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Receptor Antagonism/Agonism Can Be Uncoupled from Pharmacoperone Activity

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Abstract

Pharmacoperones rescue misrouted mutants of the vasopressin receptor type 2 (V2R) and enable them to traffic to the correct biological locus where they function. Previously, a library of nearly 645,000 structures was interrogated with a high throughput screen; pharmacoperones were identified for V2R mutants with a view toward correcting the underlying mutational defects in nephrogenic diabetes insipidus. In the present study, an orthologous assay was used to evaluate hits from the earlier study. We found no consistent relation between antagonism or agonism and pharmacoperone activity. Active pharmacoperones were identified which had minimal antagonistic activity. This increases the therapeutic reach of these drugs, since virtually all pharmacoperone drugs reported to date were selected from peptidomimetic antagonists. Such mixed-activity drugs have a complex pharmacology limiting their therapeutic utility and requiring their removal prior to stimulation of the receptor with agonist.

Keywords

pharmacoperone; high throughput screening; antagonism; receptor trafficking; receptor misfolding; therapeutic targeting of trafficking

Introduction

Protein trafficking from the endoplasmic reticulum (ER) to the plasma membrane (PM) or other intracellular loci is a relatively new therapeutic target and is effective since misfolded

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and misrouted proteins can be rescued and returned to function by protein-specific pharmacoperone drugs (1–3). These drugs diffuse into cells, serve as structural templates and guide the folding (or refolding (4)) of otherwise misfolded and misrouted mutants of receptors and enzymes (2). Rescued mutants pass the scrutiny of the cellular quality control system and are routed to their correct intracellular locus where they function appropriately. This approach is effective since the cellular quality control system is non-specific, recognizing motifs such as exposed hydrophobic surfaces, unpaired Cys bonds and other general indicators of misfolding. The assessment by the quality control system addresses structural not functional failures, so misfolded proteins are sequestered even if they retain function. Accordingly, mutant enzymes, receptors and ion channels can be rescued and restored to function (1, 2).

The WT V2R is a GPCR that is coupled to both cAMP and IP production and normally resides in the plasma membrane of the distal convoluted tubule and collecting ducts of the kidney. In response to arginine vasopressin, diuresis is regulated. When this function is lost, nephrogenic diabetes insipidus results (5, 6).

There are a large number of disease-causing mutants of the V2R (4) that do not properly traffic to the plasma membrane, frequently being retained in the endoplasmic reticulum; here they cannot perform their biological function of transducing a signal from vasopressin to the G-protein. Pharmacoperone drugs can rescue the V2R mutants and restore function. For the V2R and other GPCRs (7, 8), drugs that rescue one mutant frequently rescue most mutants of an individual protein and this suggests that drugs may be identified that are generally effective yet retain target specificity.

One problem in reducing the approach to identify novel pharmacoperones to practice is that peptidomimetic antagonists were initially selected by many laboratories, including our own, for demonstration of pharmacoperone activity because these were small, hydrophobic molecules that bound to the V2R with high specificity and did not evoke agonism. While effective as pharmacoperones, drugs with both antagonist and pharmacoperone activity present a complex pharmacology *in vivo* since they need to be removed after rescue is effected so that the agonist can occupy the active site. Accordingly, determination of whether pharmacoperone activity and antagonism can be uncoupled is an important consideration for the development of therapeutic activity. Accordingly, we evaluated hits from a previous large HTS campaign with a view toward answering this question.

MATERIALS AND METHODS

General

SR121463B, a V2R peptidomimetic antagonist used in the current study as a known pharmacoperone drug, was generously provided by Dr. Claudine Serradeil at Sanofi-Aventis and used as received. Test compounds used in this study were prepared at the screening facility and stored as 10 mM DMSO stock solutions at −20 °C in sealed polypropylene plates and these stocks were also used for the orthologous assay. 3-Isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, St. Louis, MO), vasopressin (Tocris Biosciences, Bristol, England UK) and fetal bovine serum (FBS, Hyclone, Logan, UT) were obtained as

indicated. The V2 receptor antagonist, d(CH_2)5[D-Ile(2),Ile(4),Tyr-NH_2(9)]AVP was a kind gift of Maurice Manning (9, 10) and was radiolabeled using Pierce Pre-Coated Iodination Tubes (Thermo Fisher Scientific, Waltham, MA) and 125-Iodine (NEZ033L; PerkinElmer, Waltham, MA), DMEM, PBS (GIBCO, Invitrogen). pTRE2-Hygromycin vector (Invitrogen, San Diego, CA), myo-[2-^{3}H(N)]-inositol (NET-114A; PerkinElmer, Waltham, MA) were obtained as indicated.

Creation of Stable (tTA + Mutant V2 Receptors) HeLa Cells

The stable HeLa (tTA; tetracycline-controlled transactivator) cell line was a kind gift from Peter Seeburg (11). The cells were maintained in growth medium (DMEM/10% FBS/20 μg/ml gentamicin) and grown at 37°C, 5% CO_2 in a humidified atmosphere until the density reached about 90%.

The human WT V2R and the mutant L83Q were cloned into pTRE2-Hygromycin vector (the response vector) and then transfected into the stable HeLa (tTA; tTA binds the TRE and activates transcription in the absence of doxycycline) cell line. Selection antibiotics were used at 400 μg/ml G418 + 200 μg/ml hygromycin. Single colonies were selected and screened for expression of the WT V2R and mutant L83Q receptors in separate, stably transfected cells. Immunofluorescence has been used to show that mutant L83Q is rescued and restored to the plasma membrane by SR121463B (7). In addition, rescue results in access of the receptor mutant to the ligand and resultant biological function (present study).

uHTS Optimized Primary V2R Pharmacoperone Assay (“the HTS assay”)

An HTS campaign was completed (12) and identified potential pharmacoperones from a chemical library of approximately 645,000 structures. The assay relies on HeLa cells stably expressing mutant L83Q of the hV2R under the control of a tetracycline-controlled transactivator. A robotic system enables identification of structures that rescue the mutant, enabling it to traffic to the plasma membrane where it can bind the native agonist and couple to G-proteins. Such structures are then re-assayed in the presence of doxycycline, which shuts off the gene expressing the mutant and serves as a negative control, identifying false positive structures. SR121463B is a known pharmacoperone and antagonist of the V2R and is used as an internal control. 147 active structures were identified.

Pharmacoperone Evaluation (“the orthologous assay”)

For the present studies a different orthologous assay was used to confirm the hits in the uHTS assay (12) and to compare pharmacoperone activity to agonism, antagonism and radioligand binding. This orthologous assay depends on radioimmunoassay of cyclic AMP produced. This orthologous assay was methodologically different than the uHTS screening procedure since that other assay had been optimized for screening procedures. The HTS, for example, does not include washing of cells to accommodate the needs of the screen. Ninety-six active compounds, representative of the 147 hits, were obtained and used for further evaluation. The chemical structures are shown in supplementary data.

Briefly, for the orthologous assay used in the present work, ten thousand cells of the stable HeLa line (containing tTA + L83Q) were plated per well in 96-well plates. The cells were

cultured in the presence or absence of 1 μg/ml doxycycline during all phases of the experiment. Fifty-four hours after plating, the cells were washed twice with DBG (DBG: DMEM/ 0.1% BSA/ 20 μg/ml gentamicin) then the 96 structures, prepared in DBG plus 1% DMSO were added to the wells in triplicate and allowed to incubate for 16 h at 37 C. After 16 h, the cells were washed 3 times with DBG + 1% DMSO to wash out the structures. As part of the washing, the cells were incubated for 10 min at 37 C twice, then once for 20 min at 37 C. The cells were then stimulated with 1 μM vasopressin in DBG containing 0.2 mM IBMX for 30 min at 37 C. After stimulation, the medium from each well was collected in 96-well plates containing 10 mM theophylline (final 1 mM). The samples were heated at 99 C for 5 min and cAMP was determined by RIA (13).

Antagonist Evaluation

Antagonism was evaluated in the WT cell line. Ten thousand cells of the stable HeLa line (containing tTA + hV2R WT) were plated per well in 96-well plates. Seventy hours after plating, the cells were washed twice with DBG. The cells were stimulated with 5 nM vasopressin (approximately ED50) containing the 96 structures (10 μM in DMSO), DMSO or the known antagonist control structure SR121463B, prepared in DBG + 0.2 mM IBMX for 30 minutes at 37 C. Cyclic AMP release was measured by RIA (13). The antagonism by 10 μM SR121643B or to carrier (DMSO) alone (each in the presence of 5 nM vasopressin) is described in the Results. The Ki for SR121643B for WT V2R is 3.4 ± 1.9 nM (14).

Agonist Evaluation

Agonism was evaluated in the same cell line. Ten thousand cells of the stable HeLa line (containing tTA + WT hV2R) were plated per well in 96-well plates. Seventy hours after plating, the cells were washed twice with DBG. The cells were stimulated with 1 μM vasopressin (positive control), DMSO or each of the 96 structures (10 μM in DMSO), and the control structure SR121463B, prepared in DBG + 0.2 mM IBMX for 30 minutes at 37 C. Cyclic AMP release was measured by RIA (13). The response to 10 μM SR121643B or to carrier (DMSO) alone is described in the Results

Inositol phosphate (IP) assays

The L83Q mutant and hV2R WT stable cell lines were plated at 20,000 cells per well. Fifty four hours after plating, DMSO or SR121463B was added to the cells in quadruplicate and allowed to incubate for 18 h and “preloaded” with 4 μCi/ml myo-[2-3H(N)]-inositol in inositol-free DMEM. After the “preload”, the cells were washed for 10 min at 37 C twice then once for 20 min at 37 C with DMEM (inositol free) containing 5 mM LiCl plus 1% DMSO and treated for 2 h with media alone or 1μM vasopressin in the same medium. Total IP was then determined as previously described (15).

Binding assay

Human V2R WT cells were cultured and plated in growth medium as described above, except 30,000 cells in growth medium were added to 24-well Costar cell culture plates. Seventy hours after plating, the cells were washed twice with DMEM/ 0.1% BSA/ 10 mM HEPES, then 8×10^6 cpm/ml of [^{125}I-Tyr]-AVP [d(CH2)5[D-Ile2, Val4, Tyr-$NH_2$9], was

added in the same medium in the presence of 100 μM of each structure and allowed to incubate at room temperature for 90 min, consonant with maximum binding (9, 10). New receptor synthesis during this period is negligible during this period and at room temperature (9, 10, 16). After 90 min, the media was removed and radioactivity was measured as previously described. To determine nonspecific binding, the same concentration of radioligand was added to cells in the presence of 5 μM unlabeled vasopressin. This is greater than 1,000 fold in excess of the mass of the tracer and sufficient to decreased binding of tracer with vasopressin to background radioactivity. Buserelin (5 μM), an irrelevant peptide, SR121463B (100 μM) and vasopressin (5 μM) were used as controls.

Results

In the present study, we evaluated pharmacoperone activity, agonism and antagonism of hits that were generated from a previous large scale high throughput campaign for pharmacoperone drugs (12) that rescue mutants of the V2 receptor and restore them to function. In the present work, the determination of pharmacoperone activity relied on an orthologous assay that did not have the constraints required for HTS (e.g. washing out drugs) and determined cAMP by an RIA. Binding of radioligand of the rescued receptors was also evaluated. First, we assessed the coupling to Gs (cyclic AMP production) of the WT V2R and the L83Q mutant that had been rescued (or not) by SR121463B. The data indicate (figure 1) that, while WT receptor couples to both Gs and Gq/11, the rescued mutant couples only to Gs. This observation was initially surprising but suggests that, once rescued, this GPCR mutant reestablishes its original conformation which couple to Gs but does not couple to Gq/11. We believe this G-protein bias is a function of the mutation itself. Because the mutant is misrouted, the bias is not until rescue occurs, of course. Before rescue, the mutant is not accessible to the agonist and so rescue is needed to observe this effect of the mutation. There are several observations that support the view that the bias observed with the rescued mutants is due to the mutation itself and not the effect of the rescue with a pharmacoperone drug: First, treatment of cells expressing the WT, when treated with the SR pharmacoperone, does not change the G-protein to which WT is coupled. Thus, the pharmacoperone drug itself does not evoke bias. Second, the position of the mutation (see red colored amino acid in figure 2) could influence the positioning of an intracellular loop that has been associated with alterations in G-protein coupling (17–19). Third, in the case of another rescued mutation GnRHR[E90K], the rescue of the receptor reveals constitutive activity that could not be seen in the absence of rescue (20). This observation also presents the possibility of an additional use of particular pharmacoperone drugs, such a SR121463B. Specifically, it may be possible to rescue particular V2R mutants so that only coupling to a single G-protein occurs, in essence, producing a fully biased receptor mutant for experimental purposes. While this may be problematic in restoring function in vivo (particular functions may require coupling to both Gs and Gq/11), this is not likely to be expected with all mutants since most mutations do not create receptor bias for GPCRs. Because of this observation, we used measurements of cAMP as a measure of mutant receptor activation.

At the completion of screening a library of 644,951 structures including concentration response analysis, 147 were identified as both active (in the absence of doxycycline) and

selective (based on loss of activity in the presence of doxycycline) in the uHTS (12). Of these, 96 were also active and selective in the orthologous assay for pharmacoperone activity (see Table 1 and supplemental data), using evaluation at a fixed concentration of 10 μM (figure 3), a concentration selected from the concentration-response curves. The structures of these 96 follow-up compounds are shown in the supplemental data. When doxycycline was included in the negative screen, the activity values associated with the 96 compound structures all scored at or around a line of no activity.

From those 96, 14 structures and SR121463B were selected for a full concentration-response curve to measure pharmacoperone activity; this curve ranged from 0.1 – 100 μM. These structures were selected to be representative of all the chemical classes in the 96 structures. A bimodal pattern was common (figure 4), frequently producing optimal activity at 10 μM. This optimal dose was used in subsequent studies. In the case of structure number 50, 1 μM provided the best activity. The bimodal pattern appears to reflect toxicity of drugs at high doses that can be observed visually as crenation.

The 96 follow-up compounds (structures in the supplemental data) were evaluated at a concentration of 10 μM for agonist activity using WT V2R stably expressed in HeLa cells. This assay can distinguish 0.1 nM vasopressin from background and has an EC_{50} of 5 nM vasopressin using cAMP as an endpoint. None of the structures evaluated showed measurable agonist activity in this assay (figure 5).

Binding was assessed for these structures using a metabolically stable, iodinatable V2R antagonist (figure 6). A surprising finding was that some of the structures produced levels of binding that exceeded binding in the presence of dimethylsulfoxide only (no competition). This observation suggested that these structures may serve as inverse agonists of the V2R and a pre-existing receptor dimer or oligomer, which is consistent with earlier findings (21–23). Alternatively, the data may suggest an allosteric effect of these molecules. Distinguishing between these possibilities is outside the scope of this study, which focuses on identifying non-antagonist pharmacoperones.

Antagonism to WT was assessed in the presence of 5 nM vasopressin (figure 7). The structures showed a range of antagonism. Correlation of antagonism (from figure 7) and pharmacoperone activity (from figure 3) is shown (figure 8 and Table 1). There were strong and weak antagonists which showed comparable levels of pharmacoperone activity, suggesting that pharmacoperone and antagonist activity are not coupled (statistics in figure legends).

Discussion

Mutation of the V2 receptor is the underlying cause of some forms of nephrogenic diabetes insipidus since these mutants do not traffic to the plasma membrane and result in a cell that is refractory to endogenous vasopressin. In the prior screen, a library of 644,951 chemical structures were interrogated by measuring rescue (G-protein coupling) of a L83Q regulated by a TET-off transactivator in HeLa cells stably expressing this mutant. Absent rescue, the mutant is retained in the endoplasmic reticulum where it is unable to bind ligand or couple

to its cognate G-protein (7). When rescued, immunofluorescence shows that the mutant migrates to the plasma membrane (7) and can couple to Gs (11). When the assay is conducted in the presence of doxycycline, the gene for the mutant is not expressed and pharmacoperones that act by refolding the mutant are without effect. This provides a means of measuring false positives in the HTS and orthologous assays.

In the prior HTS, structures were evaluated for their ability to rescue this mutant (pharmacoperone activity) by means of the cAMP-Glo Max, which measures cAMP accumulation of this Gs coupled receptor mutant in response to vasopressin.

Structures which rescue the mutant were re-evaluated in the presence of doxycycline, a treatment which extinguishes the mutant gene by way of the TET-off transactivator and, accordingly identifies false positives. 147 active structures which enabled the mutant to couple to Gs and generate cAMP in the absence but not presence of doxycycline were identified (12).

In the present work, selected “hits” were assessed in an orthologous assay which measured cAMP accumulation by RIA. This assay, described in Methods, differs in several important ways from the HTS assay, notably by including washes of cell treatments that cannot be accommodated in the HTS protocol.

Ninety-six structures that were positive in both the cAMP-Glo Max (HTS) and RIA-based (orthologous) assays and represented all chemical classes of hits from the HTS assay, were subjected to evaluation for agonism, antagonism and pharmacoperone activity. Rescued V2 receptor mutants were evaluated for radioligand binding. None of these structures showed agonism in a cell-based assay and antagonistic activity did not correlate with pharmacoperone activity. This latter observation is important since most prior demonstrations of pharmacoperone rescue were performed with drugs that were known to be peptidomimetic antagonists. These were selected because they were known to interact with mutants with high specificity, not evoke an agonistic response and were readily available. The question of the existence of non-antagonist pharmacoperone drugs has not been addressed and the early selection of antagonists for pharmacoperone active drugs has caused some to believe that the two activities are inextricably linked. The present study suggests otherwise.

The observation that pharmacoperone drugs that are not also antagonists (or agonists) of the V2R can be identified is important in the effort to exploit this class of drugs, since drugs with both activities present a complex pharmacology. In the case of drugs with pharmacoperone and antagonist activities, the mutant must first be rescued then washed out so that endogenous agonist can bind. The drug must be given in a discrete pulse, rather than simply elevated over time, making oral administration difficult or impossible. If a pharmacoperone can be identified without antagonistic activity, the drug need not be washed out and this opens the door to oral administration. Additionally, it appears that pharmacoperones that rescue one mutant of a particular protein, rescue many (7, 8), suggesting that these agents stabilize one or more regions that are needed in a particular conformation to pass the scrutiny of the cellular quality control system. The presence of

more than a single binding site that impacts on a GPCR is not novel (24), but we believe that the present study is the first to identify small molecules that impact receptor trafficking without competing for binding at the native ligand binding site.

Radioligand assays of the rescued receptors suggested that some of these hits appeared to be inverse agonists of a precoupled receptor oligomer or allosteric modulation (25).

This study suggests that allosteric ligand binding sites (i.e. sites that do not produce antagonism or agonism) exist on GPCRs that produce conformational changes. We (26) and other laboratories (27–30) have similar findings. In fact, there are data to show that the entire surface of the GPCR can be considered a potential binding site for ligands, since conformational changes occur (31–34). In addition, allosteric modulators for family A peptide receptors are very often drug-like non–peptides (31–34) consonant with the observed function of pharmacoperones as well. It has been shown and is predicted by molecular dynamics that binding is not a passive process and binding to a receptor will almost certainly change the ensemble thermodynamics (i.e. conformations) with the exception of the unlikely possibility that the ligand has identical affinities for all the members of the ensemble (35). This view is also supported by the data in this study. Luttrel and colleagues report, using beta-arrestin signaling, conformational differences that code for trafficking of the receptor once internalized. There is bias even as to whether the arrestin-bound receptor will internalize, whether it will signal, and if internalized, where it will go (i.e. become misrouted, destroyed or recycled) (36).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The present study is the first to systematically identify small molecules that impact receptor trafficking without competing for binding at the native ligand binding site.
- Antagonist and agonist activities can be uncoupled from pharmacoperone activity, enhancing the therapeutic possibilities of these drugs.
- The data may suggest that some pharmacoperones can rescue proteins acting by allosteric interactions.
- Pharmacoperone drugs can be identified that are not agonists or antagonists.

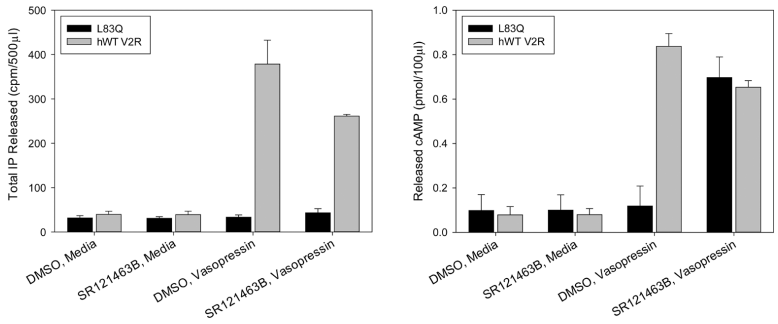


Figure 1. WT V2R is coupled to Gs and Gq/11 but (pharmacoperone-rescued) mutant L83Q is only coupled to Gs

Stably transfected HeLa cells containing WT V2R or the mutant L83Q were used to compare Gq/11 (IP) or Gs (cAMP) coupling when the SR121643B pharmacoperone structure was used at 1 µM. When cells were stimulated, 1 µM vasopressin was used. At least 3 independent experiments were performed in replicates of 3. SEMs show the variance between experiments.

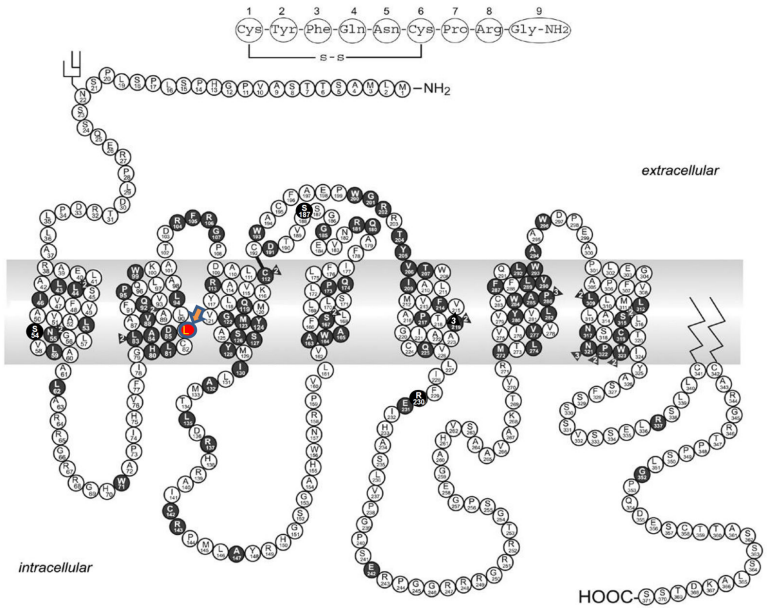


Figure 2.
Structure of the vasopressin 2 receptor (and vasopressin), showing the site of the mutation, L83Q, in red.

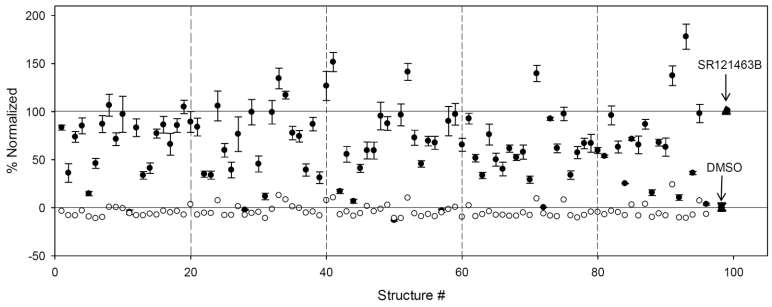


Figure 3. Pharmacoperone activity in 96 structures that are active in the uHTS and orthologous assay

Stably transfected HeLa cells containing the mutant L83Q were used to show pharmacoperone activity at 10 μM with and without 1μg/ml doxycycline and measuring cAMP release. L83Q is in a tetracycline dependent vector. When tetracycline or doxycycline are added to the cells, the L83Q gene is turned off. The data from three experiments were normalized by subtracting the basal cAMP response (DMSO, 0%, horizontal line) from the cAMP response from each drug and dividing this value by subtracting the basal cAMP response from that obtained from SR121463B cAMP treatment (100%, horizontal line). The result was expressed as a percentage, e.g., {[(cAMP response for each drug) − (basal cAMP)] / [(cAMP response with SR121463B) − (basal cAMP)]} × 100%. At least 3 independent experiments were performed in replicates of 3. The filled circles received no doxycycline, the open circles received 1 μg/ml doxycycline. DMSO only (carrier, the hourglass symbol) and SR121463B (upward pointing triangle) were present as indicated. In some cases the error bars are smaller than the symbols. A high number indicates high pharmacoperone activity.

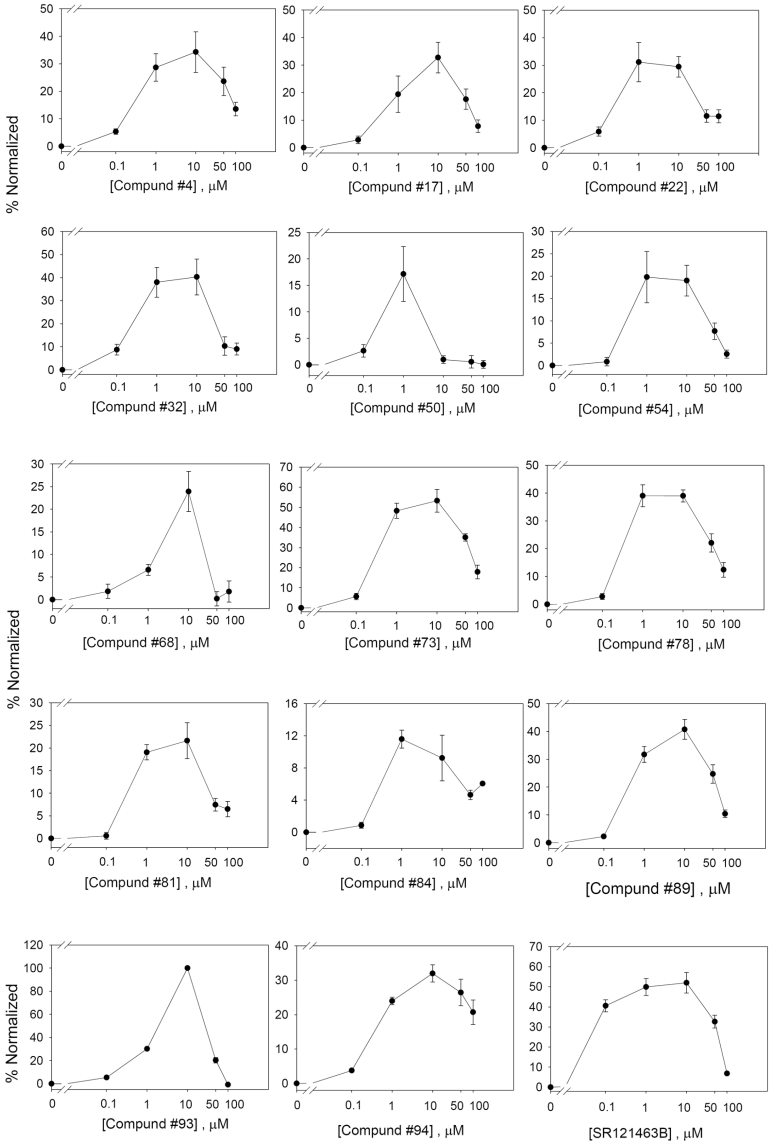


Figure 4. Full concentration response curves for 14 selected pharmacoperone drugs representing the range of structures evaluation and SR121463B, a known pharmacoperone-antagonist

Stably transfected HeLa cells containing the V2 mutant hL83Q were used to show pharmacoperone concentration response curves. Cyclic AMP release was measured. The data were normalized using the same method as described above for figure 3. At least 3 independent experiments were performed in replicates of 3. SEMs show the variance between experiments. A high number indicates high pharmacoperone activity.

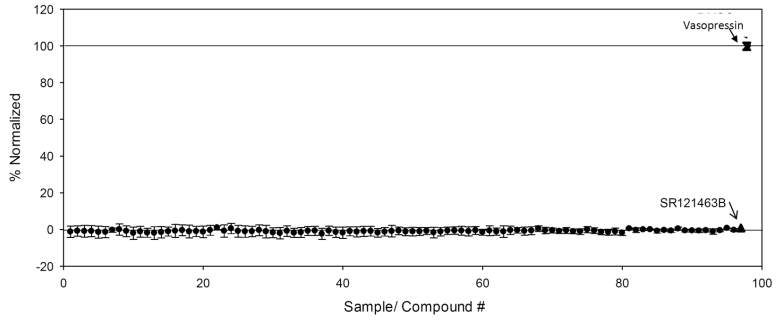


Figure 5. Agonist activity was evaluated for each of the 96 structures

Stably transfected HeLa cells containing hWT V2R were used to check the agonist activity of the chemical structures at 10 μM. Cyclic AMP release was measured. The data were normalized by subtracting the basal cAMP response obtained in the presence of antagonist (SR121463B, 0% response, horizontal line) from the cAMP response obtained from the presence of each structure and dividing that value by the cAMP response obtained in the presence of vasopressin- only (100% response, horizontal line) after subtracting basal cAMP. See equation in figure 3. The result was expressed as percentage. At least 3 independent experiments were performed in replicates of 3. Vasopressin only (1 μM, a positive control for agonism, the hourglass symbol) and SR121463B (upward pointing triangle) were present as indicated. In some cases the error bars are smaller than the symbols.

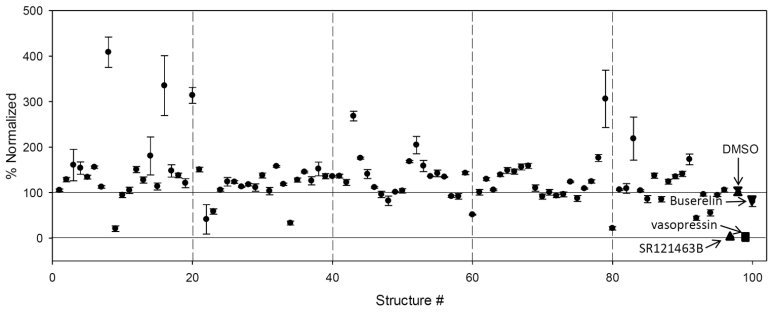


Figure 6. Binding displacement for each of the 96 structures

Stably transfected HeLa cells containing hWT V2R were used to check binding / displacement of each structure (100 μM) incubated with an iodinated V2R antagonist. The data were normalized from three experiments by subtracting the non-specific binding (5 μM non-iodinated vasopressin, 0% binding, horizontal line) from the binding in the presence of each drug binding and then dividing by the binding result of radioligand in DMSO (100% binding, horizontal line). Data are expressed as percentages. At least 3 independent experiments were performed in replicates of 3. 1% DMSO only (carrier control, the hourglass symbol), Buserelin (5 μM, an irrelevant peptide agonist of the gonadotropin releasing hormone receptor, downward triangle), vasopressin (5 μM, filled square) and SR121463B (100 μM, upward pointing triangle) were present as indicated. In some cases the error bars are smaller than the symbols. A high number indicates a high amount of radioligand binding.

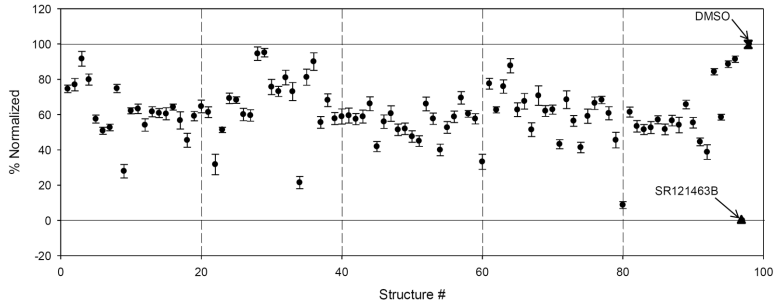


Figure 7. Stably transfected HeLa cells containing hWT V2R were used to evaluate the antagonist activity of each structure (10 μM) in the presence of 5nM vasopressin

The data were normalized from four experiments by subtracting the basal cAMP response (in the presence of antagonist SR121463B alone, 0%, horizontal line) from the cAMP response to each drug in the presence of 5 nM vasopressin and dividing that number by the response in the presence of vasopressin alone less basal. The horizontal line (100%) shows cAMP (less basal) to vasopressin alone, without competing peptide. The data are expressed as percentages. At least 4 independent experiments were performed in replicates of 3. DMSO only (carrier, the hourglass symbol) and SR121463B (upward pointing triangle) were present as indicated. A high number means low antagonism activity.

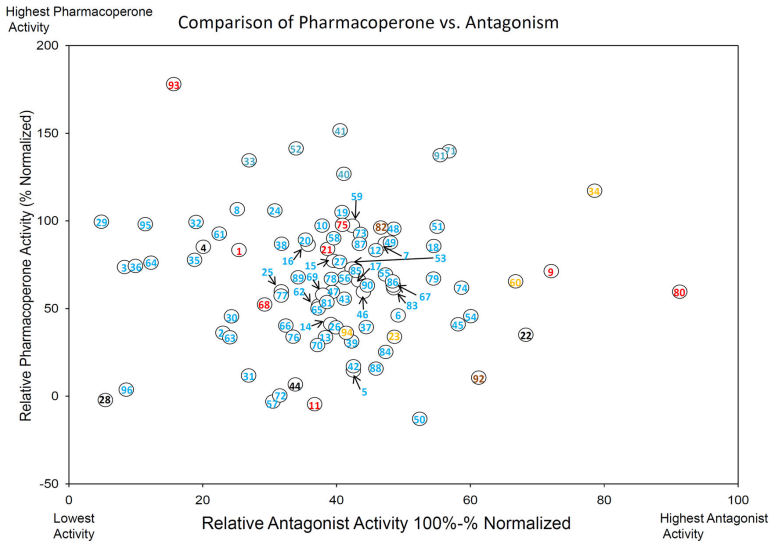
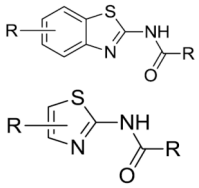
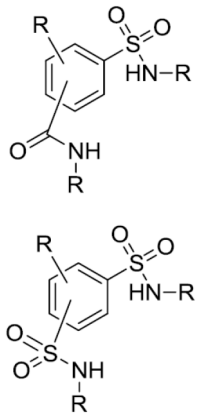
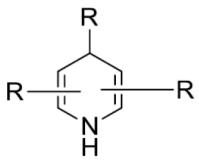
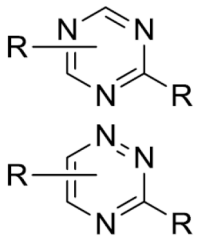
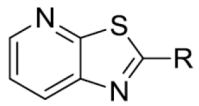
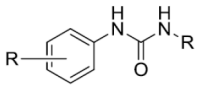
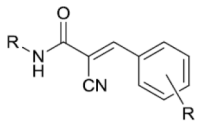
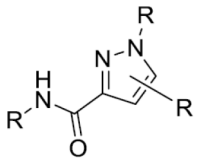


Figure 8. Comparison of relative pharmacoperone activity versus relative antagonist activity

The normalized pharmacoperone activity was plotted against the normalized antagonist activity to determine the relation between pharmacoperone and antagonist activity in the 96 structures. In this graph, the normalized value for antagonism was subtracted from 100% so that high numbers are both high pharmacoperone and high antagonism. Measured antagonism is not predictive of pharmacoperone activity. We calculated the Pearson product-moment correlation coefficient ($r = -0.013$), which informed us of a 0.016% common variance between antagonist and pharmacoperone activities. Accordingly, the correlation can be considered negligible, almost null, meaning that the antagonist activity of these drugs cannot systematically predict their pharmacoperone activity, since each one has a different behavior.

Table 1

#	name(s)	gen structure(s)	compound numbers (bold = pharmacoperone, italics = antagonist
1	aminobenzothia-zole amides, aminothiazole amides		2,3,5,6,7,**8**,10,12,13,14,15,16,17,*18*,**19**,20,**24**,25,26, 27,29,30,31,32,**33**,35,36,37,38,39,**40**,**41**,42,43,*45*,46, 47,48,49,*50*,*51*,**52**,53,*54*,55,56,57,58,59,61,62,63,64, 65,66,67,69,70,***71***,72,73,*74*,76,77,78,*79*,81,83,84,85, 86,87,88, 89,90,***91***,95,96 (color blue in the figure)
2	amide sulfonamides (meta and para) and bissulfonamides		1,*9*,11,21,68,75,*80*,**93** (note: compounds 1, 11, and 75 also have the structural features of class 1 compounds) (color red in the figure)
3	dihydropyridines		23,*34*,*60*,94 (color orange in the figure)
4	triazines		82,*92* (color brown in the figure)
5	azabenzothiazole		4 (color black in the figure)
	acyl urea		44 (color black in the figure)

#	name(s)	gen structure(s)	compound numbers (bold = pharmacoperone, italics = antagonist
	cyanoacrylamide		28 (color black in the figure)
	pyrazole amide		*22* (color black in the figure)

Of the 96 hits analyzed, over 80% (78 compounds) are in the two closely related structural series termed the “aminothiazole amides” and the “aminobenzothiazole amides”. This class of molecules show a wide diversity of activity. Several are moderately strong antagonists (>50% relative antagonist activity) without strong pharmacoperone activity: compounds 9, 18, 22, 34, 45, 50, 51, 54, 60, 74, 79, 80, and 92. Others are strong pharmacoperones (>100% relative activity) with weak antagonist activity: compounds 8, 19, 24, 33, 40, 41, 52, and 93. Two compounds were both strong pharmacoperones (>100% relative activity) and moderately strong antagonists (>50% relative antagonist activity): compounds 71 and 91. The remaining large majority of the compounds in these series tended to be weak-to-moderate pharmacoperones and weak-to-moderate antagonists.

The second most-represented class of compounds have phenyl rings substituted with *meta* or *para* amide and/or sulfonamide groups. These include two of the stronger antagonists: compounds 9 and 80; as well as a strong pharmacoperone: compound 93. The third class is the dihydropyridines, which includes two of the stronger antagonists: compounds 34 and 60.

The fourth class is the triazines, which includes one of the stronger antagonists: compound 92.

The remainder of the hits are “singleton” structures with only one hit in each structure type, thus lumped together as class 5. These include an azabenzothiazole, an acyl urea, a cyanoacrylamide, and a pyrazole amide, the latter of which is one of the stronger antagonists: compound 22.