ACS Medicinal Chemistry Letters

TECHNOLOGY NOTE

pubs.acs.org/acsmedchemlett

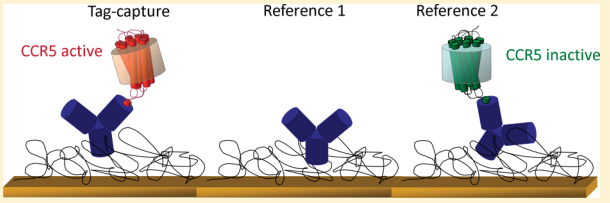
Screening for GPCR Ligands Using Surface Plasmon Resonance

Iva Navratilova,* Jérémy Besnard, and Andrew L. Hopkins

Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, United Kingdom

Supporting Information

ABSTRACT: G-protein coupled receptors (GPCRs) are a class of drug targets of primary importance. However, receptor assays are based on measurement of either ligand displacement or downstream functional responses, rather than direct observation of ligand binding. Issues of allosteric modulation, probe dependence, and functional selectivity create challenges in selecting suitable assays formats. Therefore, a method that directly measures GPCR−ligand interactions, independent of binding site, probe, and signaling pathway would be a useful primary and orthogonal screening method. We have developed a GPCR biosensor assay protocol that offers the opportunity for high-throughput label-free screening that directly measures GPCR−ligand interactions. The biosensor-based direct screening method identifies the interaction of both orthosteric and allosteric ligands with solubilized, native GPCRs, in a label-free and cell-free environment, thus overcoming the limitations of indirect and displacement assay methods. We exemplify the method by the discovery of novel ligands for the chemokine receptor, CCR5, that are ligand efficient fragments.



KEYWORDS: Surface plasmon resonance, G-protein coupled receptors, CCR5, allosteric, fragments

The potential therapeutic benefit of allosteric modulation of G-protein-coupled receptors (GPCRs) is increasingly being recognized.[1,2] Allosteric modulation can be an attractive mechanism of action for GPCR drugs for several reasons. First, distinct allosteric binding sites may be less conserved than orthosteric sites and thus offer different selectivity profiles. Second, allosteric and orthosteric ligands often occupy different areas of chemical space with different physicochemical properties; thus, potentially, an allosteric site may be more druggable. Third, allosteric ligands do not directly compete with the endogenous agonists; therefore, they may exhibit insurmountable kinetics and thus offer the possibility of lower drug doses or prolonged pharmacodynamic profiles. Fourth, allosteric ligands may offer the possibility of modulating pharmacology by exhibiting cooperativity with orthosteric ligands or selectively modulating the signal from an orthosteric ligand.

However, the discovery of allosteric ligands can be challenging with conventional GPCR assay formats. Some allosteric antagonists are known to disrupt agonist signaling without necessarily disrupting the binding of the agonist to the receptor. Competitive displacement assays with an endogenous ligand may fail to detect the binding of a noncompetitive ligand to a novel binding site. The use of radiolabeled ligands in displacement assays also introduces expensive manufacturing and disposal costs. Allosteric modulators can also exhibit "probe dependence". For example, the CCR5 antagonist, aplaviroc, blocks the binding of ^{125}I-MIP-1α but not ^{125}I-RANTES; thus, a radioligand displace screen with ^{125}I-RANTES would have failed to find this compound.[3] A range of probe dependencies have been observed for synthetic CCR5 ligands: from compounds that block chemokine binding but not HIV-1 gp-120 binding[4] to compounds that block HIV-1 binding but partially spare CCR5 function through chemokine signaling.[5]

To overcome some of these issues with displacement assays, indirect signaling assays are commonly used in drug discovery, where the downstream response of a signaling pathway is used to detect functional binding to a receptor. Common receptor signaling assay formats include fluorescence-based systems that detect levels of calcium (Ca^{2+}) mobilization, cyclic adenosine monophosphate (cAMP), inositol phosphates (IP_1 and IP_3), and ERK signaling. Functional assays are often unable to distinguish between different mechanisms without more detailed deconvolution and displacement assays. Small molecule synthetic ligands may even mimic the function of endogenous agonists. The initial high-throughout screening hit (UK-107,543) that was optimized into the drug maraviroc is a small molecule agonist of CCR5 discovered by the screening of the displacement of radiolabeled MIP-1β.[4,6] Modification of the agonist UK-107,543 resulted in compounds that are antagonists. However, despite their widespread use, significant limitations of indirect signaling-based assays are emerging, as ligands can possess "functional selectivity",[7] where a ligand can induce differential signals toward different pathways. Thus, the "efficacy" of a GPCR−ligand complex is dependent on the context of the downstream components present in a cell type[8] where a ligand can demonstrate dual and opposite efficacies on different signaling pathways while binding to the same target: That is, the same compounds

Received: January 2, 2011
Accepted: May 16, 2011
Published: May 16, 2011

ACS Publications © 2011 American Chemical Society

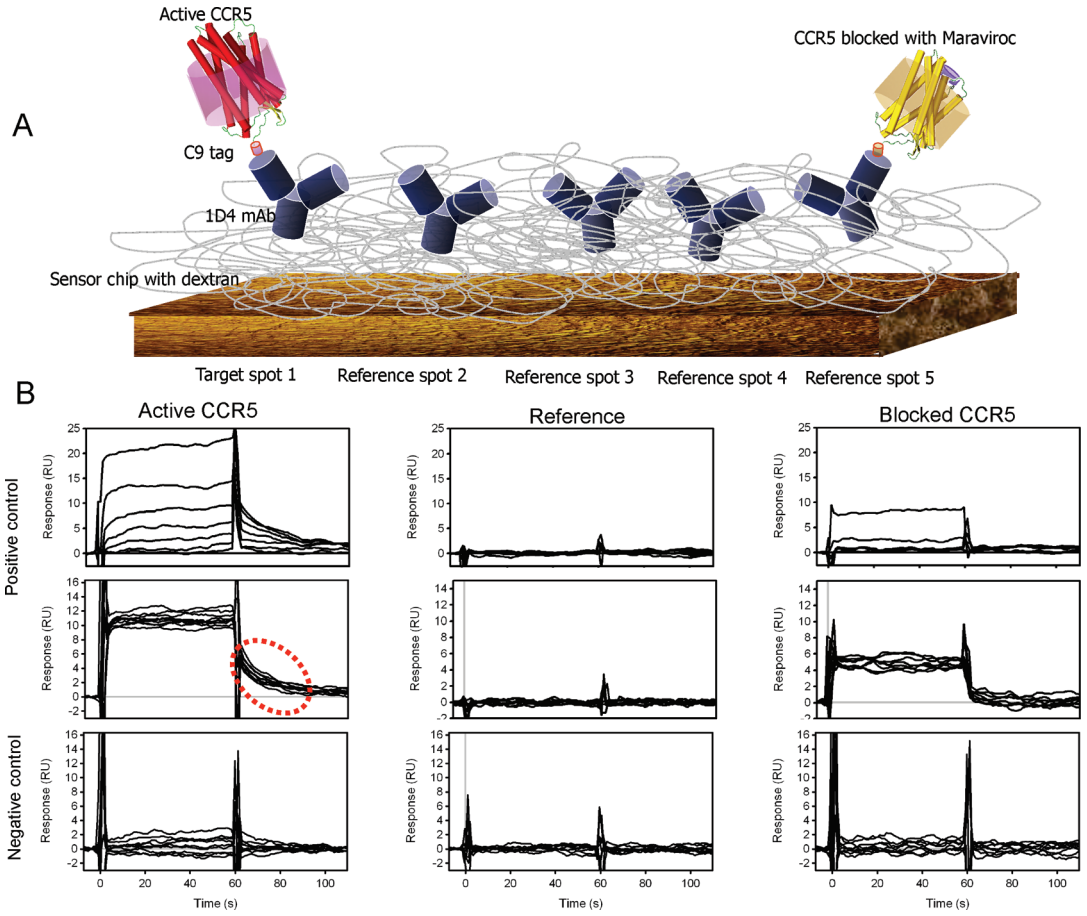


Figure 1. (A) Assay design: 1D4 mAb is immobilized on the dextran surface of the CM4 chip, CCR5 is captured on spots 1 and 5, and maraviroc is injected on spot 5 only to block the active site of CCR5. (B) Sensorgrams collected on surfaces: active CCR5, 1D4 surface, and blocked CCR5. Binding of control compound UK-107543 at 3-fold concentration series 0.0045−10 μM and overlay of positive (UK-107543) and negative (sulpiride) control at 5 μM injected during screen.

can be an agonist against one pathway but an antagonist or inverse agonist against another pathway.[9] These caveats suggest that some signaling assays might not detect allosteric modulators if only one signaling pathway is measured. The binding of some allosteric ligands may modulate receptor internalization and thus also fail to be detected by many functional assays and indirect signaling screens.

A further complication is the putative intracellular location of several newly discovered allosteric binding sites, which may remain undetected in cell-based assays, if novel, unoptimized compounds do not possess the necessary physicochemical properties to permeate the cell membrane or the choice of cell type possesses pumps and transporters that lower the effective intracellular concentration of the compounds.[10,11]

The phenomena of allosteric modulation, probe dependence, and functional selectivity create possibilities for sophisticated pharmacology for novel GPCR drugs. However, these aspects also highlight some severe limitation with current GPCR screening formats. Therefore, there is a need for direct binding assays for GPCRs that can distinguish between binding and function, as function is often context dependent. A method that directly measures GPCR−ligand interactions, independent of binding site or signaling pathway, would be useful means of confirming high-throughput screening (HTS) hits and potentially a primary screening method in its own right.

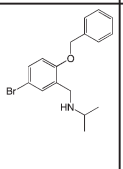
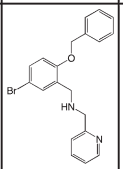
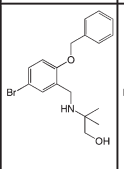
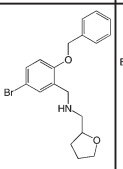
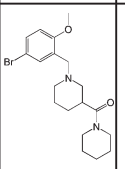
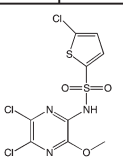
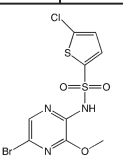
Surface plasmon resonance (SPR) is emerging as an analysis method to determine the kinetics and affinity of protein, peptide, and small molecule ligands with GPCRs.[4,12−16] SPR has several advantages as a biophysical method for measuring GPCR−ligand interactions. First, SPR is a "label-free" method that measures the direct binding of a ligand with the receptor. Second, SPR analysis occurs in real time and thus enables association and dissociation rates to be measured to determine kinetics and affinity. Third, SPR has been applied to both purified, thermostabilized receptors[15,16] and native-tagged receptors captured directly from cell pellets.[12−14] Here, we describe the development of SPR into a primary screening methodology for tagged, native GPCRs to discover novel ligands for CCR5.

The class A GPCR, chemokine receptor type 5 (CCR5), modulates several inflammatory mechanisms through the binding of the chemokines macrophage inflammatory protein (MIP) 1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5). CCR5 is an important therapeutic target as it is the major coreceptor, in cooperation with CD4, for the cell entry of HIV role. The CCR5 antagonist, maraviroc, is an approved drug for the treatment of HIV-1. Maraviroc[17] and many other chemokine antagonists are noncompetitive allosteric inhibitors respective to the orthosteric chemokine agonists.[3]

Previously, we have described the kinetic analysis of the chemokines RANTES[14] and the small molecules TAK 779[14]

dx.doi.org/10.1021/ml2000017 |ACS Med. Chem. Lett. 2011, 2, 549–554

Table 1. Chemical Structure, Affinity and Ligand Efficiency (LE) of Ligands Binding to Maraviroc Pocket of CCR5 (Compounds A−E) and an Allosteric Pocket of CCR5 (Compounds F and G)

Compound ID	A	B	C	D	E	F	F*	G	G*
Compound Structure									
K_D (μM)	10.0 (±0.2)	8.2(±0.8)	27.5 (±4)	18.4 (±0.2)	49.0 (±0.5)	17.5 (±0.4)	10.8 (±0.9)*	22.6 (±0.5)	28.6 (±0.3)*
Non-hydrogen atoms	20	24	22	23	24	20	20	19	19
LE (kcal/mol/non-hydrogen atom)	0.335	0.283	0.278	0.276	0.240	0.319	0.333*	0.328	0.320*
Bayesian score	22	17	19	12	14	NA	NA	NA	NA

*Affinity and LE measured to blocked CCR5.

and maraviroc[4] with CCR5 using SPR. In this study, we extend the SPR analysis of GPCRs into a screening method for immobilized CCR5. A Bayesian activity model for CCR5 was used to select 200 compounds from the University of Dundee's compound file of over 90 000 compounds, the vast majority of which are purchased from commercial suppliers (see the Supporting Information). The Bayesian activity model was trained using 1 166 known CCR5 ligands from 206 748 compounds in the ChEMBL database (version 2). Chemical structure information was described as extended connectivity fingerprints (ECFP) with a neighborhood size of 6.

We used a previously developed CCR5 SPR assay[12−14] where we captured C9-tagged native CCR5 receptor on the sensor chip of Biacore 4000 instrument. The Biacore 4000 fluidics system allows the assignment of five detection spots to each of four flow cells, enabling the screening of four different compounds per cycle, where one of the targets per flow cell has to be assigned as a reference or preferably left blank. In our case, we immobilized 1D4 antibody on all detection spots of all four flow cells and captured solubilized native CCR5 via C-terminal C9 tag onto two spots of each flow cell, leaving three spots per flow cell as reference with immobilized 1D4 mAb (Figure 1A). By solubilizing membrane proteins from native membranes, it is not always possible to obtain 100% active receptor captured on the sensor surface, therefore leaving some proportion of the receptor inactive or containing residuals from membrane attached to the receptor. All of these factors can contribute to a nonspecific binding when screening libraries of compounds that can act as false positives, mainly at high concentrations. Therefore, it is important, especially for membrane proteins, to find the closest reference target as possible. Suitable reference systems could be (i) unrelated membrane proteins that are solubilized and captured in the same way as target protein; (ii) deactivated membrane proteins, solubilized in detergent, which will solubilize receptor from the membrane, but does not keep active conformation; or (iii) receptors with binding sites that have been blocked with known high affinity ligands. Because the vast majority of CCR5 ligands used to train the Bayesian model that is used to select the screening library are likely to bind to the same site as maraviroc, we therefore used CCR5 receptor blocked with 5 μM maraviroc as a reference. Maraviroc binds to CCR5 with high affinity (K_D = 25 nM) and dissociates very slowly from the receptor (off-rate, $k_d = 6 \times 10^{-4}$ s^{-1}).[4] To keep the receptor blocked during screening and follow up confirmation experiment, we repeated injections of 5 μM maraviroc every 10 cycles, addressing the injection only over reference spot, while leaving active CCR5 intact. To test the activity of the receptor and also to distinguish between specific and nonspecific binding sensorgrams, we included injections of negative (sulpiride) and positive (UK-107,543)[4] control at a 5 μM concentration within the screen. Figure 1B shows concentration series of control compound and overlay of binding sensorgrams for both positive and negative controls to active CCR5, reference surface with immobilized 1D4 mAb, and CCR5 blocked with maraviroc during one experimental run collected on one of four flow cells. Negative control sulpiride does not show any binding to any of the surfaces. The positive control shows concentration-dependent binding to active CCR5 with a slow off-rate (in red circle). Binding of the positive control to blocked receptor occurs only at high concentrations and fast off-rate, suggesting that the binding is either weak or nonspecific. A clear difference in the binding modes for control compound to active and blocked CCR5 suggests this compound to be a suitable control to assess the validity of the assay and activity of receptor during the screen. Each compound was screened at three concentrations: 0.1, 1, and 10 μM. The overlay of binding sensorgrams is shown in the Supporting Information, Figure 1, together with report points read just before the end of injection for each analyte. Most of the compounds show responses at the highest concentrations for both active and blocked receptors, therefore identifying binders based only on the response in equilibrium is not a suitable method to distinguish between true and nonspecific ligands. Each sensorgram was therefore carefully inspected, and only compounds showing differences for binding modes between active and blocked receptor were selected for further confirmation. Hit confirmation was run in 3-fold concentration series at 0.3−25 μM. All concentrations were injected in duplicate and referenced for 1D4 mAb surface and blank injections of buffer to minimize nonspecific signal and possible baseline drift. A total of five hits in two chemical series were identified as binders to active form of CCR5 (Table 1). Sensorgrams and equilibrium fits for both active and blocked CCR5 are shown in Figure 2. The five hit

dx.doi.org/10.1021/ml2000017 |ACS Med. Chem. Lett. 2011, 2, 549–554

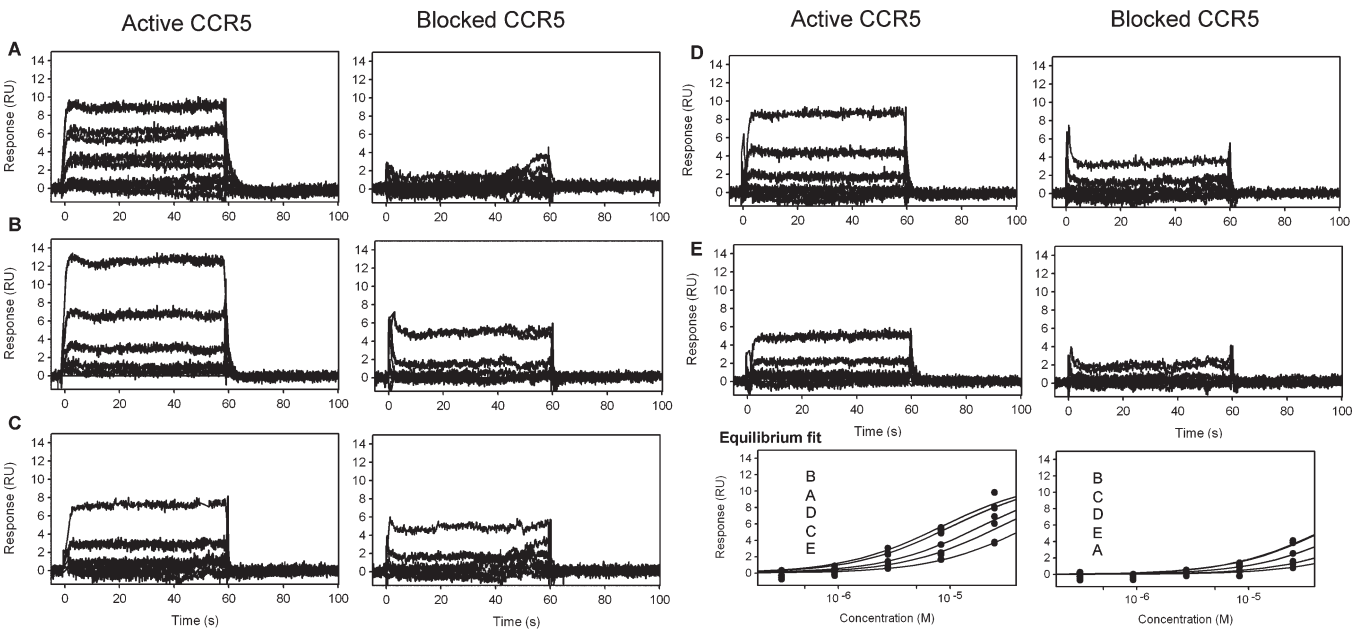


Figure 2. Sensorgrams for compounds A−E identified as hits binding to active and blocked CCR5. Each compound is injected in duplicate at 3-fold concentration series 0.3−25 μM. Equilibrium fits for affinity determination are shown as an overlay for all compounds binding to both active and blocked CCR5.

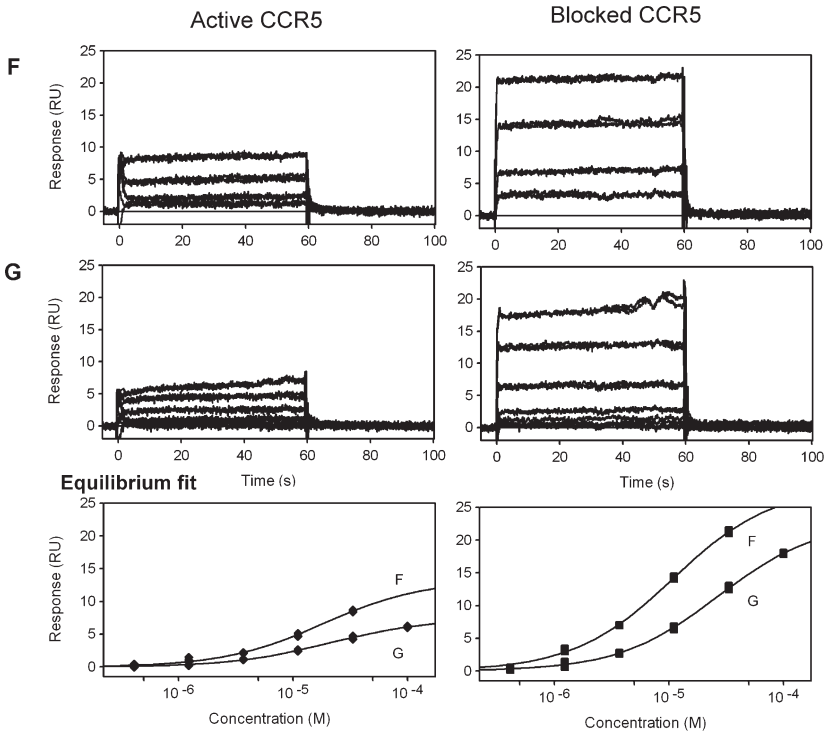


Figure 3. Binding responses for allosteric compounds F and G binding to active and blocked CCR5 and overlay of affinity fits for both compounds binding to active and blocked CCR5. Compounds were injected in duplicates at 3-fold dilutions and concentrations of 1.23−33.3 μM (compound F) and 1.23−100 μM (compound G).

compounds were counter-screened on the Biacore T100 against CXCR4 using our previously described SPR assay.[12−14] Except compound A, most of the compounds showed some response to blocked CCR5 at higher concentrations. At the highest concentration only, the five compounds also showed a small response against CXCR4 (Supporting Information, Figure 2). We observed the confirmed hits can be classified into two distinct chemical series: compounds A−D, consisting of one series, and compound E, representing second series. The ligand efficiencies (LE) for the hits are 0.24−0.335 kcal/mol/nonhydrogen atoms with an average

dx.doi.org/10.1021/ml2000017 |ACS Med. Chem. Lett. 2011, 2, 549–554

LE = 0.282. The best LE was measured for compound A (K_D = 10 μM, LE = 0.335). The LE of maraviroc is 0.275 due to its high molecular mass (MW = 514 Da). Interestingly, the average number of heavy atoms for compounds A−E is 23; therefore, the ratio of heavy atoms for maraviroc [37 nonhydrogen ("heavy") atoms] to the average hit is 1.6. The ratio of average heavy atoms of hits (23 heavy atoms) to the additional structure required for a potent compound (14 heavy atoms) is also 1.6. The ratio of 1.6 is significant as it is the Golden Ratio. An analysis of a number of fragment optimization projects reveals the ratio of the initial fragment to the final potent compound is usually the Golden Ratio.[18] Therefore, the hit compounds A−E can be considered as "fragment" ligands for CCR5 according to the golden ratio argument. Affinities, LE, and Bayesian prediction ranking are summarized in Table 1. Compound A has the highest Bayesian prediction of all of the confirmed hits.

Site-directed mutagenesis studies on CCR1,[19] CCR3,[20] and CCR5[21] have revealed a common binding pocket formed by the trans-membrane helices and exposed to the extracellular surface, equivalent to the rhodopsin binding site. However, a number of chemokine receptor antagonists and inverse agonists belong to distinct chemical classes, and evidence suggests that these may bind to distinct allosteric sites. To determine whether SPR is a suitable method to detect the binding of compounds to distinct allosteric binding sites on membrane proteins, we included compounds F and G in the screening library. The pyrazinyl sulfonamides compounds F and G are allosteric CCR4 antagonists that are reported to be weak CCR5 ligands.[10] Compounds were injected at 3-fold concentration series 1.2−100 μM over both active and blocked CCR5. Interestingly, compounds F and G showed approximately a 2-fold higher response values (R_{max}) when binding to blocked CCR5 over active CCR5, suggesting that maraviroc stabilizes CCR5 in a conformation that benefits the binding of compounds to the intracellular allosteric binding site[10] (Figure 3). The affinities of compounds F and G (Table 1) are approximately equivalent in the presence and absence of maraviroc.

We have developed a GPCR biosensor assay protocol for the Biacore 4000 platform that offers the opportunity for high-throughput label-free screens that directly measure GPCR−ligand interactions, independent of binding site. A biosensor-based direct screening method to identify interactions between orthosteric and allosteric ligands and solubilized GPCRs, in a label-free and cell-free environment, overcomes the limitations of indirect assay methods. A key advantage of the method is that it utilizes native GPCR sequences and thus does not require extensive protein engineering that is required for other more expensive and labor-intensive biophysical methods such as X-ray crystallography. The method that we have described can be a useful tool for confirming the binding and mode of action of hits from cell-based screens and a primary screening method in its own right for focused and fragment-like libraries.

■ ASSOCIATED CONTENT

Supporting Information. Details and experimental procedures for the Bayesian compound selection and CCR5 biosensor screening. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: i.navratilova@dundee.ac.uk.

Funding Sources

This work is funded by SULSA and the University of Dundee Devolved MRC DPFS Portfolio (G0900864/D037). The work benefits from the infrastructure investments from the Wellcome Trust Strategic Award (WT 083481) and the European Regional Development Fund.

■ ACKNOWLEDGMENT

We thank David Gray and Irene Hallyburton for the supply of the compound libraries and Natalia Shpiro for synthesis of the standard compounds.

■ REFERENCES

(1) Kenakin, T. Allosteric Theory: Taking Therapeutic Advantage of the Malleable Nature of GPCRs. *Curr. Neuropharmacol.* **2007**, *5*, 149–156.

(2) Keov, P.; Sexton, P. M.; Christopoulos, A. Allosteric modulation of G protein-coupled receptors: A pharmacological perspective. *Neuropharmacology* **2011**, *60* (1), 24–35.

(3) Watson, C.; Jenkinson, S.; Kazmierski, W.; Kenakin, T. The CCR5 Receptor-based mechanism of action of 873140, a potent allosteric non-competitive HIV entry-inhibitor. *Mol. Pharmacol.* **2005**, *67*, 1268–1282.

(4) Mansfield, R.; Able, S.; Griffin, P.; Irvine, B.; James, I.; Macartney, M.; Miller, K.; Mills, J.; Napier, C.; Navratilova, I.; Perros, M.; Rickett, G.; Root, H.; van der Ryst, E.; Westby, M.; Dorr, P. CCR5 Pharmacology Methodologies and Associated Applications. *Methods Enzymol.* **2009**, *460*, 17–55.

(5) Muniz-Medina, V. M.; Jones, S.; Maglich, J. M.; Galardi, C.; Hollingsworth, R. E.; Karzmierski, W. M.; Ferris, R. G.; Edelstein, M. P.; Chiswell, K. E.; Kenakin, T. P. The relative activity of "function sparing" HIV-1 entry inhibitors on viral entry and CCR5 internalisation: Is allosteric funtional selectivty a valuable therapeutic property?. *Mol. Pharmacol.* **2009**, *75* (3), 490–501.

(6) Dorr, P.; Westby, M.; Dobbs, S.; Griffin, P.; Irvine, B.; Macartney, M.; Mori, J.; Rickett, G.; Smith-Burchnell, C.; Napier, C.; Webster, R.; Armour, D.; Price, D.; Stammen, B.; Wood, A.; Perros, M. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob. Agents Chemother.* **2005**, *49* (11), 4721–4732.

(7) Urban, J. D.; Clarke, W. P.; von Zastrow, M.; Nichols, D. E.; Kobilka, B.; Weinstein, H.; Javitch, J. A.; Roth, B. L.; Christopoulos, A.; Sexton, P. M.; Miller, K. J.; Spedding, M.; Mailman, R. B. Functional selectivity and classical concepts of quantitative pharmacology. *J. Pharmacol. Exp. Ther.* **2007**, *320* (1), 1–13.

(8) Galandrin, S.; Oligny-Longpré, G.; Bouvier, M. The evasive nature of drug efficacy: Implications for drug discovery. *Trends Pharmacol. Sci.* **2007**, *28* (8), 423–430.

(9) Lane, J. R.; Powney, B.; Wise, A.; Rees, S.; Milligan, G. Protean agonism at the dopamine D2 receptor: (*S*)-3-(3-hydroxyphenyl)-*N*-propylpiperidine is an agonist for activation of Go1 but an antagonist/inverse agonist for Gi1,Gi2, and Gi3. *Mol. Pharmacol.* **2007**, *71* (5), 1349–1359.

(10) Andrews, G.; Jones, C.; Wreggett, K. A. An intracellular allosteric site for a specific class of antagonists of the CC chemokine G protein-coupled receptors CCR4 and CCR5. *Mol. Pharmacol.* **2008**, *73* (3), 855–867.

(11) Nicholls, D. J.; Tomkinson, N. P.; Wiley, K. E.; Brammall, A.; Bowers, L.; Grahames, C.; Gaw, A.; Meghani, P.; Shelton, P.; Wright, T. J.; Mallinder, P. R. Identification of a putative intracellular allosteric antagonist binding-site in the CXC chemokine receptors 1 and 2. *Mol. Pharmacol.* **2008**, *74* (5), 1193–1202.

(12) Navratilova, I.; Sodroski, J.; Myszka, D. G. Solubilization, stabilization, and purification of chemokine receptors using biosensor technology. *Anal. Biochem.* **2005**, *339* (2), 271–281.

(13) Navratilova, I.; Pancera, M.; Wyatt, R. T.; Myszka, D. G. A biosensor-based approach toward purification and crystallization of G protein-coupled receptors. *Anal. Biochem.* **2006**, *353* (2), 278–283.

(14) Navratilova, I.; Dioszegi, M.; Myszka, D. G. Analyzing ligand and small molecule binding activity of solubilized GPCRs using biosensor technology. *Anal. Biochem.* **2006**, *355* (1), 132–139.

(15) Rich, R. L.; Errey, J.; Marshall, F.; Myszka, D. G. Biacore analysis with stabilized G-protein-coupled receptors. *Anal. Biochem.* **2010**In press.

(16) Robertson, N.; Jazayeri, A.; Errey, J.; Baig, A.; Hurrell, E.; Zhukov, A.; Langmead, C. J.; Weir, M.; Marshall, F. H. The properties of thermostabilised G protein-coupled receptors (StaRs) and their use in drug discovery. *Neuropharmacology* **2011**, *60* (1), 36–44.

(17) Haworth, B.; Lin, H.; Fidock, M.; Dorr, P.; Strange, P. G. Allosteric effects of antagonists on signalling by the chemokine receptor CCR5. *Biochem. Pharmacol.* **2007**, *74* (6), 891–897.

(18) Orita, M.; Ohno, K.; Niimi, T. Two 'Golden Ratio' indices in fragment-based drug discovery. *Drug Discovery Today* **2009**, *14* (5−6), 321–328.

(19) Vaidehi, N.; Schlyer, S.; Trabanino, R. J.; Floriano, W. B.; Abrol, R.; Sharma, S.; Kochanny, M.; Koovakat, S.; Dunning, L.; Liang, M.; Fox, J. M.; de Mendonça, F. L.; Pease, J. E.; Goddard, W. A. r.; Horuk, R. Predictions of CCR1 chemokine receptor structure and BX 471 antagonist binding followed by experimental validation. *J. Biol. Chem.* **2006**, *281* (37), 27613–27620.

(20) Wise, E. L.; Duchesnes, C.; da Fonseca, P. C.; Allen, R. A.; Williams, T. J.; Pease, J. E. Small Molecule Receptor Agonists and Antagonists of CCR3 Provide Insight into Mechanisms of Chemokine Receptor Activation. *J. Biol. Chem.* **2007**, *282* (38), 27935–27943.

(21) Kondru, R.; Zhang, J.; Ji, C.; Mirzadegan, T.; Rotstein, D.; Sankuratri, S.; Dioszegi, M. Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. *Mol. Pharmacol.* **2008**, *73* (3), 789–800.