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Identification and Characterization of a Small Molecule Antagonist of Human $VPAC_2$ Receptor

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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 $VPAC_1$, $VPAC_2$, and PAC_1 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 $VPAC_2$ antagonists. Via high-throughput screening of 1.67 million compounds, we discovered a single small molecule antagonist of human $VPAC_2$, compound 1. Compound 1 inhibits $VPAC_2$-mediated cAMP accumulation with an IC_{50} of 3.8 μM and the ligand-activated β-arrestin2 binding with an IC_{50} of 2.3 μM. Compound 1 acts noncompetitively in Schild analysis. It is a specific $VPAC_2$ antagonist with no detectable agonist or antagonist activities on $VPAC_1$ or PAC_1. Compound 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 $VPAC_2$. 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 $VPAC_2$, and the only $VPAC_2$ antagonist molecule known to date that allosterically interacts with the 7TM region, will be a valuable tool in further study of $VPAC_2$ 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, $VPAC_1$ (also known as $VIPR_1$) and $VPAC_2$ (also known as $VIPR_2$). In addition, PACAP binds to PAC_1 (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 regulation 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). $VPAC_2$ 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.

$VPAC_1$, $VPAC_2$, and PAC_1 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 PAC_1 (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 $VPAC_2$ antagonist is still lacking. PG-99-465 (Moreno et al., 2000), a VIP analog that was initially reported to be a $VPAC_2$-specific antagonist with an IC_{50} of 2 nM, seems to show complex pharmacology. It also is a partial agonist at $VPAC_2$ (EC_{50} = 5 nM) and a full agonist at $VPAC_1$ and PAC_1 receptors with reported EC_{50} values of 8 and 70 nM, respectively (Dickson et al., 2006).

We carried out a high-throughput screen on human $VPAC_2$ 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 $VPAC_2$ 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 $hVPAC_2$ and $hVPAC_1$ in pCDNA3.1 vector, and selected with 800 μg/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. $hVPAC_2$-HEK stable clone 11 and $hVPAC_1$-HEK stable clone 12 were selected for all the later studies. On the day of assay, 3000 cells (4 μl/well) or 15,000 cells (25 μl/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 μl of agonist VIP in growth medium. Assay plates were returned to cell incubator for 30 min before addition of 2.5 μl/well (1536 format) or 15 μl/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

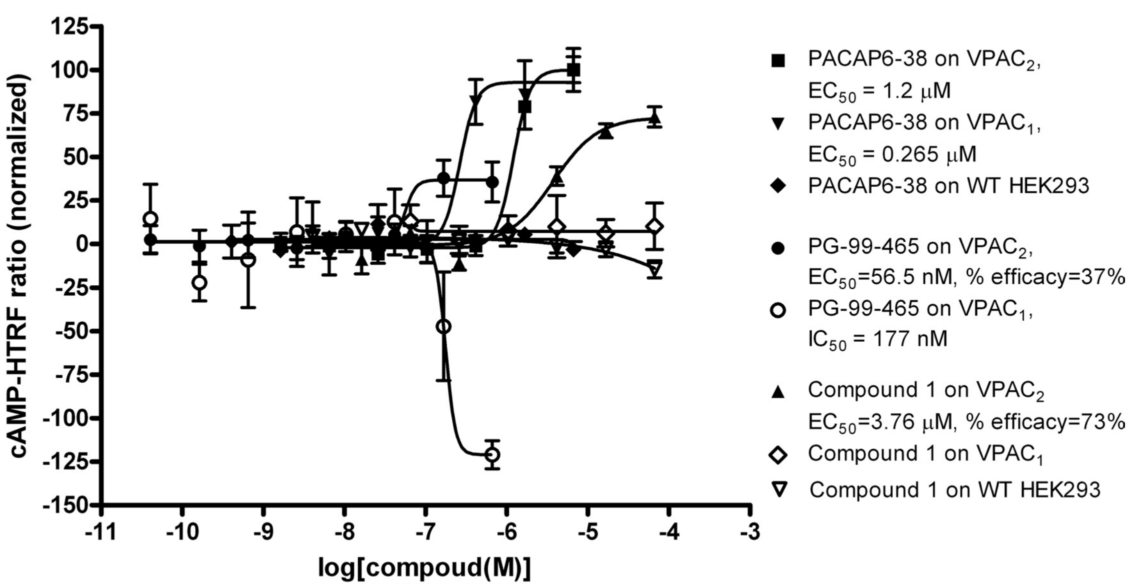

Fig. 1. Human $VPAC_2$ and $VPAC_1$ cAMP antagonist assays. Cellular cAMP responses were measured in a human $VPAC_2$-HEK293 stable cell line (clone 11), a human $VPAC_1$-HEK293 stable cell line (clone 12), and a wild-type (WT) HEK293 cell line as indicated. Varying amounts of the test compounds were added to the specific cell line before the following agonist was added: 1 nM VIP for $hVPAC_2$ cells, 5 nM VIP for $hVPAC_1$ 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 μl/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% CO_2. Flash detection reagents were added at 12.5 μl/ 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. EC_{50} or IC_{50} 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 $hVPAC_2$ Receptor Antagonist. To establish an assay for high-throughput screening, clonal-derived HEK293 stable cell lines expressing human $VPAC_2$ and $VPAC_1$ 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 EC_{50} values showed little appreciable antagonist responses, whereas the cell lines that gave weaker agonist curves with high EC_{50} 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 $hVPAC_1$-HEK293 and a $hVPAC_2$-HEK293 stable cell line, respectively. PG-99-465 demonstrated $VPAC_1$ agonist activity and partial $VPAC_2$ antagonist activity (Fig. 1). On human $VPAC_2$ 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 $hVPAC_2$-HEK293 stable cell lines with or without IBMX (data not shown). Therefore, PG-99-465 seems to be an agonist on $hVPAC_1$ and a partial agonist/antagonist on $hVPAC_2$. In comparison, PACAP6-38 showed antagonist activities on both $VPAC_1$ and $VPAC_2$ 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 $hVPAC_2$-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 $hVPAC_2$ receptor with an IC_{50} of 3.8 μM 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 IC_{50} of 2.3 μM in the β-arrestin assay (Fig. 5, top left).

Compound 1 Noncompetitively Antagonizes $hVPAC_2$-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 hVAPC2-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 pA_2 of 5.63, predicting a binding affinity of ~2.3 μM. A similar experiment with the β-arrestin Pathhunter assay was carried out. Here, compound 1 not only increased the apparent EC_{50} 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 $hVPAC_2$-mediated cAMP accumulation and β-arrestin2 binding, but it seemed to act noncompetitively.

Compound 1 is Specific to Human $VPAC_2$ and Does Not Antagonize $hVPAC_1$ or $hPAC_1$. The specificities of compounds 1 and 2 for $hVPAC_2$, $hVPAC_1$, and $hPAC_1$, the three human VIP/PACAP receptors, were determined. Although PACAP6-38 antagonized VIP-triggered cAMP accumulation by $hVPAC_1$ 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

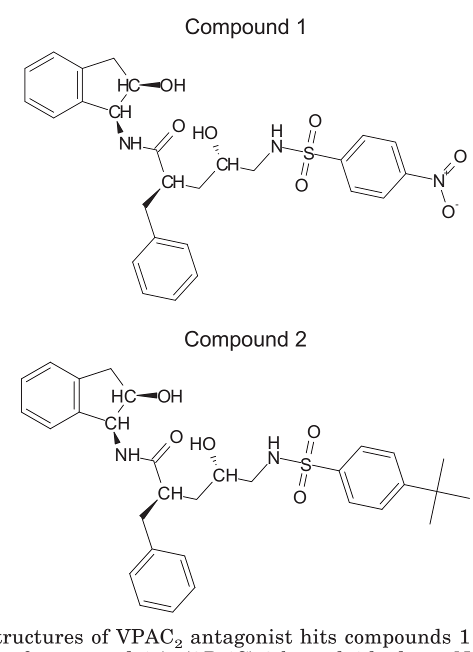

Fig. 2. Structures of $VPAC_2$ antagonist hits compounds 1 and 2. The full name of compound 1 is (2*R*,4*S*)-2-benzyl-4-hydroxy-N-((1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl)-5-(4-nitrophenylsulfonamido) pentanamide. The full name of compound 2 is (2*R*,4*S*)-2-benzyl-5-(4-*tert*-butylphenylsulfonamido)-4-hydroxy-*N*-((1*S*,2*R*)-2-hydroxy-2, 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 $hPAC_1$ transient β-arrestin Pathhunter assay (Fig. 4B), compound 1 exhibited no appreciable agonist or antagonist activities, whereas compound 2 showed agonist activity with an EC_{50} of 26.3 μM and 51% efficacy (VIP = 100%) in the absence of a ligand. PACAP6-38, a PAC_1 antagonist, showed potent antagonist activity (IC_{50} = 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 $hVPAC_2$ and no detectable activity toward $hVPAC_1$ and $hPAC_1$ receptors. Compound 2, on the other hand, albeit showing specific antagonist activity toward $hVPAC_2$, also showed a tendency toward receptor activation on $VPAC_1$ and PAC_1 at higher concentrations.

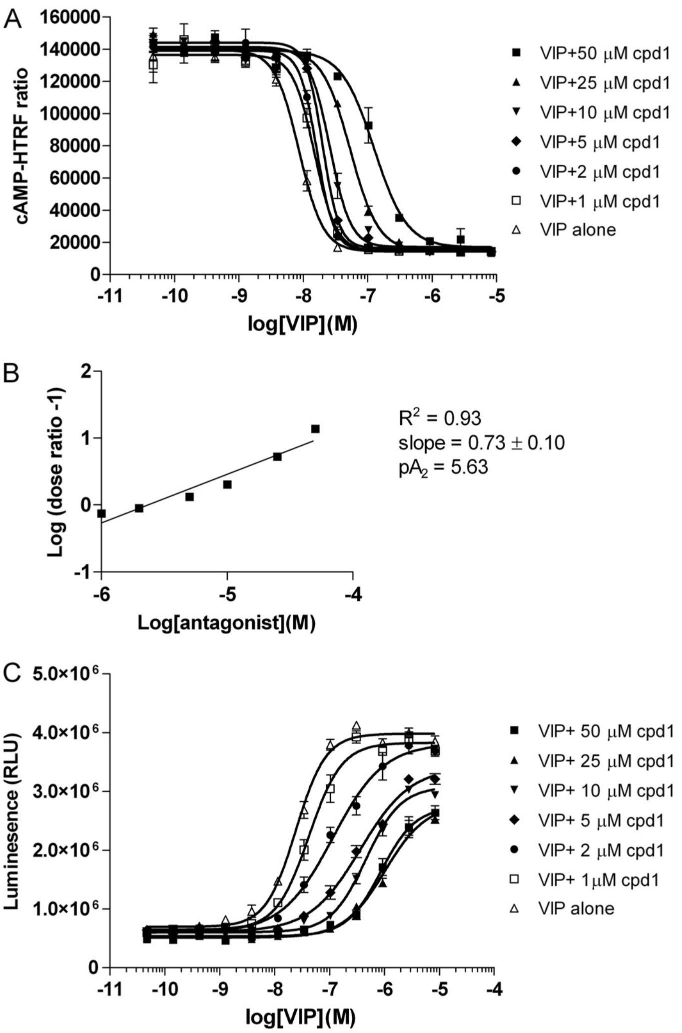

Fig. 3. Compound 1 noncompetitively antagonizes $hVPAC_2$-mediated cAMP accumulation and β-arrestin2 binding. A, cellular cAMP responses were measured in a human $VPAC_2$-HEK293 stable cell line after 30 min of compound stimulation (varying amounts of VIP and a fixed concentration of compound 1 as indicated) 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 EC_{50} of VIP in the presence of a given concentration of compound 1 over the EC_{50} of VIP in the absence of compound 1. R^2 indicates the goodness of the linear fit. C, human $VPAC_2$ was transiently transfected into HEK293-arrestin2-EA parental cell line for β-arrestin assay. β-Arrestin binding to activated $hVPAC_2$, 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.

Compound 1 Interacts with the Transmembrane Core Region of Human $VPAC_2$. Compounds 1 and 2 were tested in a transient mouse $VPAC_2$ 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 $VPAC_2$ 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 His^{49}→Gln and Asn^{66}→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-$VPAC_2$), and another one with human receptor ECD and mouse 7TM region (h-m-$VPAC_2$) (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

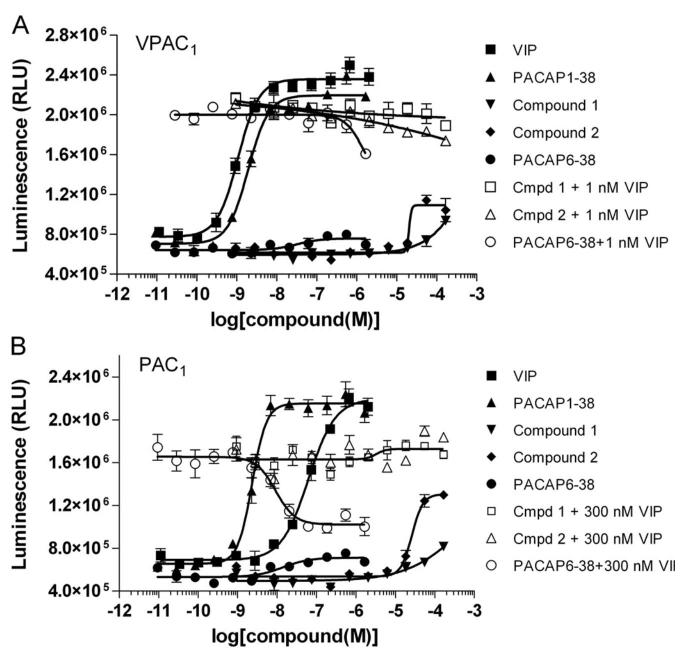

Fig. 4. $hVPAC_1$ and $hPAC_1$ transient β-arrestin Pathhunter assays. HEK293-arrestin2-EA parental cells were transiently transfected with human $VPAC_1$ (A) or human PAC_1 (B). After 16 to 24 h, test compounds were added to the cells at the indicated concentrations. Two hours later, the reaction was stopped by the addition of flash detection reagent, and β-gal activity was measured. RLUs are plotted on the y-axis and the data are expressed as mean ± S.E.

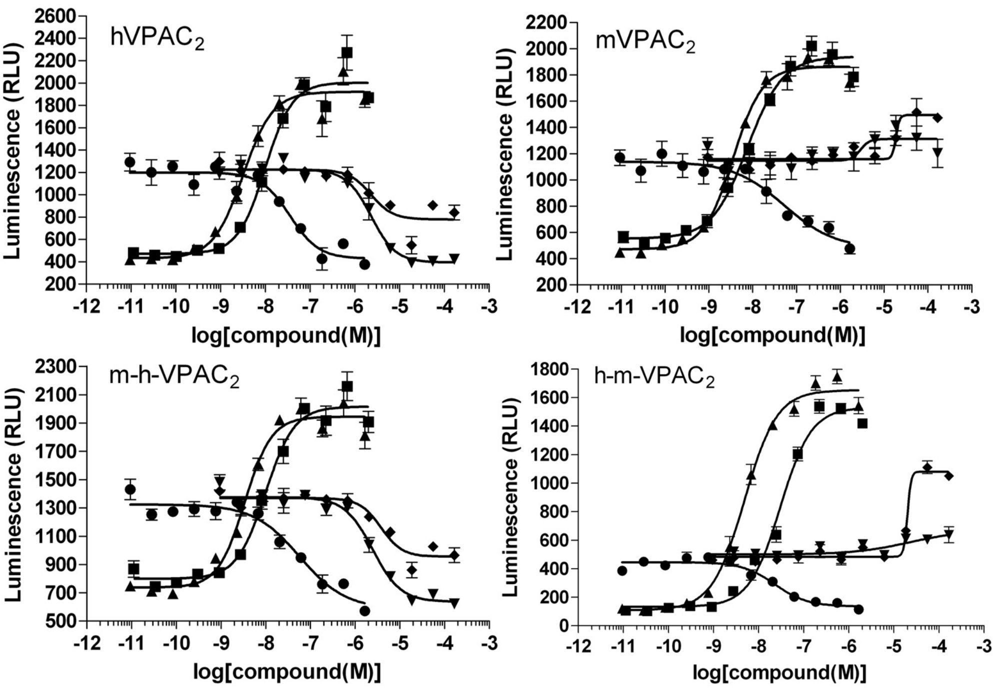

Fig. 5. Compound 1 interacts with the 7TM region of the human $VPAC_2$ receptor. Human $VPAC_2$ ($hVPAC_2$), mouse $VPAC_2$ ($mVPAC_2$), the receptor chimera m-h-$VPAC_2$ with mouse ECD (aa 1-119) linked with human 7TM region (aa 121-438), and the receptor chimera h-m-$VPAC_2$ with human ECD (aa 1-120) 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 ± S. E. ■, VIP; ▲, PACAP1–38; ●, PACAP6-38 + 5 nM VIP; ▼, compound 1 + 5 nM VIP; ♦, compound 2 + 5 nM VIP.

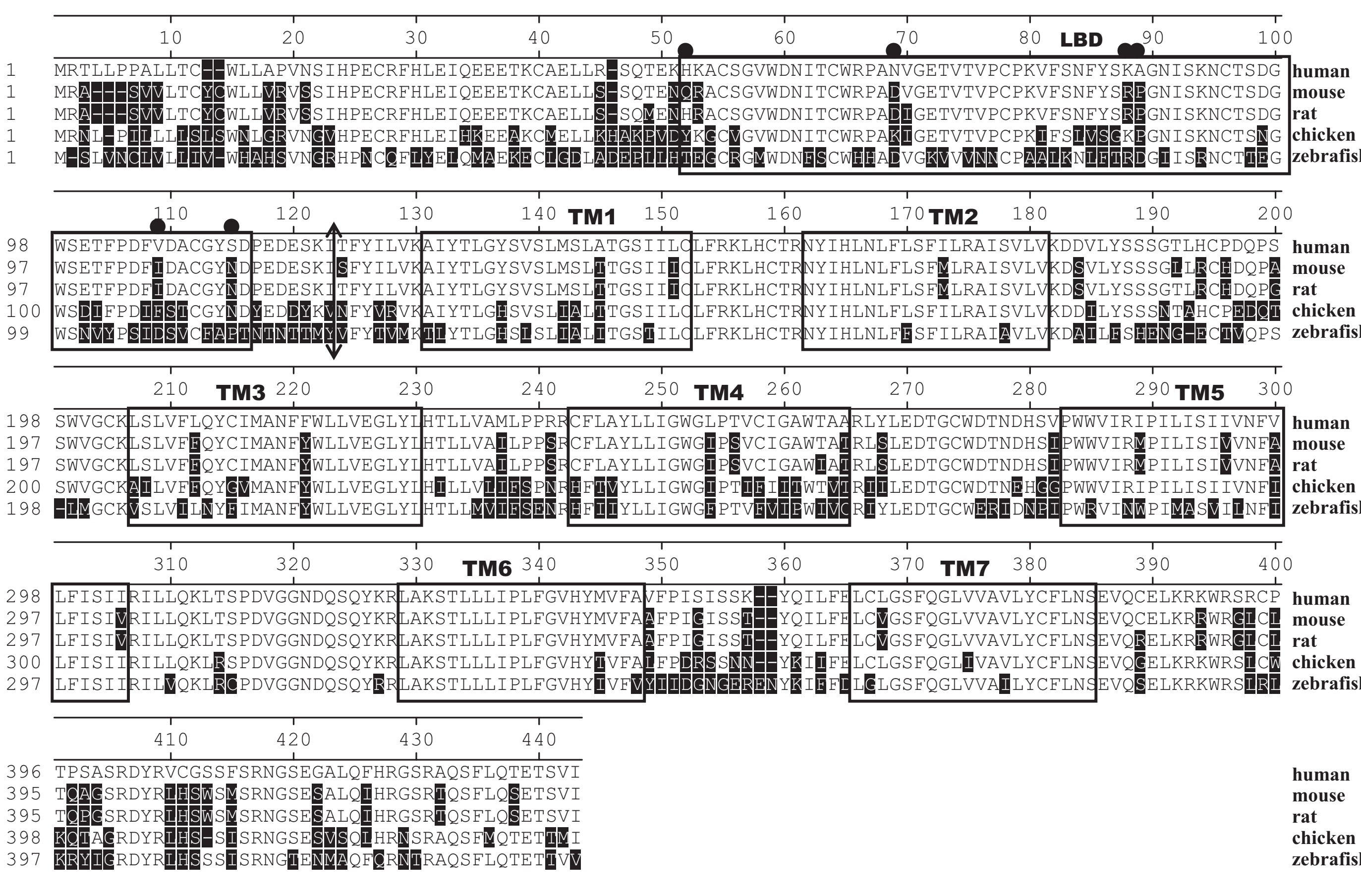

Fig. 6. $VPAC_2$ sequence alignment across species. Human, mouse, rat, chicken, and zebrafish $VPAC_2$ protein sequences were aligned using DNASTAR MegAlign software and Clustal V method. Residues that are *not* identical to the human sequence are highlighted by black background. Putative ligand binding domain (LBD) and seven transmembrane helices (TM) are boxed and labeled. The human and mouse receptor chimeras 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 $VPAC_2$ 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 $VPAC_2$ 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 $VPAC_2$ receptor antagonist. The two control $VPAC_2$ 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 $VPAC_2$ antagonist (Moreno et al., 2000), was later shown to exhibit significant $VPAC_1$ agonist activity and also $VPAC_2$ *agonist* activity (Dickson et al., 2006). Indeed, we confirmed these findings and found that the pharmacology of the compound on $VPAC_2$ 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 PAC_1 specific antagonist, when in fact it is a potent dual $VPAC_2$/PAC_1 antagonist (Laburthe et al., 2007). The binding affinities of PACAP6-38 for $VPAC_2$, $VPAC_1$, and PAC_1 were shown to be 40, 600, and 30 nM, respectively (Gourlet et al., 1995), whereas the IC_{50} values of PACAP6-38 in functional cAMP assays (with 5 nM VIP as agonist) were shown to be 170 nM, $\gg$3 μM, and 14 nM, respectively (Dickinson et al., 1997). In our transient β-arrestin assay system, PACAP6-38 exhibited IC_{50} values of 35 nM, $\gg$1 μM, and 9.3 nM on $VPAC_2$, $VPAC_1$, and PAC_1, respectively, consistent with earlier results. It should be noted that PACAP6-38 showed better potency in the cAMP $hVPAC_1$ 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 $VPAC_1$ 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 $VPAC_2$ 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-$hVAPC_2$ 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 IC_{50} of 35 nM in the β-arrestin assay (5 nM VIP as agonist) versus an IC_{50} of 1.2 μM in the cAMP assay (1 nM VIP as agonist). However, it should be noted that compound 1 had similar IC_{50} 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 $VPAC_2$ 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 $hVPAC_2$ 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 GLP_1 (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 $VPAC_2$ 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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