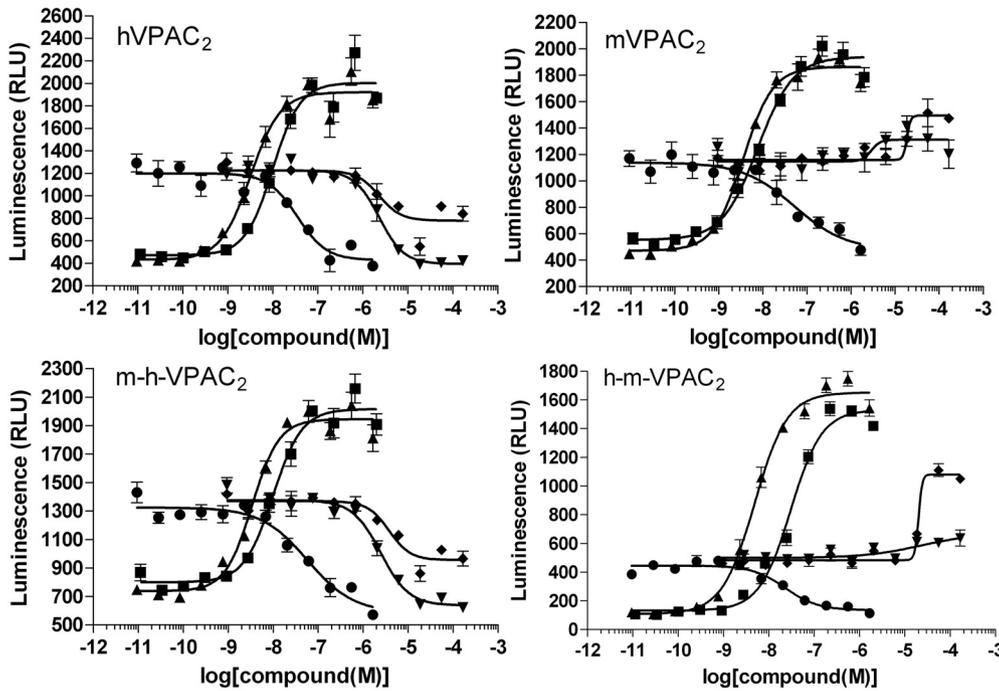


Fig. 5. Compound 1 interacts with the 7TM region of the human VPAC₂ receptor. Human VPAC₂ (hVPAC₂), mouse VPAC₂ (mVPAC₂), the receptor chimera m-h-VPAC₂ with mouse ECD (aa 1-119) linked with human 7TM region (aa 121-438), and the receptor chimera h-m-VPAC₂ with human ECD (aa 1-120) linked with mouse 7TM region (aa 120-437) were transiently transfected into HEK293-arrestin2-EA parental cell line for β -arrestin assay. Total β -arrestin binding to activated receptors was detected 2 h after compound addition to the cells. Relative luminescence unit, or RLU is plotted on the y-axis and the data are expressed as mean \pm S. E. ■, VIP; ▲, PACAP1-38; ●, PACAP6-38 + 5 nM VIP; ▼, compound 1 + 5 nM VIP; ◆, compound 2 + 5 nM VIP.

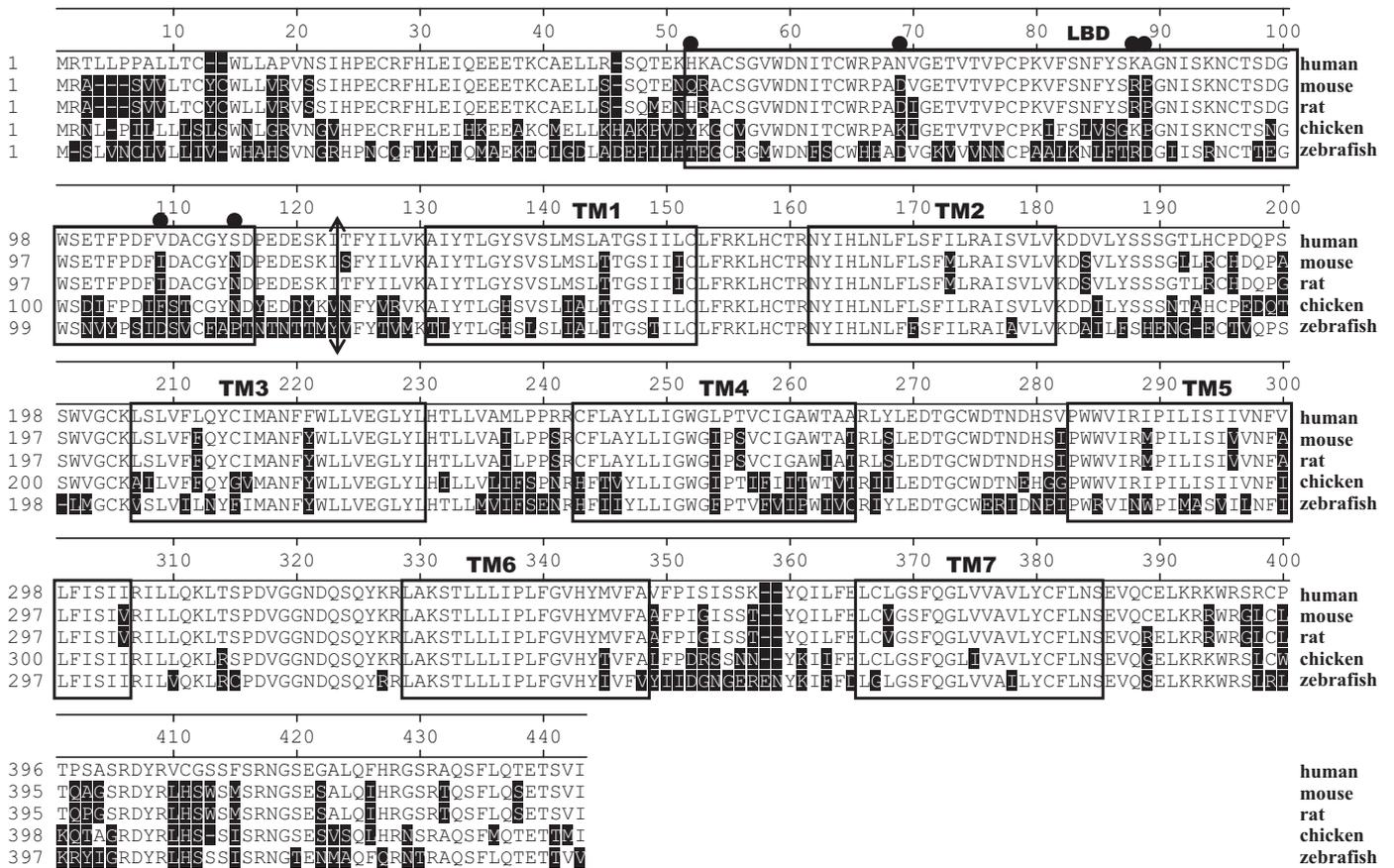


Fig. 6. VPAC₂ sequence alignment across species. Human, mouse, rat, chicken, and zebrafish VPAC₂ protein sequences were aligned using DNASTAR MegAlign software and Clustal V method. Residues that are *not* identical to the human sequence are highlighted by black background. Putative ligand binding domain (LBD) and seven transmembrane helices (TM) are boxed and labeled. The human and mouse receptor chimera switching point—the I/T or I/S junction—is marked by a vertical line with an arrow on each end. The dark circles (•) on top of the sequence mark the residues where human-to-mouse mutagenesis was performed. The six mutants made were His→Gln (mutant 1), Asn→Asp (mutant 2), LysAla→ArgPro (mutant 3), Val→Ile (mutant 4), Ser→Asn (mutant 5), and His→Gln, Asn→Asp (double mutant 6).

that it is indeed the 7TM domain of the human VPAC₂ receptor (extracellular and intracellular loops included) that interacts with the compounds, not the ECD domain. PACAP6-38 was used as a control in these assays, and it inhibited all receptor variants as expected (Fig. 5).

Discussion

Class II GPCRs have been notoriously resistant to small-molecule drug discovery. Thus far, only 5 of the 15 known class II GPCRs have small molecule modulators. Our discovery of a novel, specific, small-molecule antagonist of VPAC₂ that binds to the 7TM region, contributes significantly to the pursuit of small molecule modulators of class II GPCRs, in particular to the VPAC receptor family.

The studies of VIP and PACAP receptors have been hampered by a lack of specific VPAC₂ receptor antagonist. The two control VPAC₂ antagonist peptides that we used, PG-99-465 and PACAP6-38, are not specific. PG-99-465, although initially reported to be a 100-fold selective VPAC₂ antagonist (Moreno et al., 2000), was later shown to exhibit significant VPAC₁ agonist activity and also VPAC₂ agonist activity (Dickson et al., 2006). Indeed, we confirmed these findings and found that the pharmacology of the compound on VPAC₂ is complicated with mixed agonism and antagonism. PACAP6-38 was used in numerous studies to antagonize physiological effects of PACAP on various cell lines and tissues, and it was often assumed that PACAP6-38 is a PAC₁ specific antagonist, when in fact it is a potent dual VPAC₂/PAC₁ antagonist (Laburthe et al., 2007). The binding affinities of PACAP6-38 for VPAC₂, VPAC₁, and PAC₁ were shown to be 40, 600, and 30 nM, respectively (Gourlet et al., 1995), whereas the IC₅₀ values of PACAP6-38 in functional cAMP assays (with 5 nM VIP as agonist) were shown to be 170 nM, >>3 μM, and 14 nM, respectively (Dickinson et al., 1997). In our transient β-arrestin assay system, PACAP6-38 exhibited IC₅₀ values of 35 nM, >>1 μM, and 9.3 nM on VPAC₂, VPAC₁, and PAC₁, respectively, consistent with earlier results. It should be noted that PACAP6-38 showed better potency in the cAMP hVPAC₁ assay using a stable cell line (Fig. 1) compared with the transient transfection β-arrestin assay (Fig. 4A). It is likely that the process of VPAC₁ stable cell line selection favored detection of weak antagonists such as PACAP6-38.

In this report, we identified compound 1, a small-molecule antagonist compound for human VPAC₂ receptor, through high-throughput screening using a cell-based functional assay detecting secondary messenger cAMP concentration change. The very low hit rate (1 in 1.67 million) was quite notable. Although this is certainly related to the poor ability of the receptor to be targeted by a small-molecule drug, it is possible that the sensitivity of the primary screen assay could have been further improved. Selecting a stable HEK293-hVPAC₂ cell clone with an even lower receptor expression level and thus a less sensitive VIP-triggered cAMP response curve might help in making the antagonist assay more sensitive in the cAMP format. Alternatively, the β-arrestin Pathhunter assay that we developed later in the project had much improved sensitivity compared with the cAMP assay and might serve better as a primary screen assay. PACAP6-38 gave an IC₅₀ of 35 nM in the β-arrestin assay (5 nM VIP as agonist) versus an IC₅₀ of 1.2 μM in the

cAMP assay (1 nM VIP as agonist). However, it should be noted that compound 1 had similar IC₅₀ values in the two assays, 3.8 μM versus 2.3 μM.

Compound 1 antagonizes the receptor activation by interacting with residues in the 7TM region that are *not* conserved between human and mouse receptors. The key activation residues in the 7TM domain that interacts with the N-terminal end of the natural ligand according to the “two-domain” model (Laburthe et al., 2007), or a hidden agonist within ECD of VPAC₂ according to the “hidden agonist” model (Dong et al., 2008), are likely to be conserved residues. Our data suggest that compound 1 most likely binds to an allosteric site and acts noncompetitively to inhibit receptor function. As a negative allosteric modulator, compound 1 reduced the efficacy of the natural ligand in inducing β-arrestin2 binding. Whether compound 1 also influences the affinity of the endogenous ligand is an interesting question and will be addressed in future radioligand binding studies. In addition, it will be interesting to map the amino acids on the hVPAC₂ transmembrane helices or extracellular loop regions that cause the human versus mouse differential inhibition of compound 1 in future studies. The small structural difference between compounds 1 and 2 at the end of the benzene ring caused compound 2 to have increased tendency for receptor activation rather than inhibition, suggesting that the two compounds probably interact with an important activation/inactivation switch region of the receptor.

Nonpeptide antagonists, both competitive and allosteric in nature, have been reported for class II GPCRs. Although a series of alkylidene hydrazides were discovered as competitive glucagon receptor antagonists (Ling et al., 2001), triarylimidazole and triarylpyrrole compounds were discovered as noncompetitive antagonists that bind to the receptor 7TM region (Cascieri et al., 1999). A nonpeptide antagonist for corticotropin-releasing factor receptor 1 was found to be allosteric in that it interacts with a methionine residue on TM5 of the receptor (Hoare et al., 2006). An ago-allosteric positive modulator has been reported for GLP₁ (Knudsen et al., 2007). For calcitonin receptor-like receptor, both competitive and allosteric small molecule antagonists have been found, and they interact with the ECD and the 7TM domain of the receptor, respectively (Salvatore et al., 2006). Thus, inhibiting class II GPCRs allosterically by interacting with the 7TM domain of the receptor might be quite common for small molecule modulators. Specificity among GPCR family members might be easier to achieve with an allosteric modulator. However, cross-species specificity might also become more common, as illustrated by compound 1.

In summary, our discovery of a novel human VPAC₂ receptor antagonist is significant because it is the first small molecular modulator that has ever been identified for the VIP/PACAP receptor family. Its exquisite specificity makes it a useful tool for future functional studies of VIP/PACAP receptors. The binding mode of the compound suggests that it inhibits the conformational change that occurs at the GPCR 7TM core domain upon peptide binding to the ECD, which is different than the previously known N-terminally truncated peptide antagonists. The precise binding site requires further investigation and may provide valuable insight toward elucidation of class II GPCR receptor activation mechanism and regulation.

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Address correspondence to: Yu Alice Chen, 10675 John Jay Hopkins Drive, San Diego, CA 92121. E-mail: achen@gmf.org