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Selective agonists and antagonists of formylpeptide receptors: duplex flow cytometry and mixture-based positional scanning libraries

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Selective agonists and antagonists of formylpeptide receptors

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List of abbreviations: FPR1, formylpeptide receptor; FPR2, formylpeptide-like 1 receptor 1;
HTS, high throughput screening; SAR, structure-activity relationship.

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Abstract

The formylpeptide receptor (FPR1) and formylpeptide-like 1 receptor (FPR2) are G protein coupled receptors that are linked to acute inflammatory responses, malignant glioma stem cell metastasis and chronic inflammation. While several N-formyl peptides are known to bind to these receptors, more selective small molecule high-affinity ligands are needed for a better understanding of the physiological roles played by these receptors. High throughput assays utilizing mixture-based combinatorial libraries represent a unique, highly efficient approach for rapid data acquisition and ligand identification. We report the superiority of this approach in the context of the simultaneous screening of a diverse set of mixture-based small molecule libraries. We used a single cross-reactive peptide ligand for a duplex flow cytometric screen of FPR1 and FPR2 in color-coded cell lines. Upon screening 37 different mixture-based combinatorial libraries totaling more than 5 million small molecules (contained in 5,261 mixture samples), seven libraries significantly inhibited activity at the receptors. Using positional scanning deconvolution, selective high affinity (low nM K_i) individual compounds were identified from two separate libraries, namely pyrrolidine bis-diketopiperazine and polyphenyl urea. The most active individual compounds were characterized for their functional activities as agonists or antagonists with the most potent FPR1 agonist and FPR2 antagonist identified to date with an EC_{50} of 131 nM (4 nM K_i) and IC_{50} of 81 nM (1 nM K_i), respectively, in intracellular Ca^{2+} response determinations. Comparative analyses of other previous screening approaches clearly illustrate the efficiency of identifying receptor selective, individual compounds from mixture-based combinatorial libraries.

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Introduction

The search for novel ligands with high affinities to newly identified/poorly understood receptors remains one of the fundamental aims of biomedical research. Further understanding a receptor's structure, function and biological relevance in certain diseases and disorders can be accelerated with the identification of high affinity ligands. Synthetic combinatorial methods have been in use for the last 20 years and have fundamentally advanced the ability to synthesize and screen large numbers of compounds. One of the earliest methods described, mixture-based libraries combined with positional scanning deconvolution is the approach that enables the most rapid and economically efficient acquisition of chemical and biological information (Dooley and Houghten, 1993;Houghten, et al., 1999;Houghten, et al., 2008;Pinilla, et al., 1992). Recently, mathematical modeling (Santos, et al., 2011) has developed a theoretical foundation for the continued success of mixture-based combinatorial libraries; the Harmonic Mean model, combined with the multiple structural analogs present in mixture-based combinatorial libraries, explains how mixtures with only a fraction of a percentage of active compounds may still be differentiated in an assay of typical resolution. Additionally, it has been demonstrated that the activity of positional scanning mixtures is robust with respect to the approximate equimolarity of their constituent compounds (Giulianotti, et al., 2012).

The ability to identify specific functionalities responsible for driving the activity at each variable position of a chemical scaffold or pharmacophore is one of the strengths of mixture-based libraries. Another advantage of mixture-based libraries resides in the very high densities of compounds that can be synthesized in specific regions of chemical space (Medina-Franco, et al., 2008;Medina-Franco, et al., 2009) to quickly identify 'activity cliffs' which are defined as chemical compounds with high structural similarity but unexpectedly different biological activity (Maggiora, 2006;Medina-Franco, et al., 2009). Additionally, these libraries expand the traditional

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relevant medicinal chemistry space typically covered by current commercial screening collections as well as the Molecular Library Small Molecular Repository (MLSMR) (Lopez-Vallejo, et al., 2012; Singh, et al., 2009). When compared to existing high throughput screening (HTS) programs, in which hundreds of thousands of individual compounds are screened against therapeutically important targets, millions of compounds formatted as mixtures can be examined using substantially less material and at much reduced time and labor than if these same mixture-based diversities were made and screened as individual compounds (Houghten, et al., 1999; Houghten, et al., 2008; Pinilla, et al., 2003).

Formyl peptide receptors (FPRs) are a small family of G protein-coupled receptors known to be important in host defense and inflammation, and studies have been carried out to identify small molecule ligands in order to characterize the structure and function of these receptors (Kirpotina, et al., 2010; Ye, et al., 2009). The two receptors of the FPR family that are studied here are FPR1, which is linked to antibacterial inflammation (Le, et al., 2002) and malignant glioma cell metastasis (Zhou, et al., 2005), and FPR2 (formerly known as FPRL-1), which is linked to chronic inflammation in systemic amyloidosis, Alzheimer's disease, and prion diseases (Le, et al., 2001). These two receptors were originally shown to be primarily expressed in myeloid cells, with varying distribution among myeloid cell subsets and a wide range of tissues (Riviere, et al., 2009; Ye, et al., 2009). There have been numerous studies to identify naturally occurring and more potent small molecule agonists or antagonists for each of these receptors, reviewed in (Dufton and Perretti, 2010; Ye, et al., 2009).

We have previously reported the use of a fluorescent ligand competition assay combined with high-throughput flow cytometry to identify a series of novel small molecule ligands for FPR1 and FPR2 (Edwards, et al., 2005; Young, et al., 2005; Young, et al., 2009). The most potent ligands identified were 3570-0208 and BB-V-115, with ligand binding inhibition constants (K_i) of 95 nM and 270 nM for FPR1 and FPR2, respectively. Each was a selective antagonist of the

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intracellular Ca^{2+} response mediated by its target receptor with an IC_{50} value of 430 nM for FPR1 (3570-0208) and 940 nM for FPR2 (BB-V-115). Herein, we report the screening and deconvolution of a collection of TPIMS libraries using a HTS flow cytometry duplex receptor assay to identify novel FPR ligands. The same flow cytometric technology used for HTS is compatible for the quantitative analysis of ligand affinity. Using positional scanning deconvolution methods, FPR1 and FPR2 selective ligands with nanomolar affinities were identified from mixtures containing from thousands to tens of thousands of compounds. Importantly, this was accomplished by screening more than 5 million compounds within only 5,261 samples, an extremely efficient and cost effective process in comparison to traditional HTS approaches that typically evaluate hundreds of thousands of individual compounds. Herein we report the most potent FPR1 agonist and FPR2 antagonist identified to date. The newly identified compounds are structurally distinct from all currently described FPR ligands, illustrating the ability of the TPIMS libraries to explore novel regions of chemical space and to identify selective, high affinity compounds.

Materials and Methods

Preparation of mixture-based libraries and individual compounds. Employing solid-phase chemistry approaches, mixture-based libraries were synthesized from resin-bound amino acids, peptides, and peptidomimetics as starting materials using the simultaneous multiple synthesis and “libraries from libraries” approaches as previously described (Houghten, 1985;Houghten, et al., 1991;Houghten, et al., 1999;Nefzi, et al., 2004;Ostresh, et al., 1994a). Extensive optimization of each reaction condition was necessary for mixture library generation. The isokinetic ratios necessary for equimolar incorporation of amino acids (Ostresh, et al., 1994b) and carboxylic acids (Acharya, et al., 2002) as well as other reagents have previously been determined. Synthetic controls are prepared systematically to determine a wide range of building blocks to be incorporated into varied reactive positions of a given central scaffold

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molecule. Supplemental Table 1 contains information on 37 small molecule libraries screened against FPR1 and FPR2. Three peptide libraries (totaling 26 million compounds; libraries 6, 7, and 33 in Supplemental Table 1) were screened, but were excluded in the deconvolution or analysis of the screening efficiency (Table 1). In most cases, libraries were dissolved in DMF at 10 mg/ml and diluted in water for final testing concentrations. The synthesis of library 1344 (including 1343, 1345, 1346 and 1347) has been previously described (Reilley, et al., 2010). In brief, library 1344 was synthesized using the “libraries from libraries” approach (Nefzi, et al., 2004; Ostresh, et al., 1994a) starting with resin-bound N-acylated peptides (Hensler, et al., 2006) that were subsequently exhaustively reduced with borane-THF. No loss of chirality was found in either the reduction or subsequent steps. The resin bound polyamine was then treated with oxyalyldiimidazole to form the resin bound pyrrolidine bis-diketopiperazine before cleavage from the resin. The library was synthesized in a positional scanning format with four positions of diversity (Supplemental Table 2). Each separate sublibrary (R_1 - R_4) was synthesized representing the same diversity and differing solely by the location of the defined position, allowing for library screening and deconvolution essentially as described (Pinilla, et al., 1992; Pinilla, et al., 2003). The synthesis of library 1481 has been described elsewhere (Nefzi, et al., 2000; Schimmer, et al., 2004). In brief, library 1481 was synthesized starting with resin-bound N-acylated dipeptides that were exhaustively reduced with borane-THF followed by treatment with phenylisocyanate to afford the resin-bound polyphenyl ureas, which were subsequently removed from the solid support. Library 1481 was synthesized in a positional scanning format with three positions of diversity. The R_1 and R_2 positions were prepared with the same 48 functionalities and R_3 was prepared with 39 functionalities ($48 \times 48 \times 39 = 89,856$, see Supplemental Table 2). Each separate sublibrary (R_1 - R_3) was synthesized representing the same diversity and differing solely by the location of the defined position, allowing for library screening and deconvolution essentially as described (Pinilla, et al., 1992; Pinilla, et al., 2003). Individual compounds derived from libraries 1344 and 1481 were synthesized by the same

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solid-phase methods used for the libraries and analyzed by LCMS to confirm identity and purity. The individual compounds shown in Table 2 were all purified by RP-HPLC and had a final purity of >99% as determined by integration of the absorbance at 254 nm. (The LCMS data for these compounds can be found in the Supplemental Figure 1 and Supplemental Table 3). Additional analytical information based on LCMS for all 106 TPI compounds can be found in Datasets S1 and S2. It should be noted that for the compounds that contain a phenyl group in the R₃ position (such as Table 2 compound a: 1754-113) the final product is close to 50:50 racemic mixture of the *S,R*-phenyl.

Flow cytometry receptor binding assay. The assay was performed in a “duplex” format in which U937/FPR1 cells were tested together with RBL/FPR2 cells as previously described (Young, et al., 2009)(PubChem Summary AIDs 805 and 1202). The FPR1-expressing cells were stained with a red-fluorescent dye, FuraRed™ allowing them to be distinguished from the FPR2-expressing cells during flow cytometric analysis. A fluorescein label was conjugated to the lysine residue of the WPep peptide (WKYMM_m) to produce a fluorescent ligand (WPep-FITC) that bound both FPR1 and FPR2. Dissociation constants (K_d) for binding of WPep-FITC to FPR1 and FPR2 were determined to be 1.2 nM and 1.8 nM, respectively. Assays were performed in polystyrene 384-well plates with small volume wells (Greiner #784101). Additions to wells were in sequence as follows: (1) test compounds and control reagents (5 ul/well); (2) the combined suspension of U937/ FPR1 and RBL/FPR2 cells (5 ul/well) and; (3) (after 30 min, 4^oC incubation) WPep-FITC (5 ul/well). After an additional 45 min, 4^oC incubation, plates were immediately analyzed using flow cytometry. The assay response range was defined by replicate control wells containing unlabeled receptor-blocking peptide (positive control) or buffer (negative control). fMLFF was used as the FPR1-blocking peptide and unlabeled WPep as the FPR2-blocking peptide. Final concentration of WPep-FITC was 5 nM. The assay was homogeneous in that cells, compounds and fluorescent peptide were added in sequence and the wells

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subsequently analyzed without intervening wash steps. The HyperCyt™ high-throughput flow-cytometry platform (Kuckuck, et al., 2001; Ramirez, et al., 2003) was used to sequentially sample cells from wells of 384-well microplates (2 ul/sample) for presentation to a CyAn flow cytometer (Beckman-Coulter) at a rate of 40 samples/min. Fluorescence was excited at 488 nm and detected with 530/40 and 680/30 optical bandpass filters for WPep-FITC and FuraRed™, respectively. Test compound inhibition of fluorescent peptide binding was calculated as percent inhibition. WPep-FITC fluorescence intensity measurements of cells from control wells were also used to calculate Z' factors (Zhang, et al., 1999), which ranged between 0.6 and 0.8 for each assay.

Intracellular calcium mobilization assays. Intracellular calcium mobilization assays were performed as previously described with modifications (Edwards, et al., 2005). Briefly, cells were collected by centrifugation (200xg, 10 min, 24°C) and suspended at 10⁶/ml in 1 ml PBS (Ca²⁺, Mg²⁺ free). Cells were added with 210 nM Fluo4 (FPR2 cells) or with a combination of 210 nM Fluo4 and 210 nM FuraRed (FPR1 cells). After incubation for 30 min at 24°C cells were washed with 1 ml PBS, suspended in 1 ml TCM (RPMI, 10%FBS) and stored in the dark at 24°C until used in assays. For test compound agonist determinations, 50 µL from FPR1 and FPR2 cell suspensions were combined, analyzed 10 s in an Accuri C6 flow cytometer (BD Biosciences) to determine baseline Fluo4 fluorescence in the FL1 channel (530/20 nm), then added with 50 µL containing test compounds and analyzed an additional 110 s to evaluate changes in Fluo4 fluorescence intensity. During the analysis FPR1 cells were distinguished from FPR2 cells on the basis of FuraRed fluorescence intensity detected in the FL3 channel (>610 nm). The innate calcium response was quantified as the maximum % of cells that exceeded the baseline Fluo4 fluorescence intensity over the course of the analysis. These responses were normalized to the innate calcium responses observed in the presence of 11 nM control peptides: 4pep for FPR1 cells and Wpep for FPR2 cells as follows:

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Normalized Calcium Response (%) = 100 x innate response % to test compound / innate response % to control peptide.

For test compound antagonist determinations, 10 μ L test compound was added to the combined FPR1/FPR2 cell mixture and incubated 5 min at 24^oC prior to assessing the innate calcium response to subsequent addition of fMLFF or Wpеп peptide. In these determinations, fMLFF and Wpеп were used at concentrations that produced 90% of the maximal intracellular calcium response (EC₉₀), and responses were normalized with respect to the innate response to each control peptide observed in the absence of test compounds.

Dose response determinations. Fluorescent ligand competition dose response assays were performed essentially as described for single concentration assays except that test compounds were initially tested at a starting concentration of 10 mM in DMSO and serially diluted 1:3 to produce final concentration ranging from 67 μ M to 0.1 nM. The resulting ligand competition curves were fitted by Prism software (GraphPad Software, Inc., San Diego, CA) using nonlinear least-squares regression in sigmoidal dose response model with variable slope, also known as the four parameter logistic equation. Two parameters, the top and bottom of the fitted curves, were fixed at 100 and 0, the expected upper and lower boundaries of normalized data. Curve fit statistics were used to determine the concentration of added test compound competitor that inhibited fluorescent ligand binding by 50 percent (IC₅₀).

FPR expression ranged from 100,000 to 200,000 receptors per cell in different assays as determined by comparison to standard curves generated with Fluorescein Reference Standard Microbeads (Bangs Laboratories, Fishers, IN). This corresponds to total FPR concentration of 0.6 to 1.2 nM. To account for effects of possible ligand depletion at the higher receptor concentrations, K_i values were calculated from IC₅₀ estimates by the method of Munson and Rodbard (Munson and Rodbard, 1988): $K_i = K_d \times [y_0/(y_0+2)] + IC_{50}/\{1 + [p^* \times (y_0+2)]/[2 \times K_d \times$

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$(y_0 + 1)] + y_0$ in which y_0 is the initial bound-to-free concentration ratio for the fluorescent ligand, p^* is the added concentration of fluorescent ligand and K_d is the dissociation constant for the fluorescent ligand.

Intracellular Ca^{2+} dose response curves were also fitted using nonlinear least-squares regression in a sigmoidal dose response model with variable slope. Curve fit statistics were used to determine the concentration of added test compound competitor that inhibited Ca^{2+} responses by 50 percent (IC_{50}) for antagonists or effected increases in Ca^{2+} response by 50% (EC_{50}) for agonists. Since responses were normalized relative to control stimuli the top and bottom of the fitted curves were fixed at 100 and 0.

Results

In the search for novel FPR ligands we have used four distinctive screening programs (Table 1). For two of these programs we also evaluated FPR1 and FPR2 ligand binding activity in parallel. All four studies used competitive displacement of a fluorescent ligand as the primary screening methodology but each differed with respect to the composition of the compound library and conceptual design of the screening program. The earliest (Young, et al., 2005) was a screen of a commercial collection of 880 off-patent drugs and alkaloids, the Prestwick Chemical Library (PCL), in which we confirmed the previously reported FPR1 binding activity of sulfinpyrazone (Levesque, et al., 1991) but failed to detect novel ligands of greater potency (Table 1: PCL). The next involved the screening of a focused library containing 4,234 small molecule compounds. The library was constructed on the basis of a preliminary computational screen of 480,000 compounds from the commercial ChemDiv collection using an FPR homology model and pharmacophore (Edwards, et al., 2005). From several chemically distinct families of FPR1 ligands identified, the most potent was an antagonist with a K_i of 1 μM (Table 1: Focused). In the third approach we screened a subset of the NIH Molecular Libraries Small Molecule

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Repository (MLSMR), a small molecule diversity library of 24,304 compounds (Young, et al., 2009). Computational SAR analysis of active compounds from the first round of screening resulted in the commercial procurement and testing of an additional 1,446 small molecules. Seven FPR1 ligands and 1 FPR2 ligand with submicromolar potency were identified and all were antagonists (Table 1: MLSMR). In each of these first three screening approaches the physical screening component involved analysis of 1 test compound/well.

The fourth screening approach is presented here and involved the screening of a collection of mixture based combinatorial libraries, of which more than 5 million are classic small molecule compounds (Table 1: TPIMS Library). This collection is made up of 5,261 mixtures ranging from 48 to 216,000 compounds per mixture. Based on the screening results for FPR1 and FPR2 described below, two different libraries were selected for deconvolution to identify individual compounds. From one library, 106 individual compounds (Table 1: 1754) were synthesized and evaluated, of which 56 compounds had K_i values $\leq 1 \mu\text{M}$ for FPR1 and 38 had K_i values $\leq 1 \mu\text{M}$ for FPR2. Additionally from the deconvolution of a second library, 8 individual compounds (Table 1: 1753) were evaluated, of which 7 had K_i values $\leq 1 \mu\text{M}$ in FPR1.

As is evident from the results elucidated in Table 1, the set of active compounds ($K_i < 1 \mu\text{M}$) generated from the mixture-based combinatorial libraries contains compounds which are more active and more selective towards each target than those sets previously obtained from any of the other screening methods performed. In addition to the superiority of these results, positional scanning of combinatorial libraries offers additional advantages to the alternative screening methods attempted. The first is efficiency, as assessed solely by the number of samples tested; given a hit rate in MLSMR of 8/24,304, the probability of randomly testing only 5,375 samples (5,261 mixtures + 114 individual compounds, Table 1) and having at least 100 total positive hits is essentially zero ($p \leq 4.2 \times 10^{-133}$). Another way to assess screening efficiency is the number of

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active compounds identified relative to the total number of samples initially tested. In our previous screen of the MLSMR collection we evaluated 24,304 samples to identify 7 hit compounds for FPR1 and 1 for FPR2 (Table 1), hit frequencies of ~0.03% and ~0.004%, respectively. In the present study, 5,261 samples were initially screened to obtain hit frequencies of 1.1% for FPR1 and 0.6% for FPR2. In comparison with the MLSMR screen this represented a 37-fold and 150-fold increase in hit identification efficiency, respectively.

Primary screening of mixtures. The TPIMS collection of mixture based combinatorial libraries containing 37 different small molecule scaffolds were formatted on 19 separate 384-well plates (1 mixture sample per well) and screened in the duplex flow cytometry assay for inhibition of the FITC-labeled Wpep peptide binding to FPR1 and FPR2 receptors. The results of the screen in both receptors are shown in Figure 1. Each of the 37 libraries is numbered and grouped by color, and the inhibitory activity for each of the 5,261 mixtures is shown. Supplemental Table 1 contains detailed information for the 37 mixture based libraries. This includes the library synthesis number, the number of mixture samples tested, the number of compounds per mixture, the total number of compounds in each library, the library name and its chemical structure. A number of libraries showed inhibitory activity for both receptors. Library 21 was one of the most active libraries for both receptors, whereas library 36 was the most active in FPR1 alone. These two libraries were selected for further testing and deconvolution. It is worth noting that other libraries showed modest activity that could also be pursued. Thus, even in the duplex primary screen, a range of pharmacological possibilities were revealed.

Library 21 is a positional scanning library with four positions of diversity. It is a pyrrolidine bis-diketopiperazine scaffold (library 1344 in Supplemental Table 1). The mixtures for R_1 (1-26), R_2 (27-52), and R_3 (53-78) were each defined with one of 26 functionalities and each mixture was composed of 28,392 compounds ($26 \times 26 \times 42 = 28,392$). The mixtures for R_4 (79-120) were each

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defined with one of 42 functionalities and each mixture was composed of 17,576 compounds (26x26x26=17,576). The building blocks and the resulting functionalities of each of the mixtures are shown in Supplemental Table 2. Each of the 120 mixtures for this library was retested in a series of confirmatory screens for their inhibitory binding activity of FPR1 and FPR2 receptors. The average and standard error of the mean of different screens (n=4-7) for each mixture in each diversity position is shown in Supplemental Figure 2.

Positional scanning deconvolution. Identification of the individual compounds responsible for library 21 activity was carried out using positional scanning deconvolution {Pinilla, 1992 2723 /id;Dooley, 1993 2666 /id;Houghten, 1999 10071 /id;Houghten, 2008 16970 /id}, in which the functionalities in each of the defined positions of the most active mixtures within each library were selected to design a set of individual compounds. The most active mixtures for each of the receptors were tested in a dose response manner and this information was taken into consideration in the selection of the functionalities from each position. The most differential inhibitory activity was seen in R₁, R₂ and R₃. Some of the most active mixtures (>40% inhibition) for one receptor were among the least active for the other receptor. In R₄ the overall inhibitory activity was lower and little difference was seen between receptors. The selection of functionalities for the synthesis of individual compounds was solely based on activity and not selectivity. The functionalities and the corresponding mixture number (Supplemental Table 2 and Supplemental Figure 2) used for the synthesis of individual compounds for FPR1 included: S-benzyl (#2), S-propyl (#19), and S-butyl (#21) in R₁; S-benzyl (#28), S-isobutyl (#31), S-hydroxymethyl (#40), R-propyl (#46) and R-butyl (#48) in R₂; S-2-butyl (#56), R-4-hydroxybenzyl (#69), S-phenyl (#70) and S-cyclohexyl (#77) in R₃; and cyclohexyl-methyl (#107), 4-methyl-1-cyclohexyl-methyl (#115) and 2-biphenyl-4-yl ethyl (#117) in R₄. For the individual compounds for FPR2 the functionalities were: S-isopropyl (#8), S-propyl (#19) and R-2-naphthylmethyl (#24) in R₁; R-4-hydroxybenzyl (#43), R-propyl (#46), R-2-naphthylmethyl (#50) and R-cyclohexyl

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(#52) in R₂; *R*-benzyl (#63), *R*-4-hydroxybenzyl (#69), *R*-propyl (#72), and *R*-butyl (#74) in R₃; and cyclohexyl-methyl (#107), 4-methyl-1-cyclohexyl-methyl (#115) and 2-biphenyl-4-yl ethyl (#117) in R₄. A total of 106 compounds (Table 1: 1754) were synthesized and tested at both receptors.

Figure 2 shows a scatter plot of the activity of individual compounds for both receptors. The structural information and inhibitory binding activities for all 106 compounds are given in Supplemental Dataset 1. Nineteen different compounds of the 106 pyrrolidine bis-diketopiperazines were identified with K_i values less than 100 nM for FPR1, of which 15 are FPR1 selective with K_i values more than 100 fold greater for FPR2. For the FPR2 receptor, 23 out of the 106 compounds have K_i values less than 100 nM and of those 12 were selective for FPR2 with K_i values more than 100 fold greater for FPR1.

A visualization of the SAR for the inhibitory binding activity of the 106 diketopiperazine individual compounds is shown in Figure 3. The figure summarizes the distribution of the inhibitory activity (K_i) values for compounds with a given R-group. Activity is color-coded with a continuous scale from more active (red) to less active (green). The most representative R-groups (i.e., with three or more compounds) are displayed. This visual representation of the SAR clearly shows striking differences in the general activity and selectivity pattern of R-groups. For example, Figure 3 highlights the overall increased activity for FPR2 when small and aliphatic substituents are utilized in R₁, in particular *S*-isopropyl. In general, bulky and aromatic rings in R₁ decrease the activity. In contrast, *S*-benzyl in R₁ is favorable for activity for FPR1. A large and hydrophobic moiety in R₁ such as *R*-naphthyl is not tolerated for either FPR1 or FPR2. For R₂, Figure 3 visually emphasizes the selectivity for FPR2 when *R*-4-hydroxymethyl or *R*-2-naphthylmethyl is utilized. Strikingly the selectivity switches to FPR1 when the aromatic *S*-benzyl is utilized instead at R₂. This switch in selectivity might be due to either the switch in stereochemistry or

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from the subtle structural differences in this set of aromatic substitutions. The small and polar *S*-hydroxymethyl group in R_2 leads to inactive compounds for FPR1 and FPR2.

Extended functional analysis. Two FPR1 selective compounds from the pyrrolidine bis-diketopiperazine library, namely 1754-113 and 1754-56, are shown in Table 2 along with that of compound 1754-26, which is the only compound out of the 106 tested with a K_i value less than 100 nM for both receptors. These 3 compounds acted as antagonists when tested for their ability to elicit or inhibit FPR1-mediated intracellular Ca^{2+} response (Table 2). By contrast, the two compounds exhibited opposite effects in parallel analyses of Ca^{2+} responses mediated by FPR2. 1754-56 was an agonist for FPR2, with a low micromolar EC_{50} that matched the K_i value observed in the competitive ligand displacement assay (Table 2, Supplemental Figure 3). 1754-56 and 1754-26 differ at the R_1 functionality, which is *S*-butyl and *S*-isopropyl, respectively. Thus, a subtle change in the structure plays a dramatic role in selectivity for these ligands, representing a clear example of an ‘activity cliff’.

Three FPR2 selective pyrrolidine bis-diketopiperazines (1754-20, 1754-19, and 1754-31) are also shown in Table 2. It can be seen that the only difference between the non-selective inhibitor, 1754-26, and the FPR2-selective inhibitor, 1754-20, is the R_2 functionality, which is *R*-propyl for the former and *R*-4-hydroxybenzyl for the latter. Another significant functional disparity resulted when the *S*-isopropyl R_1 functionality of 1754-31 was replaced with an *S*-propyl functionality in 1754-49. Not only was there a decrease in FPR2 selectivity (>10,000 to ~70) but the antagonist (1754-31) was converted to partial agonist (1754-49) in the FPR2-mediated Ca^{2+} response determinations (Table 2, Supplemental Figure 3).

As shown in Figure 1, library 36 (Library 1481 in Supplemental Table 1) was clearly more active at FPR1 than FPR2. This library is made up of a polyphenylurea scaffold having three positions of diversity. Each mixture contains 1,777-2,304 compounds while the entire library contains

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85,248 compounds. Eight individual compounds (Table 1: 1753) were designed based on the deconvolution of the library screening data in FPR1, and the results of the 4 most active and selective compounds (K_i values 1-24 nM) are shown in Supplemental Dataset 2. 1753-101, 1753-102 and 1753-103 (Table 2) were determined to be selective partial agonists for FPR1 in intracellular Ca^{2+} response determinations, as illustrated in a representative dose-response profile for 1754-103 in Supplemental Figure 3.

Discussion

The rapid and efficient identification of potent and selective FPR ligands presented in this work is in accord with our previous findings that screening of mixture-based libraries enables the rapid exploration of novel regions of the chemical space not typically covered by commercially available screening compounds. The results of screening mixture-based combinatorial libraries over traditional HTS of commercially available small-molecule libraries to identify potent and selective FPR ligands are compared in Table 1. These results clearly show that the use of mixture-based libraries in a duplex HTS system was a more efficient and robust approach for the primary screening stage than the other approaches involving the screening of 1 compound/well. In the present study, 5,261 samples were initially screened to obtain hit frequencies of 1.1% for FPR1 and 0.6% for FPR2. In comparison with the MLSMR screen this represented a 37-fold and 150-fold increase in hit identification efficiency, respectively. This indicates that positional scanning of mixture-based combinatorial libraries was significantly more efficient than random testing when evaluated by virtually any standard of significance chosen. It should be noted that the number of total compounds evaluated by the mixture libraries in this study (including 5 million small molecule compounds) is beyond the range of feasibility for most single-compound screening efforts.

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Screening of the highly dense region of chemical space covered by the active libraries tested herein led to the identification of potent and highly selective ligands and also provided substantial and useful structure-activity relationship (SAR) information. The individual compounds generated from a positional scanning deconvolution are especially well-suited to SAR analysis, since one can immediately analyze the effect that single- or double-substitutions at individual positions have on the activities of these compounds (see more below). This SAR allows for the identification of the causes of activity cliffs that cannot be readily accomplished in libraries of individual compounds of disparate scaffolds. Thus, the exhaustive exploration of the SAR of the most active libraries readily revealed the presence of activity cliffs, chemical compounds with highly similar structures, but unexpectedly very different biological activities.

Recently, two separate groups have identified potent small molecule FPR1 antagonists with Ca^{2+} response IC_{50} values of 398 nM (Unitt, et al., 2011) and 4 nM (Morley, et al., 2012). Small molecule FPR1 agonists have also been identified in a number of recent studies (Cilibrizzi, et al., 2009; Kirpotina, et al., 2010; Schepetkin, et al., 2007), the most potent of which had a Ca^{2+} response EC_{50} value of 630 nM (Schepetkin, et al., 2007). Potent FPR2 agonists have also been reported with Ca^{2+} response EC_{50} values in the 30-40 nM range (Burli, et al., 2006; Frohn, et al., 2007). The compounds reported here include the most potent FPR1 agonist (1753-103) and FPR2 antagonist (1754-31) identified to date. These compounds are structurally distinct from all previously described FPR ligands. In intracellular Ca^{2+} response determinations the FPR1 agonist had an EC_{50} of 131 nM (binding affinity 4 nM K_i) and the FPR2 antagonist had an IC_{50} of 81 nM (binding affinity 1 nM K_i).

The SAR findings summarized in Table 2 and Figure 3 for the FPR1 and FPR2 ligands suggest the importance of *S*-butyl in the R_1 functionality for FPR1 active and selective ligands and *R*-4-hydroxybenzyl in the R_2 functionality for FPR2 active and selective ligands. More extensive

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structural activity analysis of the 106 compounds supports the importance of these functionalities. The functionalities that are more frequently found in the active and selective compounds for each of the receptors are: 1) For FPR1, in R₁ *S*-butyl and *S*-benzyl and in R₂ *R*-propyl and *S*-benzyl; and 2) For FPR2, in R₁ *S*-isopropyl followed by *S*-propyl and in R₂ *R*-4-hydroxymethyl and *R*-2-naphthylmethyl. It is important to note that even in the primary screen the mixtures with these functionalities show clear selectivity. For example in library mixture 1344-8 R₁ is defined with *S*-isopropyl and is active for FPR2 while library mixture 1344-21 is defined with *S*-butyl and shows significantly greater activity for FPR1 than FPR2 (Supplemental Figure 2 and Supplemental Table 2). Although there are specific R-groups with marked differences in activity for FPR1 and FPR2, Figure 3 and the activity data for individual compounds in Table 2 also indicate that there are specific combinations of R-groups that determine the activity and selectivity of the individual ligands. In order to explore in detail the SAR of the entire data set, a systematic pairwise comparison of the structure and activity of the 106 molecules was conducted using the principles of activity landscape modeling. Results of the systematic analysis indicated that the compound pair 1754-26/1754-56 (Table 2) represents an “activity switch” where the substitution of an *S*-isopropyl with *S*-butyl at R₁ increases the activity for FPR1 by 1.36 log units but decreases the activity for FPR2 by 1.28 log units. The analysis also rapidly revealed that the compound pair 1754-20/1754-56 (Table 2) is a “selectivity switch” because changes in the R-groups have a large and opposite effect on the activity for FPR1 and FPR2. These and other conclusions from the comprehensive SAR analysis based on the structure of the 106 ligands are reported elsewhere (Medina-Franco, et al., 2013). The structure-activity analysis derived from the deconvolution of positional scanning libraries provides useful information about the importance of the functionalities at each position of the compound, which can then be used as a starting point for a more detailed characterization of the functionalities required for activity and selectivity.

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Discrimination between agonists and antagonists in this study was based on elevation of intracellular calcium. We have shown previously that in these assay systems, the cellular response reflects a distribution of responding and non-responding cells which are captured in the current analysis and can distinguish among antagonists as well as full and partial agonists. Thus, the collection of FPR1 and FPR2 agonists and antagonists we report here adds to the FPR1/FPR2 ligands identified and characterized by different groups. The ligands identified cover divergent structural classes, but in some cases the ligands within structural classes display similar functional activity, i.e., piperazines as selective FPR2 agonists (Kirpotina, et al., 2010). However there are several examples from these studies, including our own, in which compounds derived from similar scaffolds have divergent functional activity against FPR1 and FPR2, such as an apparently slight modification in structure changes a ligand from a selective agonist to a selective antagonist. For instance t-Boc peptides have been reported as FPR1 antagonists while the N-formyl peptides of the same or similar composition are FPR1 agonists (Ye, et al., 2009). The synthetic, nonpeptide FPR2 agonist (Quin-C1) differs from the FPR1 antagonist Quin-C7 only in the *para* position of the phenyl ring, methoxy (Quin-C1) to hydroxyl (C7) (Zhou, et al., 2007). In the current study modification of the R₁ propyl functionality of the FPR2 agonist (1754-49) to an isopropyl yielded an FPR2 antagonist (1754-31). Additionally there are examples of what appear to be small structural modifications that change a ligand from a target selective functionality to a non-selective functionality against FPR1 and FPR2. For instance, Khlebnikov et al. report that by modifying the *p*-COOCH₃ group on their benzimidazole selective FPR1 agonist (AG-11/05) to a *m*-COCH₃ group they obtained the dual FPR1/FPR2 agonist (AG-11/06) (Khlebnikov, et al., 2012). In a similar manner we observed that the modification of the phenol portion of the R₂ functionality of the FPR2 selective antagonist (1754-20) to a methyl group yielded the dual FPR1/FPR2 antagonist (1754-26) (Supplemental Figure 4). The accumulated SAR to date supports the high degree of homology between the FPR targets. The ligands in which slight structural modifications produce a

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dramatic shift in functionality holds promise for the identification of therapeutically relevant, target/functionality specific compounds. Additionally the breadth of structural classes reported to date for FPR1 and FPR2 indicates that many structural motifs are available for these targets, expanding the range of medicinal chemistry space available for optimization of leads.

The use of positional scanning libraries with a duplex HTS approach enabled the identification of compounds with diverse functional activities (selective agonist, selective antagonist, dual antagonist, mixed agonist/antagonist) containing a wealth of SAR from the screening 5,261 samples. It is important to note that more than 5 million small molecules were assessed in this approach. Such a wealth of data lends itself to a broad analysis and quantification of the overall screening process, which will be reported elsewhere (Santos, et al., 2013). We anticipate that the combined approaches illustrated herein, in which mixture-based positional scanning libraries are screened in a variety of differing targets and assay formats, such as the duplex flow cytometry utilized in the current studies, will facilitate and accelerate a wide range of translational drug discovery efforts. Finally, it is worth noting that while the exploration of two libraries has revealed extraordinary pharmacological diversity, other opportunities within the TPIMS chemical space, such as libraries 8, 22, 23, 24, and 28, remain to be explored.

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Authorship contributions

Participated in research design: Pinilla, Edwards, Young, Sklar, Houghten

Conducted experiments: Pinilla, Edwards, Appel, Yates-Gibbins, Giulianotti, Young, Medina-Franco, Santos

Contributed new reagents or analytic tools: Pinilla, Edwards, Appel, Yates-Gibbins, Giulianotti, Medina-Franco, Santos

Performed data analysis : Pinilla, Edwards, Appel, Yates-Gibbins, Giulianotti, Young, Medina-Franco, Santos

Wrote or contributed to the writing of the manuscript : Pinilla, Edwards, Appel, Giulianotti, Medina-Franco, Santos, Sklar, Houghten

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Footnotes

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C. Pinilla and B.S. Edwards contributed equally

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Legends for Figures

Figure 1. Activity profiles for FPR1 and FPR2 screened against 37 different mixture-based small molecule libraries. Each library screened is numbered (see Supplemental Table 1 for library details) and color coded, and each bar represents the activity (% inhibition of labeled ligand binding to receptor) for a given mixture within each library.

Figure 2. Scatter plot representation of 106 individual compounds derived from pyrrolidine bis-diketopiperazine library screened against FPR1 and FPR2. The activities (K_i , μM) of each compound for each receptor are shown, their selectivity is denoted and the number of compounds with different activities and selectivities are shown. See Supplemental Dataset 1 for structure and activity data on each compound. Data labeled a-g refer to compounds in Table 2 and Supplemental Figure 1.

Figure 3. Visual representation of the SAR of 106 individual compounds screened against FPR1 and FPR2. The distribution of the activity (K_i) values for compounds containing an R-group is color-coded using a continuous scale from more active (red) to less active (green). The most populated R-groups, with three or more compounds, are shown. The number of compounds with a given substituent is represented with the number of slices in each pie. For visual clarity, the maximum number of slices shown is 16. The figure was generated with the Structure-Activity Report application of Molecular Operating Environment (version 2011.10; Chemical Computing Group Inc.: Montreal, Quebec, Canada)(Clark and Labute, 2009).

Tables

Table 1. Comparison of FPR1 and FPR2 ligands identified by 4 different screening programs.

| Library | # Samples Tested | # Compounds Evaluated | FPR1 | | | | FPR2 | | | | Ref. |
|-------------|------------------|-----------------------|----------------------------|----------------|---------|-------------|----------------------------|----------|---------|-------------|-----------------------|
| | | | # Compounds Ki < 1 μ M | Best | | | # Compounds Ki < 1 μ M | Best | | | |
| | | | | ID | Ki (nM) | Selectivity | | ID | Ki (nM) | Selectivity | |
| PCL (1) | 880 | 880 | 0 | Sulfinpyrazone | 14,000 | ND | ND | ND | ND | ND | Young, et al., 2005 |
| Focused (2) | 4,324 | 4,324 | 1 | 1910-5441 | 1,000 | ND | ND | ND | ND | ND | Edwards, et al., 2005 |
| MLSMR (3) | 24,304 | 24,304 | 7 | 3570-0208 | 95 | >187 | 1 | BB-V-115 | 270 | >20 | Young, et al., 2009 |
| TPIMS (4) | 5,261 | 5 million | | | | | | | | | Herein |
| 1754 | 106 | 106 | 55 | 1754-113 | 3 | >3,333 | 38 | 1754-31 | 1 | >10,000 | |
| 1753 | 8 | 8 | 7 | 1753-101 | 1 | >10,000 | 0 | | | | |

1 Prestwick Chemical Library, a commercial collection of 880 off-patent drugs and alkaloids.

2 FPR-focused, small molecule library based on a computational screen.

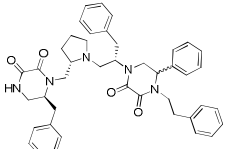
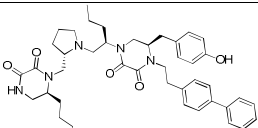
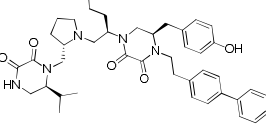
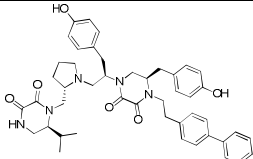
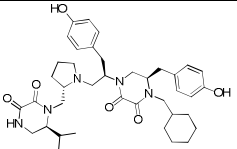
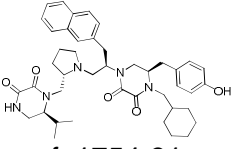
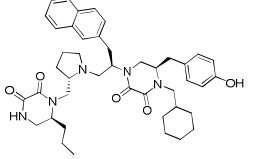
3 Small molecule diversity library (NIH Molecular Libraries Small Molecule Repository).

4 TPIMS small molecule mixture based combinatorial libraries. 1754 and 1753 are two sets of individual compounds derived from the deconvolution of selected mixture based libraries.

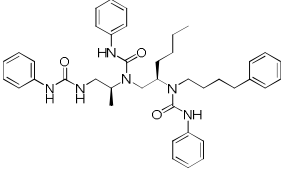
ND not determined.

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Table 2. Examples of active compounds for FPR1 and FPR2.

| Compound # | FPR1 | | | FPR2 | | |
|---|--------------------|-------------------------|-------------------------|--------------------|-------------------------|-------------------------|
| | Binding Ki (nM) | Agonist EC50 (nM) | Antagonist IC50 (nM) | Binding Ki (nM) | Agonist EC50 (nM) | Antagonist IC50 (nM) |
| Pyrrolidine bis-diketopiperazine scaffold | | | | | | |
|  a: 1754-113 | 3 | ** | 1,070 | * | ** | *** |
|  b: 1754-56 | 2 | ** | 139 | 1,320 | 1,300 | N.A. |
|  c: 1754-26 | 46 | ** | 2,680 | 70 | ** | 3,480 |
|  d: 1754-20 | * | ** | *** | 15 | ** | 452 |
|  e: 1754-19 | 3,370 | ** | *** | 6 | ** | 1,410 |
|  f: 1754-31 | * | ** | *** | 1 | ** | 81 |
|  g: 1754-49 | 2,320 | ** | *** | 33 | 144 | N.A. |
| Polyphenylurea scaffold | | | | | | |

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| | | | | | | |
|--|---|-----|------|---|----|-----|
|  h: 1753-103 | 4 | 131 | N.A. | * | ** | *** |
|--|---|-----|------|---|----|-----|

* K_i greater than 10,000 nM; **No agonistic activity at up to 12 μM ; *** No antagonistic activity at up to 10 μM ;
N.A. not applicable.

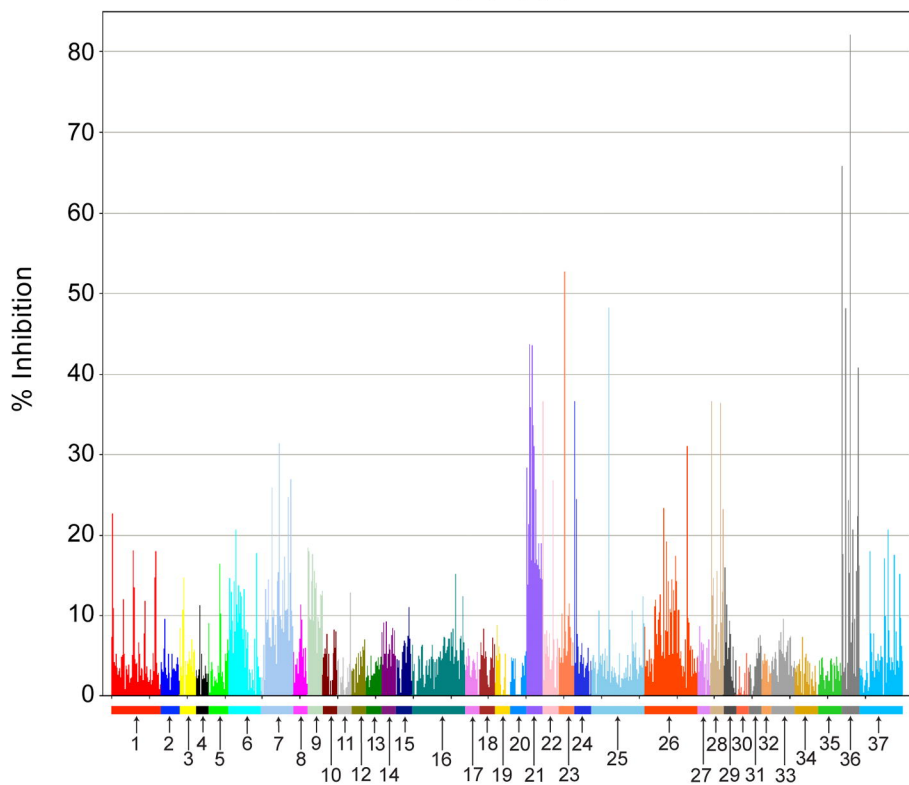
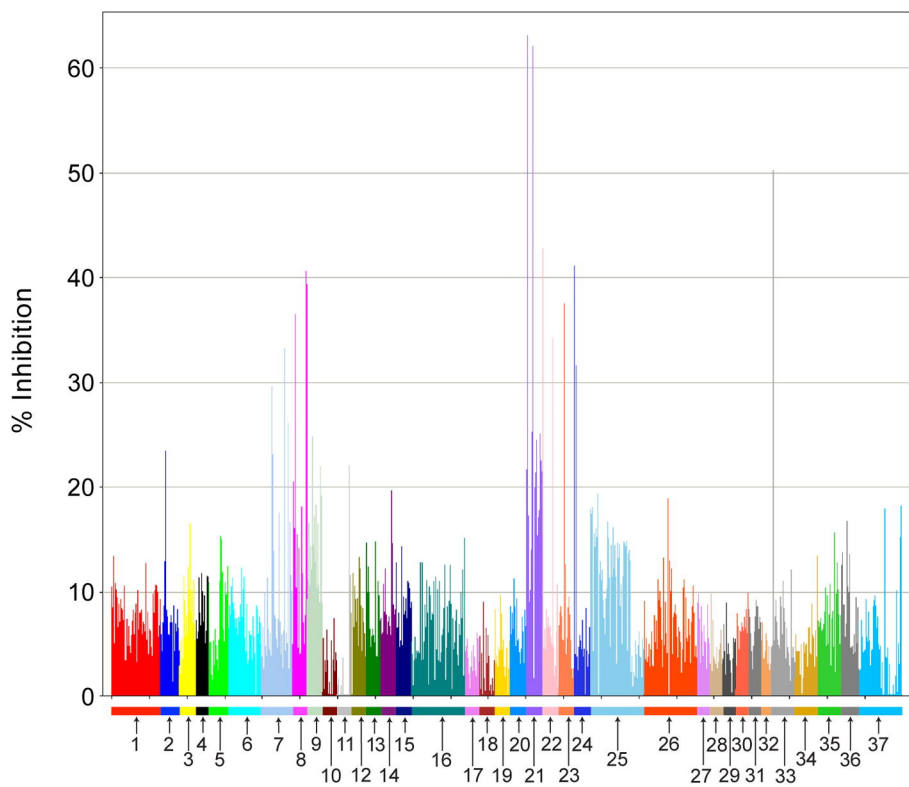
Figure 1**FPR1****FPR2**

Figure 2

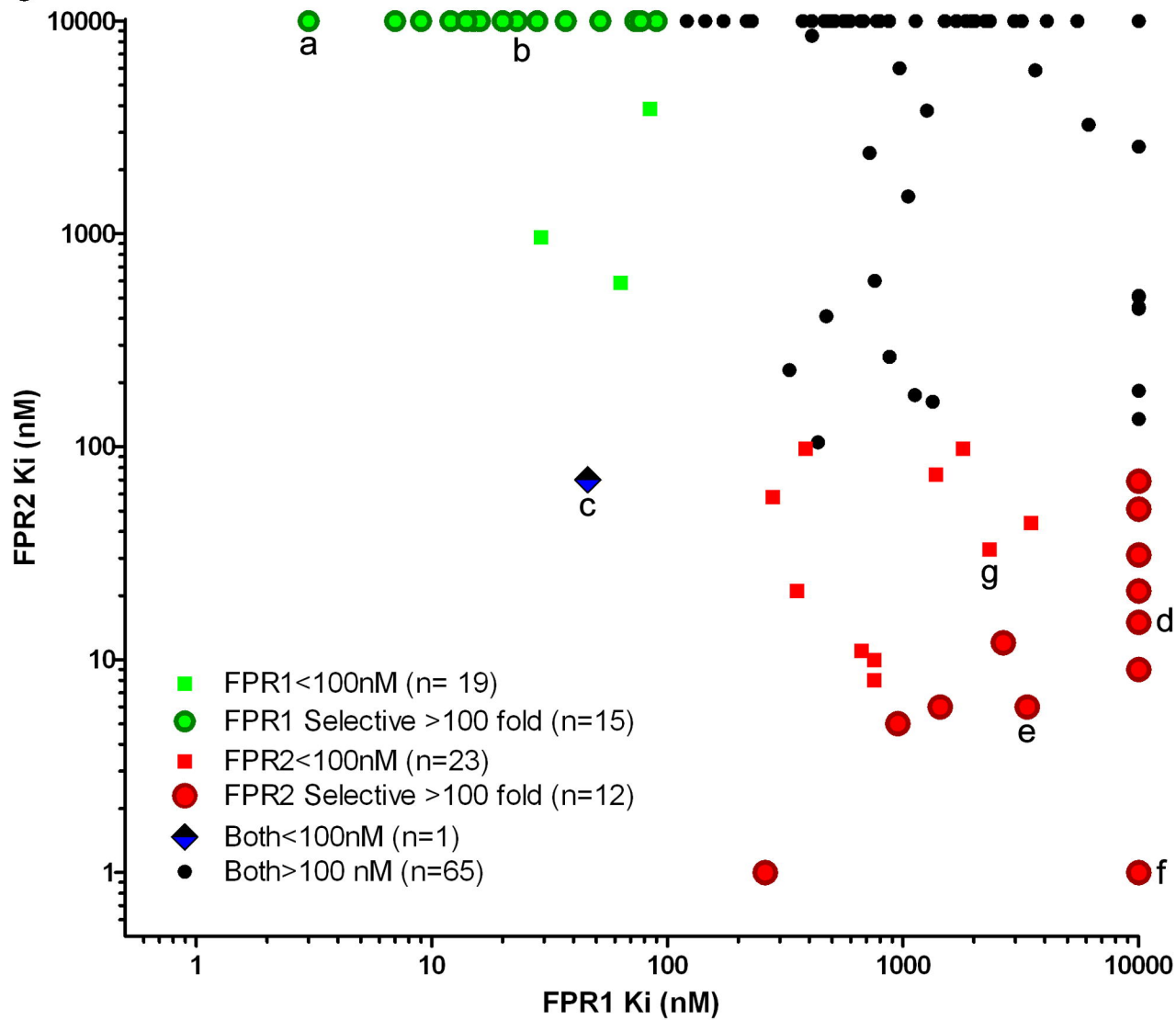
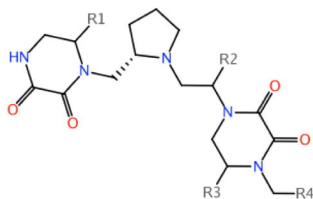
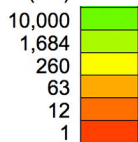
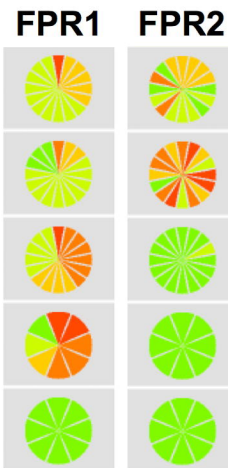
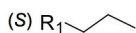
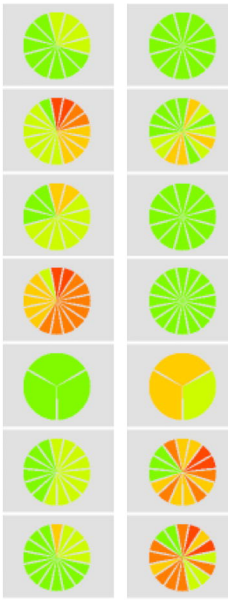
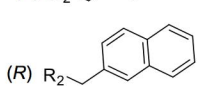
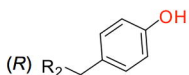
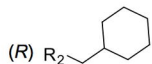
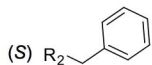
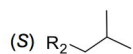
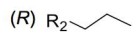
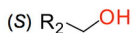
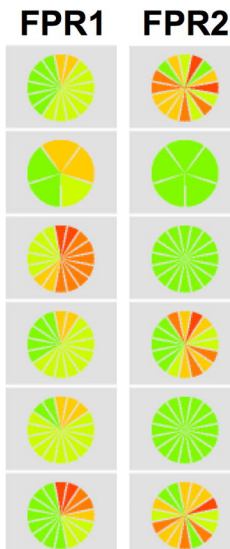
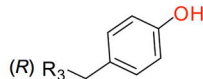
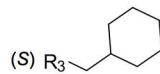
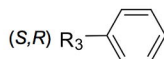
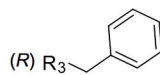
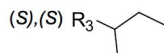


Figure 3

Ki (nM)

**R1****R2****R3****R4**