Small-Molecule Vasopressin-2 Receptor Antagonist Identified by a G-Protein Coupled Receptor ‘Pathway’ Screen

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ABSTRACT

G-protein coupled receptors (GPCRs) such as the vasopressin-2 receptor (V2R) are an important class of drug targets. We developed an efficient screen for GPCR-induced cAMP elevation using as read-out cAMP activation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channels. Fischer rat thyroid cells expressing CFTR and a halide-sensing yellow fluorescent protein (YFP-H148Q/I152L) were transfected with V2R. Increased cell Cl⁻ conductance following agonist-induced cAMP elevation was assayed using a platereader from cell fluorescence following solution I⁻ addition. The Z’-factor for the assay was ~0.7 with the V2R agonist dDAVP (1 nM) as positive control. Primary screening of 50,000 small molecules yielded a novel, 5-aryl-4-benzoyl-3-hydroxy-1-(2-arylethyl)-2H-pyrrol-2-one class of V2R antagonists that are unrelated structurally to known V2R antagonists. The most potent compound, V2Rinh-02, which was identified by screening of 35 structural analogs, competitively inhibited V2R-induced cAMP elevation with Ki ~ 70 nM, and fully displaced radiolabeled vasopressin in binding experiments. V2Rinh-02 did not inhibit forskolin or β2-adrenergic receptor-induced cAMP production, and was >50-times more potent for V2R vs. V1aR. The favorable in vitro properties of the pyrrol-2-one antagonists suggests their potential utility in aquaretic applications. The CFTR-linked cAMP assay developed here is applicable for efficient, high-throughput identification of modulators of cAMP-coupled GPCRs.
INTRODUCTION

G-protein coupled receptors (GPCRs) represent the largest and most versatile group of cell surface receptors (Hill, 2006). GPCRs are an important class of targets for identification of clinically useful agonists and antagonists, constituting ~15% of the total ‘druggable’ genome and ~25% of marketed drugs (Hopkins et al., 2002). GPCRs are coupled to the G-proteins Gs or Gi, which alter cAMP concentration, or Gq, which increases cytoplasmic calcium concentration. Out of ~800 GPCR genes identified in the human genome, 190 have known function (Wise et al., 2004), of which ~30 are the targets of available drugs (Chalmers et al., 2002). The vasopressin-2 receptor (V2R) is of considerable interest because V2R antagonists have aquaretic effects in the kidney for therapy of hyponatremias associated with inappropriately high levels of the antidiuretic hormone vasopressin (Schrier et al., 2006).

Several types of functional assays of cAMP concentration have been developed for high-throughput identification of modulators of Gs- or Gi-coupled GPCRs. The three available assay strategies include: (i) competition assay, which is based on competition between endogenous and radiolabeled cAMP for cAMP antibody binding (William, 2004); (ii) reporter gene assay, where cAMP drives the expression of a reporter gene containing a cAMP response element (Hill, 2001); and (iii) cAMP-dependent protein activity assay, where the cAMP-dependent activity of a target protein is measured (Rich et al., 2005). The merits and limitations of these assays are described in the Discussion section. For Gq-coupled GPCRs, assays of phosphatidylinositol, inositol triphosphate, and intracellular calcium have been developed (Horstman et al., 1988; Monteith et al., 2005).

In this study we report an efficient assay of GPCR activity for Gs and Gi-coupled GPCRs in which cAMP is assayed from halide conductance of the cystic fibrosis transmembrane

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conductance regulator (CFTR) protein. CFTR halide conductance is increased following cAMP-dependent phosphorylation mediated by protein kinase A. The principle of the assay is diagrammed in Figure 1A. Cell expressing wildtype CFTR and a yellow fluorescent protein-based halide sensor (YFP-H148Q/I152L) are transfected with a GPCR. In the case of the Gs-coupled GPCR V2R, agonist-induced cAMP elevation activates CFTR, which is assayed from the kinetics of I- entry into cells following solution I- addition. YFP-H148Q/I152L fluorescence is strongly quenched by I-, with 50% reduction in fluorescence at 2-3 mM I- (Galietta et al., 2001a). We reported previously that Fischer Rat Thyroid (FRT) cells express CFTR and YFP strongly and stably after transfection, and have other properties suitable for high-throughput screening, including low basal halide transport, rapid growth on plastic, and high electrical resistance (Galietta et al., 2001b). FRT cells expressing YFPs and wildtype or mutant CFTRs were used by our lab to identify CFTR activators for potential therapy of cystic fibrosis (Pedemonte et al., 2005) and CFTR inhibitors for potential therapy of secretory diarrheas and polycystic kidney disease (Ma et al., 2002a; Muanprasat et al., 2004). Here we validate the CFTR-linked GCPR assay as applied to the identification of V2R antagonists, and report the discovery and characterization of a 5-aryl-4-benzoyl-3-hydroxy-1-(2-arylethyl)-2H-pyrrol-2-one class of V2R antagonists.
MATERIALS AND METHODS

Plasmids

Plasmids encoding human wildtype and mutant (W164S) V₂Rs were kindly provided by Dr. Daniel Bichet (U. Montreal). N-terminus c-myc-tagged V₂Rs were created by PCR introduction of a c-myc sequence after the methionine initiation sequence. PCR products were subcloned into plasmid pCDNA3.1Hyg+ (Invitrogen) at XbaI and HindIII restriction sites to give plasmids wV₂R-Hyg (wildtype) and mV₂R-Hyg (mutant). cDNAs were confirmed by sequence analysis. The pCDNA3.1 plasmids encoding HA-tagged human β-adrenergic and vasopressin V₁a receptors were purchased from the UMR cDNA Resource Center.

Cell culture and transfection

Fisher rat thyroid cells expressing CFTR and YFP-H148Q/I152L, as described previously (Maunprasat et al., 2004), CHO-K1 cells, and Calu-3 cells were cultured in F-12 Modified Coon’s Medium (Sigma), F-12 Ham’s Nutrient mix, and MEM Eagle & Earle’s BSS medium, respectively. Media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium for FRT cells was supplemented with 2 mM glutamine, 500 µg/ml zeocin, and 500 µg/ml geneticin. The medium for Calu-3 cells was supplemented with 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 1.5 g/l NaHCO₃ and non-essential amino acids. Cells were grown at 37 °C in 5% CO₂ / 95% air.

For stable transfection, cells at ~80% confluence were transfected with wV₂R-Hyg, HA-tagged human V₁aR, or mV₂R-Hyg using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions (Invitrogen). Twenty-four hours after transfection the cells were
selected for 2 weeks with 350 µg/ml hygromycin (Roche) for V2R, or with 750 µg/ml geneticin (Gibco) for V1aR. Remaining colonies were grown at clonal density and screened for receptor expression by immunofluorescence and immunoblot analysis. Stably transfected cell lines were maintained in the same medium used for selection. Transient transfection of CHO-K1 cells with the HA-tagged human β2-adrenergic receptor was done in a similar manner to the stable transfections. Cells were used at 48 h after transient transfection.

**Immunofluorescence and immunoblot analysis**

For c-myc immunostaining, non-permeabilized cells were washed three times with phosphate buffered saline (PBS) and incubated for one hour with anti-c-myc antibody (1:1000, Roche) in PBS containing 1% bovine serum albumin (BSA), washed three times with PBS, and fixed for 10 min in 4% paraformaldehyde. After three washes with PBS, cells were incubated for 1 h with Cy3-conjugated anti-rabbit IgG (Zyme), washed, and mounted for fluorescence microscopy. Immunostaining of permeabilized cells was done similarly, except that cells were permeabilized with 0.1% Triton-X100 in PBS prior to fixation, and blocked for 15 min in PBS containing 1% BSA.

For immunoblot, cells were homogenized in 250 mM sucrose, 1 mM EDTA and 1% protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 5000g for 10 min and the supernatant was assayed for protein concentration (Bio-Rad DC kit). Proteins (20 µg/lane) were resolved by SDS-PAGE (NuPAGE 4-12% Bis-Tris gel; Invitrogen) and transferred to a PVDF membrane. The PVDF membrane was blocked overnight in 5% skim milk in PBS containing 1% Tween-20, washed three times, and incubated for 2 h with anti-c-myc antibody (1:1000). The membrane was then washed three times, incubated for 1 h with HRP-conjugated anti-rabbit IgG.
YFP fluorescence measurement of I⁻ influx

Transfected FRT cells were plated in black-walled, 96-well plates with transparent plastic bottom (Corning-Costar), cultured overnight to confluence, washed three times with PBS, and treated with specified compounds in a final volume of 60 µl. YFP-H148Q/I152L fluorescence was measured using a commercial plate-reader (FluoStar Optima; BMG Lab Technologies) equipped with custom excitation and emission filters (500 nm and 544 nm, respectively, Chroma). Fluorescence intensity in each well was measured for a total of 14 seconds. In each well 100 µl of PBS/I⁻ (PBS with 100 mM Cl⁻ replaced by I⁻) was injected by a syringe pump at two seconds after the start of data collection.

CFTR Cl⁻ current measurement

Cells were cultured on Snapwell filters (Costar 3801) until confluence (transepithelial resistance >500 ohms). Apical membrane current was measured in an Ussing chamber (Vertical diffusion chamber; Costar) with Ringer’s solution bathing the basolateral surface and half-Ringer’s bathing the apical surface. The composition of Ringer’s solution was: 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Na-HEPES, 10 mM glucose, pH 7.3. Half-Ringer’s solution was the same, except that 65 mM NaCl was replaced with Na gluconate, and CaCl₂ was increased to 2 mM. Chambers were bubbled continuously with air. Apical membrane current was measured using a DVC-1000 voltage-clamp apparatus (World Precision Instruments).
High-throughput screening

The compound library for screening contained 50,000 chemically diverse, drug-like small molecules (ChemDiv, San Diego, CA). Stock compounds were stored in 96-well plates at 2.5 µM in DMSO. Compounds occupied 80 wells with the remaining 16 wells containing only DMSO (for positive and negative controls). Screening was done using an automated apparatus (Beckman Coulter) containing a CO2 incubator, carousels for compound plates and pipette tip boxes, plate washer (Elx405; Bio-Tek instrument), liquid handling station (Biomek FX), and two plate readers (FluoStar Optima; BMG lab Technologies). Robotic operations were controlled by SAMI software (version 3.3, Beckman Coulter).

For high-throughput screening cells expressing human wildtype V2R were plated in 96-well plates using a LabSystems Multidrop Dispenser. After overnight growth to confluence, cells were washed with PBS, and dDAVP (1 nM; Ferring Pharmaceuticals) was added together with test compounds (20 µM). The first and last columns of each plate were used for positive (PBS) and negative (dDAVP, no test compound) controls. I- influx was assayed as described above after a 30 min incubation at 37 °C in a CO2 incubator.

Data analysis

I- influx (d[I-]/dt at t=0) was computed from fluorescence time course data as described (Muanprasat et al., 2004). Percent inhibition was computed as: % inhibition = 100 x (negative control-compound) / (negative control-positive control). Positive and negative control values denote d[Γ]/dt obtained from the first and last columns of each plate. Primary screening data was subjected to histogram analysis for ‘hit’ selection.
Synthesis procedures

2,4-Dioxo-4-phenyl-ethylbutylate 1. A solution of anhydrous benzene (50 ml) and acetophenone (1.2 g, 0.010 mol) was added to a suspension of NaH in oil (60%; 0.8 g, 0.020 mol) and the mixture was stirred for 30 min. To a solution of diethyl oxalate (2.19 g, 0.015 mol) benzene (10 ml) was added dropwise and the reaction mixture was stirred for 6 h. The mixture was filtered over Celite and purified by chromatography to give 2,4-dioxo-4-phenyl-ethylbutylate 1 (1.76 g; 85% yield).

4-benzoyl-5-(4-fluorophenyl)-3-hydroxy-1-(4-hydroxyphenylethyl)-2,5-dihydro-2-pyrrolone (V2Rinh-02). Tyramine (93 mg, 0.675 mmol) and 4-fluorobenzaldehyde (84 mg, 0.675 mmol) were heated to 110 °C for 10 min. Water was removed during reflux with a cotton swab. Compound 1 (135 mg, 0.614 mmol) was added dropwise in dioxane (5 ml) and stirred overnight at 100 °C. The 2-pyrrolone was purified by column chromatography and recrystallized to give V2Rinh-02 (153 mg, 60 % yield). \(^1\)H NMR (400 MHz, CD\(_3\)OD): \(\delta\) 7.72 (d, J = 7.6 Hz, 2H), 7.59 (t, J = 7.2 Hz, 1H), 7.48 (d, J = 8.0 Hz, 2H), 7.24 (m, 2H), 7.10 (t, J = 8.8 Hz, 1H), 7.03 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 5.26 (s, 1H), 3.91 (m, 1H), 3.36 (s, 1H), 2.97-2.68 (m, 2H), 2.71 (m, 1H). LC-MS: m/z 418.1 [M+ H]+ (Nova-Pak C\(_{18}\) column, 99%, 200-400 nm).

cAMP measurement

Cells were grown in 24-well plates, treated with test compounds for 30 min, lysed by sonication, centrifuged to remove cell debris, and assayed for cAMP according to manufacturer’s instructions (R&D systems). For CHO-K1 cells, 24 h after transfection with \(\beta_2\) adrenergic receptors cells were trypsinized and plated onto 24-well plates overnight before cAMP assay.
Receptor binding assay

Radiolabeled vasopressin binding was measured in intact FRT cells stably expressing human V2R or V1aR. Confluent cells in 24-well plates were washed twice with ice-cold binding buffer (PBS containing 0.1% glucose, 0.2% bovine serum albumin). Cells were incubated for 2 h at 4 °C with binding buffer containing 1 nM [3H]AVP (Perkin Elmer) and specified concentrations of V2Rinh-02, washed twice with ice cold PBS, and lysed in 0.1 N NaOH containing 0.2% SDS. Radioactivity was measured with a scintillation counter. Non-specific binding, determined by radioactivity with dDAVP (for V2R) or SR 49059 (for V1aR) incubation, was subtracted. dDAVP and SR 49059 (Sanofi Pharmaceutical) bind selectively to V2R and V1a receptor, respectively (Serradeil-Le Gal et al., 1993).

RESULTS

Expression of the wild-type and the mutant V2Rs in FRT cells

Stably transfected FRT cell lines were generated that coexpress human wild-type CFTR, YFP-H148Q/I152L, and c-myc-tagged wild-type V2R or the mutant V2R-W164S. The c-myc-tag was inserted at the external-facing V2R N-terminus. Wild-type V2R showed a plasma membrane distribution by c-myc staining (Figure 1B), whereas no membrane staining was seen in non-transfected cells or cells expressing V2R-W164S that has a defect in cellular processing with retention at the endoplasmic reticulum (Oksche et al., 1996). Immunoblot analysis with c-myc antibody showed bands at ~40 and 64 kDa, corresponding to non-glycosylated and glycosylated V2R, respectively (Innamorati et al., 1996; Sadeghi et al., 1997). These results indicate stable surface expression of wild-type V2R in FRT cells.

Forskolin, an adenylyl cyclase activator, and dDAVP, a V2R-selective vasopressin receptor...
agonist (Chang et al., 2005), increase cytoplasmic cAMP concentration and hence activate CFTR. CFTR activity was assayed from the kinetics of decreasing YFP-H148Q/I152L fluorescence following external I⁻ addition. Forskolin treatment increased CFTR activity in wild-type, mutant and non-transfected cells, whereas dDAVP increased CFTR activity only in cells expressing wild-type V₂R (Figure 2A). Increased CFTR activity in response to forskolin or dDAVP was inhibited by the CFTR blocker CFTRₐₙ₉₋₁₇₂ (Ma et al., 2002a). Inhibition of dDAVP-stimulated I⁻ influx was found with the partial V₂R agonist [1-ß-mercapto-ß, ß-cyclopentamethylenepropionyl¹, O-ET-TYR², VAL⁴, ARG⁸]-vasopressin (Sigma) and the V₂R antagonist SR121463B (Sanofi Pharmaceutical) (Serradeil-Le Gal et al., 1996; Manning et al., 1997). Concentration-activation data are summarized in Figure 2B. The IC₅₀ for CFTR activation by dDAVP of ~ 0.1 nM is less than that observed in V₂R radioligand binding experiments, but comparable to that reported in other functional assays (Saito et al., 1996). Figure 2C shows the forskolin and dDAVP concentration-dependent increase in apical membrane Cl⁻ current in FRT cells expressing wild-type V₂R, providing a direct measure of CFTR function. In each case the current was fully inhibited by CFTRₐₙ₉₋₁₇₂. These results confirm functional cell surface expression of wild-type V₂R in the stably transfected FRT cells.

**High-throughput screening**

To establish a screening assay using the V₂R-transfected cells, we first investigated possible effects of agonist-induced receptor desensitization/internalization, which for GPCRs depends on incubation time and agonist concentration (Robben et al., 2004). Figure 3A shows incubation time- and concentration-dependent reduction in the cellular response to dDAVP, as assayed by CFTR activation. At 1 µM dDAVP, the response was maximal but decreased rapidly with time.
Similar maximal responses were found for 1 and 10 nM dDAVP, but with little time-dependent desensitization. For primary screening, we used 1 nM dDAVP to maximize sensitivity for detection of weakly active, small-molecule competitive antagonists. To prove that receptor desensitization rather than downstream processes was responsible for the time-dependent reduced response to dDAVP, a similar study was done using a high concentration of forskolin in place of dDAVP. Forskolin (100 µM) fully activated CFTR, but without a measurable reduction in activity over 45 min, supporting a desensitization mechanism for dDAVP.

The suitability of the assay for high-throughput screening was evaluated by experimental determination of the Z’-factor, a quantitative measure of assay ‘goodness’ that depends on the difference in positive and negative control signals and their standard deviations (Seethala et al., 2001). Figure 3B (left) shows original fluorescence data from individual wells of 96-well plates incubated for 30 min with PBS or 1 nM dDAVP. The distribution of Γ influx rates in individual wells, d[Γ]/dt, shows well-separated positive and negative controls (Figure 3B, right), giving a Z’-factor of 0.71. A Z’-factor greater than 0.5-0.6 is considered excellent such that a single primary screen is predicted to be informative in identifying ‘hits’. Test compounds did not quench YFP-H148Q/I152L fluorescence directly as indicated by similar cell fluorescence prior to Γ addition.

A primary screen of 50,000 small molecules was done using the FRT cells expressing wild-type human V₂R, CFTR and YFP-H148Q/I152L. Cells were incubated with 1 nM dDAVP and test compounds (20 µM final concentration) for 30 min prior to fluorescence assay of Γ influx. Figure 3C (left) shows representative original data for positive and negative controls, along with an example of an active test compound. Figure 3C (right) summarizes percentage inhibition values as a frequency histogram. The majority of the compound groups (12456 groups of 4 different compounds) did not reduce d[Γ]/dt (< 30% inhibition at 20 µM). Two hundred and seventy-two
compound groups were classified as weak inhibitors (30-90% inhibition), and 27 groups were considered strong inhibitors (> 90% inhibition). These definitions of weak vs. strong inhibitors are arbitrary.

The ‘strong’ inhibitor groups identified in the primary screen were further evaluated to confirm their inhibition activity at low micromolar concentration and to identify individual compounds responsible for activity out of the groups-of-four used in the primary screen. Approximately 70% of the hits identified in the primary screen were confirmed in subsequent secondary screening by the fluorescence platereader assay. Three potent classes of compounds were found, one of which was a V2R antagonist (Figure 4A), as judged by selective inhibition of dDAVP-induced cAMP production (see below). The V2R antagonist is of the 5-aryl-4-benzoyl-3-hydroxy-1-(2-arylethyl)-2H-pyrrol-2-one chemical class, which is not structurally similar to known V2R antagonists (OPC 31260 and SR 121463A structures shown in Figure 4A). The other (non-V2R antagonist) hits identified in the screen are being characterized and are not discussed further in this paper. These compound inhibited forskolin-induced cellular responses, thus they act at downstream sites.

Analysis of structure-activity relationship (SAR)

SAR analysis was done by screening a small set of 81 commercially available 2,5-dihydro-2-pyrorlone analogs. Table 1 lists V2R inhibition data of active compounds, and Figure 4B (left) summarizes the SAR analysis in terms of the functional groups conferring V2R antagonist activity. At position R1, phenyl and 4-substituted phenyl conferred greatest inhibition; inhibition was lost when R1 was methyl or furanyl. Notably, V2Rinh-13, which contains a thiophenyl ring at R1, was also active. The greatest diversity in the collection of 2-pyrrolone analogs was in R2. Activity was
seen primarily for 4-halogen or 4-nitro substituted phenyl. Exceptions to this were the two 2-fluorophenyl derivatives [V2Rinh-04 and V2Rinh-11] and the 3-nitrophenyl derivative [V2Rinh-08]. Phenyl substitutions at R2 that led to inactive compounds were generally electron donating substituents including methoxy, hydroxy, and alkyl; exceptions to this were the two 4-methoxyphenyl derivatives [V2Rinh-03 and V2Rinh-07]. Methyl formate esters and other heterocycles were not tolerated at R2, including furyl, thiophenyl, and 3-pyridinyl rings. R3 as phenyl and 4-hydroxy phenyl gave active compounds; 3,4-dimethoxyphenyl and 4-hydroxy-3-methoxyphenyl analogs were inactive. The 4-hydroxylphenyl derivative gave the greatest inhibition potency, with the most potent compound being 4-benzoyl-5-(4-fluorophenyl)-1,5-dihydro-3-hydroxy-1-[2-(4-hydroxyphenyl)ethyl-2H-pyrrol-2-one (V2Rinh-02). This compound was synthesized in pure form on a large scale and further characterized.

Synthesis and characterization of V2Rinh-02

The synthesis of V2Rinh-02 was accomplished in two steps. The dioxoethyl butylate 1 was synthesized by reaction of the enolate of acetophenone with diethyloxylate to give 2,4-dioxo-4-phenyl-ethylbutylate 1 (Figure 4C). Following reaction of 4-bromobenzaldehyde and tyramine to form the imine, the addition of 2,4-dioxo-4-phenyl-ethylbutylate 1 in dioxane gave V2Rinh-02 (Figure 4C), which was purified by crystallization. Its pKa was 4.76 as measured by spectrophotometric titration (absorbance 346 nm) of 100 µM V2Rinh-02 in aqueous solution containing citric acid, sodium acetate, HEPES, sodium borate, tris, and sodium carbonate (each 5 mM) titrated to indicated pH using HCl and NaOH. Deprotonation of the hydroxyl group on the pyrrolone ring occurs at low pH, such that V2Rinh-02 contains a single negative charge at physiological pH. The aqueous solubility of V2Rinh-02 in PBS was 377 µM as measured by optical
absorbance of a saturated solution after appropriate dilution. The high aqueous solubility of V2Rinh-02 is a consequence of its polarity and charge.

V2Rinh-02 identity was confirmed by 1H NMR and mass spectrometry. Purity was determined to be 99% by liquid chromatography. Figure 4B (right) shows the predicted 3-d structure of V2Rinh-02 based on energy minimization computations. An interesting finding from 1H NMR spectra was the magnetic nonequivalence of the two CH2 groups of the 1-(4-hydroxyphenethyl) fragment. From the computed structure, we conclude that this magnetic nonequivalence is due to the proximal 4-fluorophenyl group interacting with the H on the -CH2-N group, accounting for the AA′BB′ splitting pattern and the downfield chemical shift ~3.9 ppm of the proximal proton. This interpretation is supported by published structure data for 5-aryl-1-benzyl-2,5-dihydro-2-pyrrolones (Aliev et al., 2003).

V2Rinh-02 was first tested for its inhibitory potency in apical membrane current measurements of CFTR Cl− channel activity. Figure 4D, which is representative of 3 separate experiments, shows V2Rinh-02 concentration-dependent inhibition of Cl− current induced by 1 nM dDAVP, with IC50 of 0.5 ± 0.1 µM (mean ± SD, n=3).

Several possible steps in the signal transduction cascade for CFTR activation could be modulated by V2Rinh-02, including: (i) V2R, (ii) G-proteins, Gs or Gi, (iii) adenylyl cyclase, (iv) phosphodiesterase, (v) protein kinase A, and (vi) CFTR. The failure of V2Rinh-02 to inhibit forskolin-induced Cl− current, as seen in Figure 4D (left), indicates that V2Rinh-02 is unlikely to act on Gi, adenylyl cyclase, or more distal components of the signal transduction cascade. To distinguish action of V2Rinh-02 as a V2R antagonist versus a Gs inhibitor, short-circuit current was measured in Calu-3 cells, which natively express the β2-adrenergic Gs-coupled receptor. Figure 4D (center) shows inhibition of isoproterenol-induced short-circuit current in Calu-3 cells by the β2
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antagonist propranolol. V2Rinh-02 did not inhibit the isoproterenol-induced response in Calu-3 cells (Figure 4D, right). Together these findings suggest that V2Rinh-02 is a V2R antagonist.

Measurements of cAMP concentration were made in the V2R-expressing FRT cells in order to verify the action of V2Rinh-02 at the vasopressin-2 receptor. Figure 5A (left) shows dDAVP concentration-response data for elevation of cAMP concentration. V2Rinh-02 inhibition of cAMP concentration following stimulation by 1 nM dDAVP is shown in Figure 5A (right). The IC50 was ~60 nM. To verify action of V2Rinh-02 on the V2R, cAMP concentration was measured in β2-receptor transfected CHO cells (Figure 5B). Cellular cAMP was increased by isoproterenol, as expected. The increased cAMP concentration was inhibited by propranolol but not by SR 121463B or V2Rinh-02, supporting V2R inhibition by V2Rinh-02.

**V2Rinh-02 is a competitive V2R antagonist**

Figure 6A shows a functional ‘competition study’ in which V2Rinh-02 reduction of cAMP concentration was measured in V2R-expressing FRT cells after stimulation by different concentrations of dDAVP. The dDAVP dose-response curves shifted in a parallel manner to the right with increasing V2Rinh-02 concentration, indicative of a competitive binding mechanism. A Schild plot is shown in the inset to Figure 6A in which [V2Rinh-02] is plotted against dDAVP dose-ratio (Kenakin, 1997). The slope of 1.3 of the fitted line supports a competitive inhibition mechanism with a Ki of ~ 70 nM.

Binding displacement assays were done to prove V2Rinh-02 competition with vasopressin at the V2R. Cell-associated radiolabeled vasopressin was measured after binding to V2R-expressing FRT cells. The V2R-selective binding component was determined by subtracting cell-associated radioactivity in the presence of a high concentration of non-radioactive dDAVP. As summarized in Figure 6B, V2Rinh-02 at 1 µM reduced cell-associated radioactivity to near zero with 50% competition at ~70 nM, supporting a
competitive V2Rinh-02 binding mechanism. To investigate V2R vs. V1aR binding selectivity, similar binding displacements experiments were done in FRT cells stably expressing V1aR. Fifty percent competition of V2Rinh-02 to [3H]AVP was seen at ~5 µM (Figure 6B), indicating ~70 times greater affinity of V2Rinh-02 to V2R than V1aR.

**DISCUSSION**

We report a novel, cell-based functional assay of Gs- or Gi-coupled GPCR modulators based on cAMP-dependent activation of CFTR Cl− channels. The assay is technically simple, inexpensive, and readily adaptable to fluorescence-based, high-throughput screening formats. The assay used an epithelial cell line suitable for both fluorescence and electrophysiological measurements. The cells have low basal halide conductance and cAMP concentration, excellent transfection efficiency, and rapid growth on uncoated plastic. The Z’-factor for the assay was ~0.7, indicating very good sensitivity and specificity in a single primary screen. The assay was applied to identify V2R antagonists in a screen of 50,000 small molecules in a 96-well plate format. Our assay was designed as a ‘pathway screen’, so that compounds inhibiting CFTR I− influx could function as V2R antagonists, Gs inhibitors, Gi activators, adenylyl cyclase inhibitors, phosphodiesterase activators, protein kinase A inhibitors, or CFTR inhibitors. Such a pathway screen has the advantage of identifying small-molecule modulators of multiple targets in a single screen, with target identification done in small-scale secondary assays.

Functional assays of cell responses are widely used in high-throughput screening, in part because they are technically easier than radioligand binding assays. Binding assays are unable to distinguish between agonists and antagonists, and have low sensitivity for detection of allosteric modulators. Several functional assays have been developed based on cAMP measurement, as
mentioned in the Introduction. An advantage of the standard cAMP antibody competitive assay is that it does not require the generation of a stable cell line if cells are available with endogenous expression of the GPCR of interest. Disadvantages of this assay include the long incubations with cAMP antibody, the need for multiple washing steps, and the relatively high cost. Reporter gene assays, utilizing green fluorescent protein (GFP), luciferase or β-lactamase, are generally more sensitive than competition assays, in part because reporter transcription is downstream from cAMP, which can produce effective signal amplification. However, disadvantages of reporter gene assays include the requirement of a stable cell line expressing a reporter gene driven by a cAMP response element and long incubation time for transcription, where receptor down-regulation can occur. Also, for some reporter assays cell lysis is required and costly signal-producing reagents are needed. The functional assay described here is suitable for discovery of modulators of Gs- or Gi- coupled GPCRs. Our assay is homogeneous and very sensitive, and requires only 3 handling steps before measurement. The assay does not require lysis or reagent addition steps. The total cost of medium, reagents and disposable supplies is ~4 U.S. dollars per one 96-well plate. However, our assay does require a specialized cell line expressing CFTR, YFP and the GPCR of interest. To our knowledge the only assay of this general type was developed by Atto Bioscience (Rich et al., 2005), where calcium influx was used as a read-out of cyclic nucleotide-sensitive ion channels.

Our study applied the GPCR pathway screen to identify inhibitors of vasopressin stimulated cAMP acting through the V₂R. Clinical indications V₂R antagonists include the treatment of hyponatremias associated with increased or normal total body water, such as congestive heart failure, cirrhosis, and the syndrome of an inappropriate ADH secretion (Paranjape et al., 2001). Water retention is a common clinical problem that increases the mortality and
morbidity of underlying cardiovascular and hepatic diseases. The current main strategy in treating water retention is the use of diuretics, which increase excretion of both electrolytes and water. V$_2$R antagonism causes an increase in water excretion without significant electrolyte loss, and so is a superior treatment strategy. Early attempts to develop V$_2$R antagonists focused on peptide analogs of vasopressin (Allison et al., 1988), though they had very low bioavailability. The first small molecule V$_2$R antagonist, OPC 31260, was a derivative of the vasopressin V$_{1a}$ receptor antagonist, OPC 21268 (Thibonnier et al., 2001). A second V$_2$R antagonist was SR 121463 (Serradeil-Le Gal et al., 1996). These V$_2$R antagonists were originally identified by Otsuka Pharmaceutical Co. (Japan) and Sanofi Recherche (Toulouse, France), respectively. Both V$_2$R antagonists have a diuretic effect in humans (Ohnishi et al., 1995; Serradeil-Le Gal et al., 2001), with IC$_{50}$ values in the nanomolar range. Subsequently, several pharmaceutical companies modified the structures of the original V$_2$R antagonists to obtain more potent V$_2$R antagonists (Yamamura et al., 1998; Gunnet et al., 2006), a dual V$_2$R/V$_{1a}$R antagonist (Tahara et al., 1997), and a V$_{1b}$R antagonist (Serradeil-Le Gal et al., 2002). Currently, only Conivaptan (YM 087), a dual V$_2$R/V$_{1a}$R antagonist, has been approved for treatment of euvolemic hyponatremia (Lemmens-Gruber et al., 2006). SR 121463, Tolvaptan (OPC 41061), and Lixivaptan (VPA 985) are in phase 3 clinical trials (Wong et al., 2003; Ghali et al., 2006; Schrier et al., 2006). However, the tricyclic antagonists are quite hydrophobic (logP > 4) and have limited aqueous solubility (< 1 mg/ml) (Matthews et al., 2004), which are potentially problematic in further development for therapeutic purposes. New classes of V$_2$R antagonists could provide useful lead compounds that might overcome these concerns.

Our small-molecule screen of 50,000 compounds identified a novel chemical class of V$_2$R antagonists that are unrelated to known V$_2$R antagonists. Seventy percent of the compounds...
identified by the primary screening were confirmed in the secondary screening. The failure to add dDAVP by the robotic system because of the clogged pipette tips might account for these false positives. After confirming the class of 5-aryl-4-benzoyl-3-hydroxy-1-(2-arylethyl)-2H-pyrrol-2-one were bona fide V2R antagonists, a small screen of commercially available structural analogs was done, identifying 4-benzoyl-5-(4-fluorophenyl)-1,5-dihydro-3-hydroxy-1-[2-(4-hydroxyphenyl)ethyl]2H-pyrrol-2-one (V2Rinh-02) as the most potent compound of the 3-hydroxy-2-pyrrolones. Comparing the structure of the active and inactive compounds gave insight to the structural determinants of compound activity. In general, R1 tolerated various substituted phenyl rings, R2 was more limited in the fact that primarily 4-halophenyl and 4-nitrophenyl derivatives gave active compounds, and R3 tolerated phenyl and 4-hydroxyphenyl substitution. A more focused 3-hydroxy-2-pyrrolone library incorporating the functional groups of the most active V2Rinh-02 antagonists and having more diversity in R1 and R3 should yield more potent V2R antagonists as well as compounds with different V2R vs. V1R selectivities.

Although, the displacement assay of radiolabeled vasopressin can not distinguish between competitive and allosteric antagonists, it did confirm that V2Rinh-02 was a V2R antagonist. V2Rinh-02 was found to be a competitive antagonist of dDAVP/vasopressin binding to the V2R, as demonstrated in cAMP measurements following cell incubations with different V2Rinh-02/dDAVP concentrations. The existing, chemically unrelated V2R antagonists OPC 31260 and SR 121463, are also competitive antagonists (Serradeil-Le Gal et al., 1996; Thibonnier et al., 2001). The K_i of V2Rinh-02 was ~70 nM, as estimated from competition data. V2Rinh-02 was ~70 times more selective for V2R than V1a, as demonstrated by radioactive binding assay.

In conclusion, we have established a novel high-throughput screening ‘pathway’ assay for identification of modulators of Gs or Gi-coupled GPCRs. The assay is very sensitive, and
technically simple and inexpensive compared to existing cell-based GPRC assays. The 5-aryl-4-
benzoyl-3-hydroxy-1-(2-arylethyl)-2H-pyrrol-2-one V₂R antagonists discovered in a small-
molecule screen using the assay have favorable properties to support their further evaluation for
aquaretic therapy.

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REFERENCES


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LEGENDS FOR FIGURES

Figure 1. Assay and cell lines used for V2R antagonist screening. A. Principle of the assay, showing increased I- influx following cAMP activation of plasma membrane CFTR Cl- channels. B. Staining of non-transfected FRT cells (FRT), FRT cells stably expressing wild-type human V2R (FRT-V2R), and FRT cells stably expressing the W164S mutant or V2R (FRT-V2R-W164S). Anti-c-myc staining shown in red, with YFP in green and nuclei (DAPI) in blue. C. Immunoblot of a crude protein extract from FRT (left lane), and FRT-V2R (right lane) cells.

Figure 2. Functional characterization of FRT-V2R-expressing FRT cells. A. YFP fluorescence following I- addition in FRT, FRT-V2R, and FRT-V2R-W164S cells. Cells were pre-incubated with 20 µM forskolin (left) or 1 nM dDAVP (right) for 20 min prior to I- addition. Indicated inhibitors were present before and during measurements: CFTRinh-172 (20 µM); [1-β-mercaptop-β, β-cyclopentamethylenepropionyl1, O-ET-TYR2, VAL4, ARG8]-vasopressin (β-ME, 10 µM); SR-21463A (10 µM). The scale bar on the y-axis indicates percent fluorescence reduction relative to baseline fluorescence (before iodide addition). Curves were displaced in the y-direction for clarity. B. Percentage maximal activity (from initial fluorescence slopes following I- addition) as a function of forskolin and dDAVP concentrations in FRT-V2R and FRT-V2R-W164S cells. The percent maximal activity was a percent of cellular response to an agonist relative to the maximal response triggered by the same agonist. The data reflected findings in a single well experiment. C. Forskolin and dDAVP concentration-response data in FRT-V2R cells measured as apical membrane current (Iap) in short-circuit current measurements. Where indicated 20 µM CFTRinh-172 was added.
Figure 3. Validation of the screening assay for identification of V₂R antagonists. A. Time course of V₂R desensitization assayed by I⁻ influx in FRT-V₂R cells exposed to indicated dDAVP concentrations. The data were shown as mean ± SE (n=4) B. (left) YFP fluorescence of FRT-V₂R cells pre-incubated with 0 or 1 nM dDAVP for 30 min with I⁻ added as indicated. (right) Histogram distribution of I⁻ influx (d[I⁻]/dt) determined from initial fluorescence slopes. C. (left) Examples of positive and negative control fluorescence data from individual wells in the primary screen, along with example of an ‘active’ test compound. (right) Histogram distribution of % inhibition from screening of 50,000 small molecules in groups of four.

Figure 4. Structure-activity of V₂R antagonists. A. Structure of V₂Rinh-02 show with previously identified V₂R antagonists, OPC-312260 and SR-121463A. B. (left) Summary of structure-activity relationship data for 35 compounds (left panel) (also see Table 1). (right) Predicted 3-dimensional structure of V₂Rinh-02. C. Synthesis of V₂Rinh-02: 4-benzoyl-5-(4-fluorophenyl)-1,5-dihydro-3-hydroxy-1-[2-(4-hydroxyphenyl)ethyl-2H-pyrrol-2-one. See text for description. D. (left) Concentration-dependence data for V₂Rinh-02 inhibition of apical membrane CFTR Cl⁻ current in V₂R-expressing FRT cells following activation by 1 nM dDAVP. Where indicated 20 µM forskolin was added. (center and right) Inhibition of isoproterenol-stimulated short-circuit current in Calu-3 cells by indicated concentrations of propranolol and V₂Rinh-02.

Figure 5. Effects of V₂Rinh-02 on cAMP concentration. A. (left) cAMP concentration in dDAVP concentration in FRT-V₂R cells after 20 min incubation with various concentrations of dDAVP. (right) Reduced cAMP following V₂Rinh-02. Cells were incubated for 20 min with 1 nM dDAVP
and various concentrations of $V_2R_{inh}$-02 (mean ± SE, n=3). B. cAMP concentration in CHO cells transiently transfected with human β2-adrenergic receptor. Cells were incubated for 20 min with 0.01 µM isoproterenol in the presence of 20 µM SR-121463A, $V_2R_{inh}$-02, or propranolol. The data were shown as mean ± SE, n=3.

**Figure 6.** Competitive binding and vasopressin receptor subtype selectivity of $V_2R_{inh}$-02.

A. Competition study showing cAMP concentration in FRT-$V_2R$ cells as a function dDAVP concentration in the presence of indicated concentrations of $V_2R_{inh}$-02 (mean ± SE, n=3). Inset: Schild plot with slope of 1.3 and extrapolated $K_i$ of 70 nM. B. $[^3H]AVP$ binding displacement assays were done in intact FRT cells stably expressing $V_2R$ or $V_{1a}R$. Cells were incubated for 2 h with 1 nM $[^3H]AVP$ in the presence of different concentration of $V_2R_{inh}$-02. Cell-associated radioactivity after incubation and washing (mean ± SE, n=3). Non-specific binding was subtracted. See Methods for details.
TABLE 1

Structure-Activity Relationships of Active V₂R Antagonists

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Ph = phenyl, Br = bromo, Cl = chloro, F = fluoro, OMe = methoxy, NO₂ = nitro
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6