Progress in Biophysics and Molecular Biology 116 (2014) 113–123

Contents lists available at ScienceDirect

Progress in Biophysics and Molecular Biology

journal homepage: www.elsevier.com/locate/pbiomolbio

Original research

Fragment screening by SPR and advanced application to GPCRs

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ARTICLE INFO

Article history:
Available online 6 October 2014

Keywords:
Surface plasmon resonance
Fragment screening
GPCRs
G-protein coupled receptors
Biophysical screening
Membrane proteins
SPR

ABSTRACT

Surface plasmon resonance (SPR) is one of the primary biophysical methods for the screening of low molecular weight 'fragment' libraries, due to its low protein consumption and 'label-free' methodology. SPR biosensor interaction analysis is employed to both screen and confirm the binding of compounds in fragment screening experiments, as it provides accurate information on the affinity and kinetics of molecular interactions. The most advanced application of the use of SPR for fragment screening is against membrane protein drug targets, such G-protein coupled receptors (GPCRs). Biophysical GPCR assays using SPR have been validated with pharmacological measurements approximate to cell-based methods, yet provide the advantage of biophysical methods in their ability to measure the weak affinities of low molecular weight fragments. A number of SPR fragment screens against GPCRs have now been disclosed in the literature. SPR fragment screening is proving versatile to screen both thermostabilised GPCRs and solubilised wild type receptors. In this chapter, we discuss the state-of-the-art in GPCR fragment screening by SPR and the technical considerations in performing such experiments.

1. Introduction to fragment screening

Fragment-based drug discovery (FBDD) has a proven ability to contribute to the discovery of approved drugs and advanced investigational drugs (Congreve et al., 2008; Schulz and Hubbard, 2009; Chessari and Woodhead, 2009). Over the past 15 years, FBDD, has become one of the primary strategies for discovering small molecule ligands, alongside analogue-based drug design (Wermuth et al., 2006), structure-based drug design (Blundell, 1996) and high-throughput screening (HTS) (Spencer, 1998; Pereira and Williams, 2007). The principles of FBDD are based on three key tenets. The first tenet is that a small number of low molecular weight compounds – (known as 'fragments') can represent large areas of chemical space (Pollack et al., 2011). The second tenet is that as the molecular weight and thus complexity of a molecule increases, the probability of an unfavourable interaction also increases (Hann et al., 2001). Thus, low molecular weight fragments are proposed to be less selective than larger compounds and are therefore capable of binding to a larger number of proteins than conventional 'drug-size' compounds (Hopkins et al., 2006). In contrast to conventional high-throughput screening, where compound libraries of 100,000 to 1,000,000 are commonly screened, FBDD usually involves screening small libraries containing only 500 to 2000 compounds. However, the primary difference between conventional HTS compound collections and fragment screening libraries is the molecular mass of the constituent compounds and their expected affinities. The compounds screened in HTS are typically of molecular mass 350 Da–500 Da, equivalent to 27 to 38 non-hydrogen atoms (Hopkins et al., 2004). The expected affinity for 'hit' compounds detected in a conventional HTS assay is in the nanomolar to low micromolar range. However, compounds selected for fragment screening are low molecular weight compounds of 8–23 non-hydrogen atoms, equivalent to 100 Da–300 Da ('fragments') with the expected affinities of 'hits' ranging from micromolar to millimolar (Hajduk and Greer, 2007). The third tenet is that despite the low binding affinity that is often observed for fragment 'hit' molecules, compounds can be optimised into high affinity ligands. To improve affinity and selectivity of the initial 'hit' fragments, the optimisation process tends to increase the molecular mass and atom count. With the need to add a significant additional number of 'heavy' atoms (*i.e.* non-hydrogen atoms) in order to optimise a fragment into a potential investigational drug candidate, the scale of the optimisation process could be considered daunting. To overcome this challenge efficiently, fragment-based drug discovery is usually informed by structure-based drug design methods. The determination of X-ray crystal structures of the fragments bound to their target protein complexes is often crucial to enable efficient optimisation of fragments. Despite the major difference in molecular mass between fragments and their resulting optimised compound, it is not uncommon in the

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http://dx.doi.org/10.1016/j.pbiomolbio.2014.09.008

field for clinical drug candidates with high affinities to be optimised in a highly efficient manner.

The low molecular mass of fragment compounds tends to result in binding interactions with low affinity, thus compounds require screening at high concentrations to be detected. High concentration screening of fragment libraries using conventional biochemical assays has been undertaken. However, secondary biophysical screening is needed to confirm and validate genuine hits, due to the large false positive hit rate encountered in high concentration biochemical assays. Therefore, in order to detect low affinity interactions, fragment screening usually employs highly sensitive biophysical techniques as primary screening methodologies. The range of biophysical technologies applied to fragment screening includes various NMR methods (Shuker et al., 1996; Fejzo et al., 1999; Mayer and Meyer, 1999; Dalvit et al., 2000; Vanwetswinkel et al., 2005), direct observation by X-ray crystallography of crystals exposed to mixtures (Congreve et al., 2003) and solutions (English et al., 1999) of fragments, mass spectrometry, isothermal titration calorimetry (Ladbury et al., 2010), protein thermal shift (Kranz et al., 2011), affinity capillary electrophoresis (Lewis et al., 2004), weak affinity chromatography (Duong-Thi et al., 2011) and label-free biosensor methods (Pröll et al., 2009; Rawlins, 2010) such as Optical Waveguide Grating (OWG, Corning Epic) and reflectometric interference spectroscopy (RIfS, ForteBio Octet). However, in recent years surface plasmon resonance (SPR) has emerged as one of the most popular and widely applied fragment screening technologies and it is application of SPR to fragment screening that we shall primarily concern ourselves within this chapter.

2. Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical biosensor detection method that measures the change in refractive index at the surface interface that occurs during a binding event (Huber and Mueller, 2006). SPR is classified as a biophysical, 'label-free' method as it quantifies the direct molecular interactions, without the need for inferring the measurement indirectly via the displacement of a 'labelled' ligand (*i.e.* radioisotope or fluorescence label). SPR biosensors measure the interactions between molecules immobilised on the biosensor surface and molecules in solution, in real time. The immobilisation of molecules to the biosensor surface can be achieved using covalent coupling methods such as amine, thiol and aldehyde coupling, for example, or by non-covalent coupling methods employing metal chelation, monoclonal antibody tags or other affinity tags, such as biotin/streptavidin.

SPR biosensor interaction analysis is employed to both screen and confirm the binding of compounds in fragment screening experiments, as it provides accurate information on the affinity, kinetics and even the thermodynamics of the fragment-protein binding interaction. SPR biosensor assays can be used to determine a number of interaction parameters that characterise the formation of a molecular complex: association rate (k_a); dissociation rate (k_d); affinity (K_D) (by equilibrium or kinetic analysis) and the stoichiometry of the interaction.

Analysis of binding interactions by SPR can identify hit compounds with slow dissociation rates (Navratilova and Hopkins, 2010). Drugs with slow dissociation rates often show superior clinical efficacy (Swinney, 2008). Therefore, the ability to identify fragments with slow dissociations rates may help prioritise starting points for lead optimisation. SPR analysis can also be used to determine the thermodynamic parameters of the interaction (ΔG, ΔH and ΔS) using the van't Hoff equation, by measuring the K_D over a temperature range (Myszka, 2000; Navratilova et al., 2007; Papalia et al., 2008). The equilibrium dissociation and thermodynamic constants determined by SPR analysis correlate extremely well with the values determined using isothermal titration calorimetry, with the use of significantly less protein (Navratilova et al., 2007). This facility has not yet been widely adopted in SPR fragment validation and confirmation studies but may provide an interesting area for development as it has been argued that fragments that bind with predominately enthalpic energies may superior starting points for optimisation (Freire, 2008).

3. SPR fragment screening

SPR biosensor assays are often used in fragment-based drug discovery as an orthogonal, complementary technique to confirm and validate the direct measurement of the kinetics and affinity of fragment hits discovered by other screening methods (Huber, 2005; Geschwindner et al., 2007; Godemann et al., 2009; Cole et al., 2010). However, in recent years SPR biosensor screening has become a primary screening method for fragment-based drug discovery (Giannetti, 2011), as it has several practical advantages over other techniques.

SPR analysis provides both the throughput and sensitivity for conducting the high throughput screening of fragment libraries as well as the confirmation and validation of the fragment hits, using a single methodology. SPR analysis is also able to provide accurate measurements of affinity and kinetics to aid the identification and prioritisation of fragment hits.

Additionally, SPR assay development and screening can often be developed quickly, compared to developing biochemical HTS assays or generating high resolution, crystallographic systems. Thus, fragment binding information can be acquired quickly and early in a drug discovery programme. Such fragments may themselves aid crystallisation. The speed of assay development is also aided by the low protein consumption of SPR fragment screens. Entire SPR fragment screening projects, including assay development, the screening campaign and hit validation require as little as 25–100 μg of protein. The protein consumption of an SPR fragment screening campaign can be 10-fold–1000-fold less than is required by other biochemical and biophysical fragment screening methods. If purified, untagged protein is available, direct coupling methods can be used to immobilise the target protein on the biosensor surface. However, the success of developing directly coupled SPR assays increases if the target protein is purified to the high standards required for other biophysical techniques, such as crystallisation. The engineering of assay protein with specific affinity tags can aid purification on the SPR chip itself by immobilisation of the target protein onto the biosensor surface, even for unpurified tagged protein from cell pellets or lysates.

The throughput of the current generation of marketed biosensor instruments enables biosensor-based screening of fragment libraries in a timeframe of around one day to two weeks. The total experimental time is determined by the number of compounds in the fragment library, number of concentration measurements screened per compound, the stability of the protein and type of instrument. For example using stable, purified or tagged protein immobilised on a high-throughput GE Biacore 4000 or a Sierra Sensors MASS-1 instrument, with appropriate test ligands for assay development, it is entirely possible to screen and confirm fragment hits from a few micrograms of protein within a few days.

4. Experimental design considerations

The objective of SPR assay design, in order to run a high throughput biophysical screen on large numbers of compounds, is

to distinguish actual binding from non-specific binding Giannetti, 2011, Danielson, 2009. In order to achieve this objective, the SPR assay needs to be designed and validated to accurately determine the binding parameters by minimising the false positive rate and the false negative rate. Details on SPR fragment screening protocols are described by Giannetti (2011), Danielson (2009) and Navratilova and Hopkins (2010).

Successful SPR fragment screening is dependent on the implementation of data quality control methods and the scaling and normalisation of the primary screening data in order to identify hits and remove false positives. Experimental design can reduce the false positive data detection rate by designing an appropriate referencing protocol for the data. An important aspect of experimental design is to distinguish between actual and non-specific binding, in the data analysis, by referencing against parallel surfaces (Navratilova and Hopkins, 2010). On instruments with multiple biosensor channels, parallel immobilisation of the target protein, reference proteins and the inclusion of a blank channel as a reference surface is recommended. The shape of the sensorgram itself also provides important information on whether a binder is likely to be genuine or non-specific.

The false negative data detection rate is determined by the sensitivity of the assay set-up to detect a true binding event and the sensitivity of the biosensor instrument itself. The latest generation of SPR biosensors instruments can detect small molecules with molecular mass as low as 50 Da. However, the detection limit is a function of the density of active protein immobilised on the surface. The activity of the surface is represented by the maximum binding response (R_{max}) of ligand bound to the immobilised protein upon saturation of all binding sites on the surface. The magnitude of R_{max} that can be recognised as the minimal detection level depends upon the signal-to-noise ratio. Modern instruments, such as the GE Biacore T200, can obtain quality responses at magnitudes below 1 RU (Response Units), due to very low baseline noise levels.

The minimum detection limit for identifying fragment hits by SPR not only depends on the sensitivity of the instrument, but also the noise of fragment binding responses. In affinity determination experiments, each compound (or fragment) is injected in a concentrations series separated by blank injections of buffer, to enable the subtraction of the injection drift of the system in order to obtain good quality responses. In the design of fragment screening experiments, blanks are usually injected at every eight or more cycles of fragment injections, to increase the overall throughput of the screening (Fig. 1). However, the lower number of blank injections can introduce issues with drift in the referencing or sample carryover and may therefore result in increased noise of responses for both binders and non-binders. Determining the behaviour of a fragment in a dose response experiment can identify compounds with super-stoichiometric behaviour, which can be a symptom of non-specific binding.

The ligand's affinity to the immobilised target protein and the molecular mass detection limit of the biosensor instrument also contribute to the sensitivity of the assay. To saturate the binding sites on the surface, the fragment has to be ideally injected at a significantly higher concentration than its binding affinity. However, as fragments may have binding affinities in the high micromolar to millimolar range it may not be practically feasible to saturate the protein surface with the fragment. A further complication that limits high concentration screening is the common observation that many fragments may aggregate and bind non-specifically at high concentrations. Therefore, for fragments, the responses may be measured at concentrations at or far below their binding affinities. The R_{eq} equation can be used to estimate the affinity cut off for fragments to be detected at equilibrium (R_{eq})

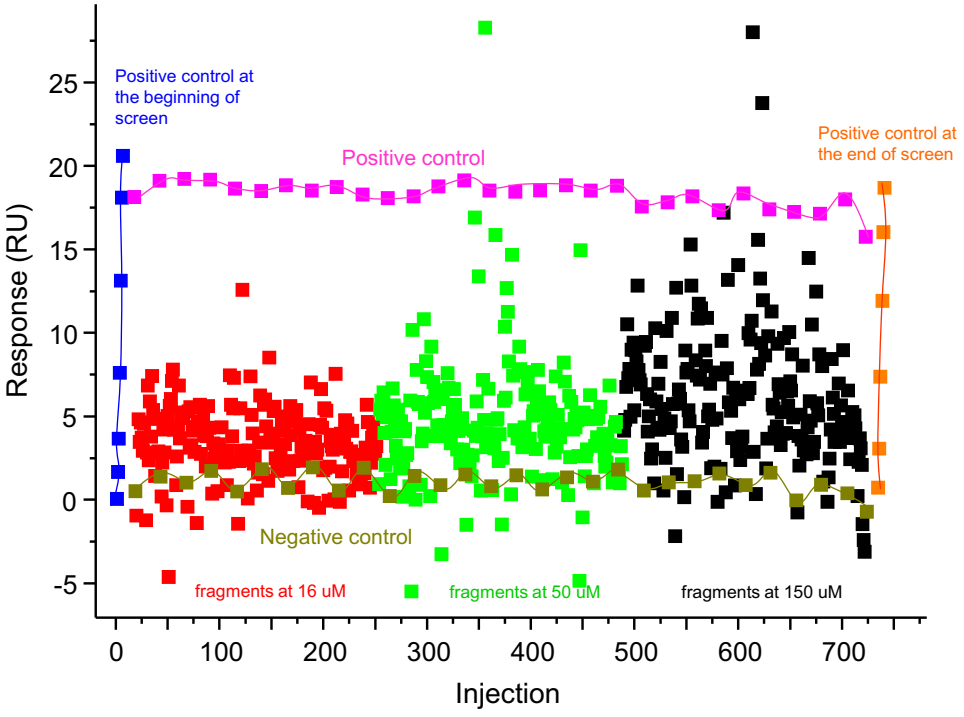


Fig. 1. Example of SPR fragment screening. Plot of responses per injection measured at equilibrium for fragments binding to the kinase SAP2K protein. Control compounds were injected during the screen (positive (SB 220025), pink, and negative (furosemide), dark yellow) and a positive control was also injected in concentration series at the beginning of the screen (blue) and end of the screen (orange). Each fragment was injected at three concentrations 16.6, 50 and 150 μM (red, green and black squares). The noise of the responses increases towards the higher fragment concentrations, however combining the data for each fragment at all three concentrations provides valuable information to distinguish between specific and non-specific binding (Data not shown).

based on R_{max} of control compound binding and the known screening concentration (c).

$$R_{eq} = \frac{R_{MAX} \times c}{K_D + c}$$

The R_{eq} equation determines that if the signal-to-noise ratio sets the cut-off for binders and non-binders at 5 RU, the screening concentration is 150 μM, and the R_{max} is 25 RU, it is possible to detect all compounds with affinities $K_D < 600$ μM with confidence. However, the detection limit can be increased by increasing the R_{max}, by immobilising a high density of active protein on the biosensor surface. The ability of well designed SPR assays to detect binders with binding affinities far above the actual screening concentration enables screens to be run at lower concentration than commonly used in many other fragment screening methods. The advantage is that, by screening fragments at lower concentrations, much of the non-specific binding events that are observed during a screening run at higher concentrations can be eliminated.

A further consideration in assay design is that some fragments have a high refractive index that can add to the noise level due to mismatch of refractive indexes between blanks and samples. However, this can be minimised by performing 'clean screens' to remove potentially problematic fragments. Additionally, the solvent dimethylsulfoxide (DMSO), which is required to maintain solubility of fragments during screening, has a high refractive index and can be major source of false positives if the concentration is not matched accurately between the samples and running buffer (Papalia et al., 2006). Overall, the minimal level of detection of fragment hits in a screen, based on the RU response, has to be adjusted for each screen separately, based on binding of positive and negative controls during the screen.

5. GPCR fragment screening

SPR fragment screening has a wide range of applications for the screening of diverse soluble proteins. However, the most advanced application is its use for fragment screening against membrane protein drug targets, such as guanine nucleotide–binding protein coupled receptors (GPCRs). GPCRs represent an important therapeutic target class as they are involved in the regulation of a variety of biological processes, such as; inflammation, neurotransmission, cellular metabolism, differentiation, growth, and secretion. In fact, around 30% of all marketed drugs target GPCRs (Overington et al., 2006). Despite their critical importance, application of biophysical and structural methods, and hence fragment based drug discovery, to GPCRs has been limited, largely because of their low expression levels and instability in detergent solutions.

Traditionally, hit identification for GPCR targets has relied upon cell-based assays measuring downstream signalling responses, or ligand binding assays measuring displacement of the orthosteric ligand, coupled with high-throughput screening (HTS) of large libraries of compounds (Houston et al., 2008). Evidently, this approach has successfully delivered compounds that have made it to the market; however the majority of these drugs act via a narrow range of receptors; mostly family A receptors that are activated by small molecules (Congreve et al., 2011a). On the other hand, hit identification for many clinically relevant and validated targets, such as peptide and protein hormone binding receptors, has been challenging. In the past decade, only ten first in class small molecule GPCR drugs have been brought to market (Congreve et al., 2011a). Furthermore, it is often difficult to achieve receptor subtype specificity; therefore the discovery of small molecules with novel mechanisms of action, such as allosteric modulators, could be beneficial.

Fragment based drug discovery could represent a powerful alternative compared to traditional HTS approaches for the discovery of novel GPCR ligands. As discussed previously, fragment screening allows greater sampling of chemical space and identifies low affinity, ligand efficient hits as starting points for the development of high-affinity compounds (Hopkins et al., 2004). High concentration screening coupled with conventional ligand displacement assays has been used for the fragment screening of several receptors (Verheij et al., 2011; Stoddart et al., 2012), and revealed remarkable similarities between the G protein-coupled receptor histamine H-4 and the ion channel serotonin 5-HT3A (Verheij et al., 2011). However, these assays often lack the sensitivity required to detect weakly binding fragments, only detect compounds that compete with the orthosteric ligand, and the high screening concentration often leads to artefacts in the data. To detect such weak binding, biophysical techniques such as NMR, SPR or X-ray crystallography are often employed. However, application of these techniques to membrane proteins is often challenging due to difficulties in obtaining sufficient amounts of pure, stable and functionally folded protein. In this context, SPR represents an attractive option for the fragment screening of GPCRs since the protein requirements are less than that of NMR or X-ray crystallography. Additionally, it is possible to capture receptors directly from crude solubilisation extracts, avoiding lengthy purification procedures during which functionality may be lost. SPR can also be used to screen for solubilisation conditions that increase receptor stability and functionality (Navratilova et al., 2005). Recently, fragment screening by SPR has been applied to both thermostabilised GPCRs (StaR®s) (Congreve et al., 2011b; Christopher et al., 2013) and wild-type tagged receptors (Navratilova et al., 2011; Aristotelous et al., 2013).

6. Fragment screening of stabilised GPCRs by SPR

Fragment screening of two thermostabilised receptors (StaR®s), the adenosine A_{2A} receptor and the β_1 adrenergic receptor, has recently been achieved using SPR (Congreve et al., 2011b; Christopher et al., 2013). StaR®s are GPCRs that have been engineered to improve their thermostability by introducing a number of point mutations (Magnani et al., 2008; Serrano-Vega et al., 2008; Shibata et al., 2009; Robertson et al., 2011). During development of a StaR, the receptor is stabilised in either the agonist or inverse agonist/antagonist conformation in complex with a suitable ligand. These receptors can be produced in greater quantities and show increased stability in detergent solution when compared with wild-type receptors. Therefore, generation of StaRs enables application of biophysical techniques, and hence FBDD, to GPCRs.

In the case of the adenosine A_{2A} receptor ($A_{2A}R$), the StaR contained four point mutations; A54L, T88A, K122A and V239A (Magnani et al., 2008). Both affinity-purified and solubilised StaRs were captured on a charged NTA sensor chip via a C-terminal His-10 tag (Congreve et al., 2011b). Binding of the antagonist, xanthine amine congener (XAC), demonstrated that capture from crude solubilisation mixtures alone produced an active $A_{2A}R$ surface that could be used to characterise antagonist binding, whilst eliminating the affinity-purification step. A panel of around 80 xanthine derivatives and unrelated compounds (136–194 Da) were screened at a single concentration of 200 μM and eight of these (~10%) were identified as potential hits with affinities ranging from 10 μM to 5 mM. This study demonstrated that SPR could be used to reliably detect fragment-sized hits and weak binders for stabilised GPCRs.

Following this proof of concept study, it was shown that fragment screening using SPR can be integrated into a true FBDD approach to identify novel high affinity leads for the β_1-adrenergic receptor (β_1AR) (Christopher et al., 2013). Thermostabilised β_1AR

contained mutations R68S, M90V, Y227A, A282L, F327A and F338M (Serrano-Vega et al., 2008). The immobilisation protocol involved capture-coupling of receptors with C-terminal His-10 tags and a "displacement regeneration" step, involving injection of a weak affinity compound, was included to regenerate the surface (Rich et al., 2011). A panel of approximately 650 fragments were screened in parallel against the β_1AR and A_{2A}R StaRs at a single concentration of 50 μM. A subset of fragments that bound more significantly to β_1AR than A_{2A}R were then re-tested in a 2-fold dilution series starting at 150 μM. Among these fragments were arylpiperazine hits 7 and 8, which were estimated to have good binding affinities and ligand efficiency (K_D = 16 μM and 5.6 μM, LE = 0.41 kcal/mol/non-hydrogen-atom and 0.48 kcal/mol/non-hydrogen-atom, respectively). A set of analogues of a similar size and complexity were then screened in an orthogonal radioligand membrane binding assay with wild-type β_1AR; all had high ligand efficiencies and moderate to high affinity.

Although recent advances in the availability of GPCR crystallographic information have enabled the use of structure based drug design strategies for GPCRs (Congreve et al., 2011a), the structure of human β_1AR has not yet been solved. Therefore, to complement the binding information, the fragments were docked into the liganded structure of turkey β_1AR (Warne et al., 2012), which is 82% identical in the transmembrane and loop regions and 100% identical in the ligand binding pocket compared to human β_1AR. It is particularly challenging to dock fragments into a receptor binding site due to their small size and numerous potential binding modes; therefore a complete druggability analysis of the binding site was carried out to identify the region most likely to be occupied by the fragment. This approach enabled a structure-based approach to select additional compounds and expand SAR in the series. Two high affinity and ligand efficient fragments were identified; indole 19 and quinolone 20. These were co-crystalized with the stabilised β_1AR and resulted in structures determined to 2.8 and 2.7 Å respectively. Interestingly, neither structures showed the change in rotamer conformation of Ser215$^{5.46}$ that is usually observed when a partial/full agonist is bound (Warne et al., 2011). This may suggest that the fragments are antagonists, although this remains to be determined in a functional assay. This example demonstrates how SPR can be used as a primary fragment screening technique for stabilised GPCRs, as well as how orthogonal assays and receptor modelling can then be used to optimise these fragments. Additionally, the use of stabilised receptors has allowed the binding mode of the resulting high affinity fragments to be determined by X-ray crystallography.

A limitation of using thermostabilised receptors is that the receptor is stabilised in the agonist/antagonist bound conformation, which alters the pharmacology of the receptor. For example, antagonist stabilised A_{2A}R has been shown not to bind agonists and the agonist stabilised receptor binds antagonists with a five-fold lower affinity than that of the wild-type receptor (Magnani et al., 2008; Robertson et al., 2011). Therefore, an understanding of the receptor's pharmacology and desired pharmacological profile is required early on in the drug discovery process. A known ligand must also be available to aid the stabilisation process; this could be limiting when there are no tool compounds available. The process of generating a StaR may also be time consuming, as hundreds of point mutations are initially tested, followed by recombination experiments to identify the optimal combination of stabilising mutations. Furthermore, reducing conformational flexibility could mean that compounds acting by novel mechanisms could be missed in the screening process. However, there is evidence that it allosteric fragments can be identified using StaRs by target immobilised NMR screening (TINS) (Chen et al., 2012).

7. Fragment screening of wild-type GPCRs by SPR

As well as utilising SPR as a primary fragment screening technique for stabilised GPCRs, two examples of SPR screening of wild-type receptors, each employing different immobilisation strategies, have been reported (Navratilova et al., 2011; Aristotelous et al., 2013). One immobilisation approach using solubilised receptors is shown in Fig. 2. Screening against the wild-type receptor could be advantageous as the receptor retains its native pharmacology; for example, immobilised wild-type CCR5 has been shown to bind agonists and antagonists with affinities in agreement with those determined for the membrane associated receptor (Fig. 3) (Mansfield et al., 2009). However, optimising solubilisation and

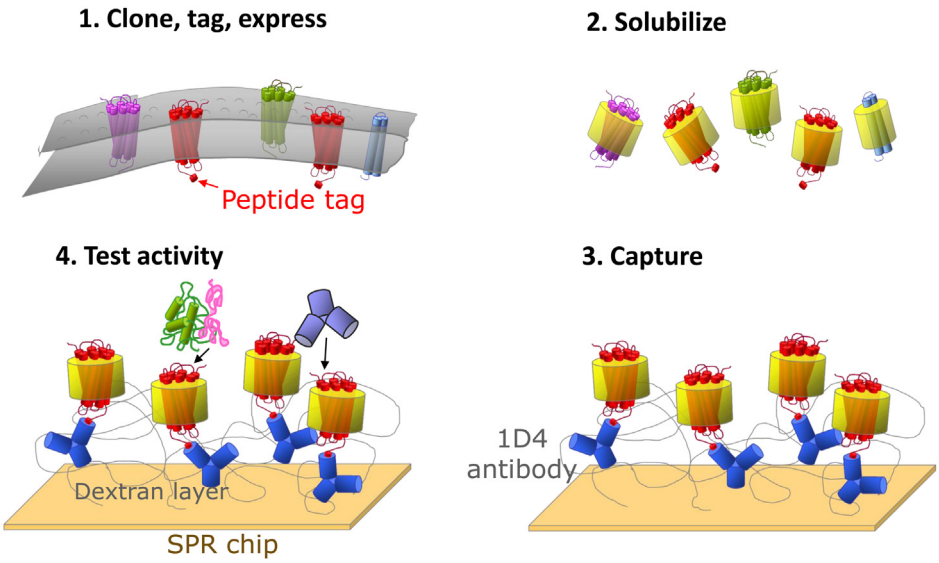


Fig. 2. Design of GPCR biosensor assay. 1) Expression of a C-terminally C9-tagged GPCR in a mammalian cell line. 2) Detergent-solubilisation of GPCR 3) Immobilisation of a 1D4-capturing antibody on the carboxymethyldextran matrix that coats the sensor chip surface and capture of a detergent-solubilised GPCR expressed with a C-terminal tag by the 1D4 antibody. 4) Activity binding tests performed using conformation-dependent probes.

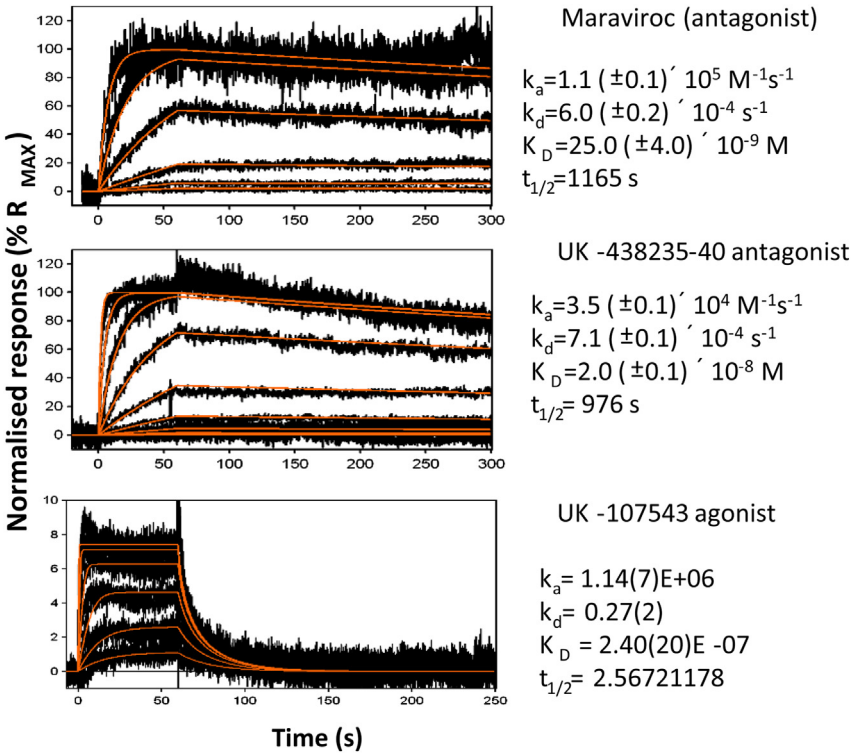


Fig. 3. SPR analysis of GPCR ligands. SPR measurement of the binding kinetics of chemokine receptor 5 (CCR5) ligands binding to wild type solubilised CCR5. Sensorgrams of the binding of antagonists (*e.g.* maraviroc and UK-438235) and agonist (UK-107543) to CCR5. Red lines represent kinetic fit.

assay conditions is far more challenging for wild-type receptors due to a lack of stability upon removal from the membrane.

Navratilova et al. have developed a high-throughput direct-binding SPR screening assay for wild-type CCR5, which provided evidence that fragment screening of wild-type receptors is technically feasible (Navratilova et al., 2011). The immobilisation strategy utilises a highly specific interaction between the final nine C-terminal amino acids of rhodopsin (C9 tag) and the 1D4 antibody (Navratilova et al., 2005). The 1D4 antibody is immobilised using amine coupling and the wild-type receptor, expressed with a C-terminal C9 tag, is captured directly from the solubilised cell pellet. Solubilisation conditions can also be optimised using SPR; a systematic detergent/lipid screen is performed to identify conditions that maximise the amount of receptor solubilised, whilst maintaining ligand binding ability (Navratilova et al., 2005). This protocol enables purification and immobilisation of the receptor in a single step.

To reduce the effect of non-specific binding, and therefore reduce the number of false positives identified, it is important to use a suitable reference surface, particularly when immobilising from a crude solubilisation mixture. Suitable surfaces may include inactive membrane protein, solubilised in a deactivating detergent; an unrelated receptor, as demonstrated by Christopher et al., or receptors where the binding site is blocked by a high affinity ligand.

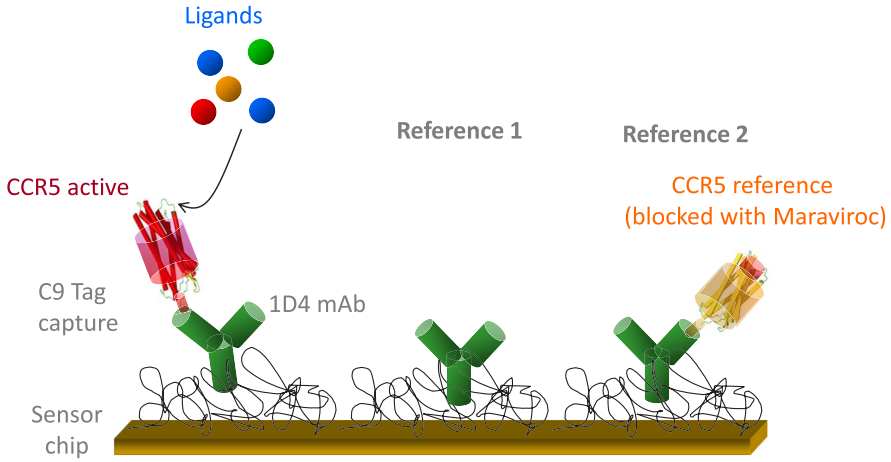


Fig. 4. Experimental set-up for ligand screening on CCR5 G-protein-coupled receptor. CCR5 was solubilised and captured via a C-terminal C9 tag onto 1D4 mAb immobilised on the sensor surface. One-flow cell carried active CCR5, reference flow-cells contained 1D4 mAb only and also CCR5 with binding site blocked by maraviroc. Ligands were then injected over all flow cells to determine binding.

However, it is also important to balance reducing the number of false positives with the risk of eliminating a compound with a novel mechanism of action; *i.e.* allosteric binders which may bind to the 'blocked' receptor or fragments that also bind to an unrelated receptor. Two reference surfaces were included in the CCR5 screen; 1D4 antibody alone and 1D4 with CCR5 captured but blocked with the tight-binding antagonist, maraviroc (Navratilova et al., 2011) (Fig. 4). Two hundred compounds were prioritised from a library containing over 90,000 compounds by Bayesian activity modelling. The majority of these compounds were expected to bind to the same site as maraviroc, making the blocked receptor a suitable reference surface. Five novel ligands were identified from the screen (K_D = 8 μM–49 μM), the binding of which was blocked by maraviroc (Fig. 5).

The ability of the assay to detect compounds binding to allosteric sites was also tested. Two pyrazinyl sulphonamide CCR4 antagonists with weak affinity for CCR5 were included in the screen; mutagenesis studies have shown that these compounds bind to a distinct intracellular allosteric site on the chemokine receptors (Andrews et al., 2008). Interestingly, these compounds showed an increased response for maraviroc bound CCR5 compared to *apo* CCR5, although the K_D was similar in the presence and absence of maraviroc (Fig. 5). This shows that the SPR direct binding assay is capable of detecting allosteric compounds, although care needs to be taken not to exclude these as 'non-specific binders' if a blocked receptor is used as a control surface.

Most of the fragments screened against CCR5 had molecular weights between 260 Da and 350 Da, so fitted the 'rule of three' concept for fragment library selection but were larger than many commercial fragment libraries (Fig. 6). The sensitivity of the assay would need to be increased to enable the detection of fragments found in typical fragment libraries, with molecular weights between 150 and 250 Da. This could be achieved by increasing the amount of active receptor on the surface by; optimising solubilisation conditions, increasing receptor expression levels or exploring alternative capture methods.

Recently, a fragment library with an average molecular weight of 187 Da was successfully screened against the β2-adrenergic receptor (β2AR) using a different immobilisation approach (Aristotelous et al., 2013). The wild-type receptor was expressed in *Sf*9 cells and purified before being captured on a charged NTA sensor-chip via a C-terminal His-10 tag (Fig. 7). The pharmacological activity of the immobilised receptors was then characterised by measuring the binding of an agonist and antagonist, fenoterol and alprenolol respectively (Fig. 7); the affinity which correlated well to

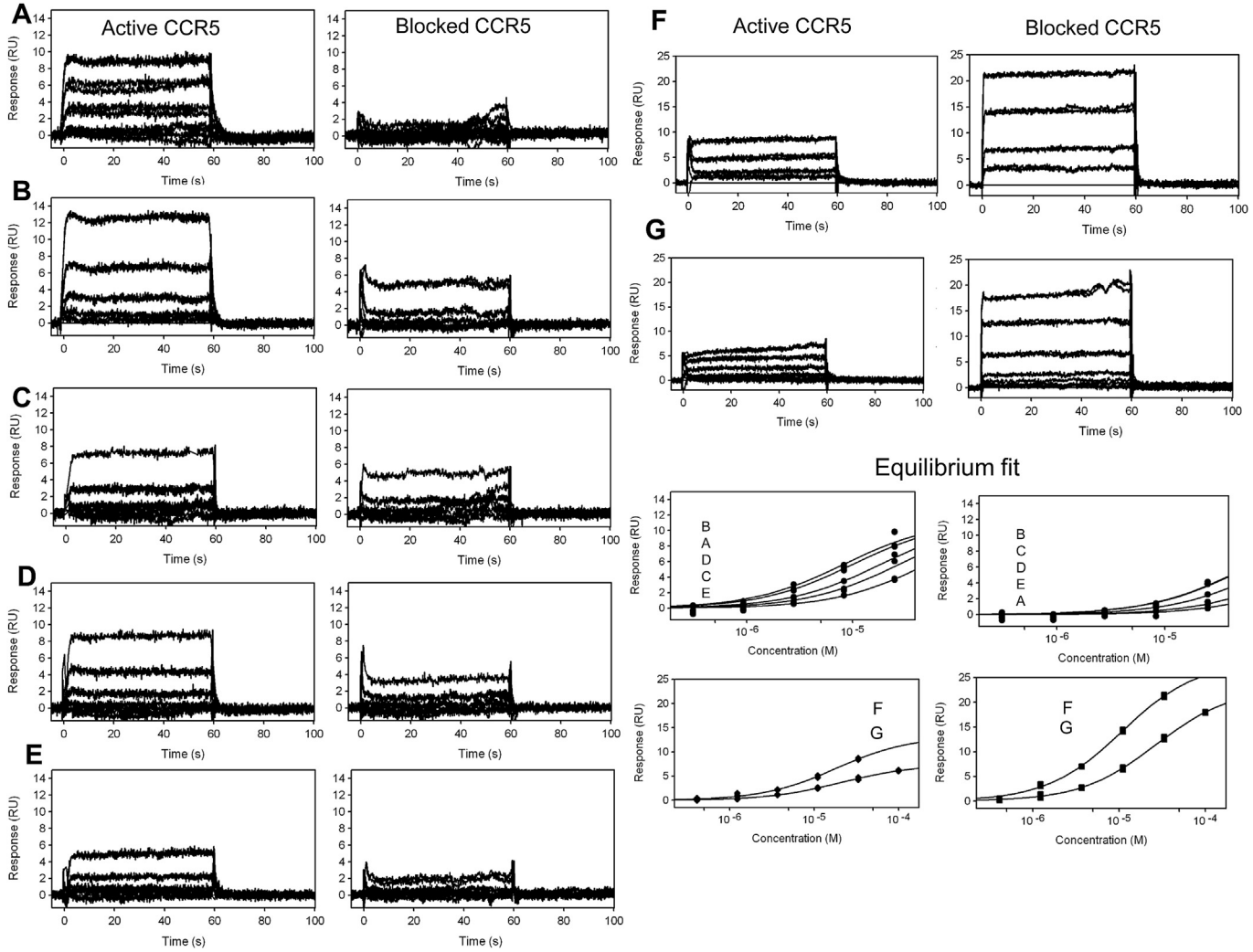
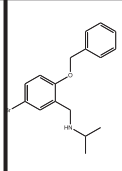
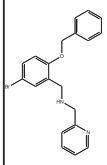
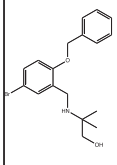
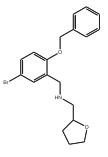
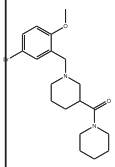
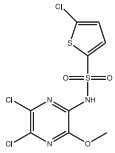
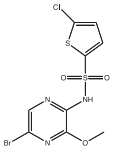


Fig. 5. SPR sensorgrams of novel CCR5 ligands. Compounds A to G identified from the SPR screen bound to active and maraviroc 'blocked' CCR5. Compounds F and G are known CCR4 ligands which show binding to CCR5. Compounds F and G show an increased response for maraviroc bound CCR5 compared to *apo* CCR5, although the K_D was similar in the presence and absence of maraviroc.

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)*

* Affinity measured to blocked CCR5

Fig. 6. CCR5 ligands. Chemical structures and binding affinities (K_D) of novel CCR5 ligands discovered during SPR screening. The average number of heavy atoms for compounds A to E is 23. The mean molecular weight of 200 compound library screened in this experiment was 362Da.

the affinity measured by radioligand binding assays (Aristotelous et al., 2013; January et al., 1997).

A fragment library containing 656 fragments with molecular weights between 94 and 341 Da (7–24 heavy atoms) was screened at a single concentration against immobilised β2AR (Fig. 8). As with CCR5, two reference surfaces were utilised to reduce the rate of false positives; a blank reference surface and β2AR pre-incubated with a slow off rate antagonist, BI-167107. Eighty one fragments were identified as potential hits and tested at six concentrations between 1.2 and 300 μM; five were confirmed with affinities ranging from K_D = 17 nM – 22 μM (Fig. 9). The effect of different detergents, DDM and MNG, on binding of these fragments was also tested and found to be minimal.

A radioligand competition binding assay suggested that all five fragment hits occupied the orthosteric binding site as they showed competitive inhibition of [^{125}I]-cyanopindolol. This may suggest that the fragments bind in the orthosteric binding pocket, however allosteric compounds can also displace the orthosteric ligand via conformational changes. The ligand binding pocket of β1AR and β2AR are highly conserved; therefore the selectivity of these fragments was examined using a radioligand competition binding assay for β1AR. Four of the fragments showed nonselective binding,

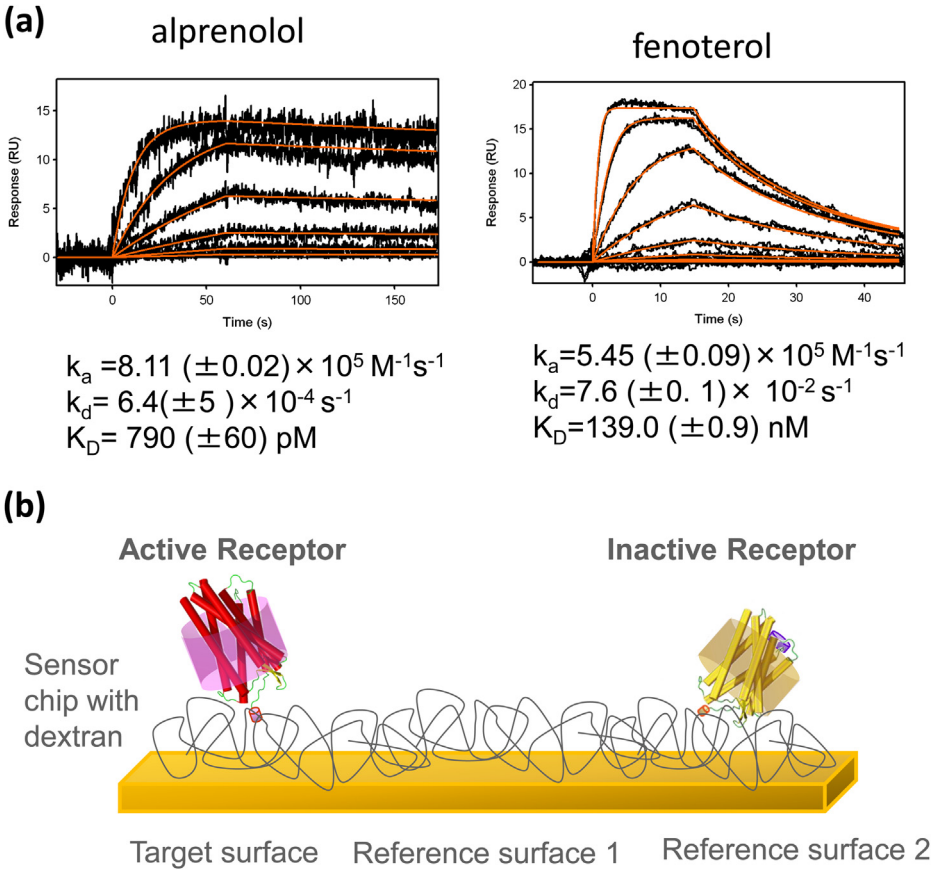


Fig. 7. β2 adrenergic receptor SPR screen. (a) SPR sensorgrams of the antagonist alprenolol and the agonist fenoterol binding to immobilised, wild-type human β2 adrenergic receptor. Red lines represent kinetic fit. (b) Experimental set-up for fragment screening on the human β2 adrenoceptor. A human β2 adrenoceptor construct containing a FLAG tag at the N-terminus and histidine 10 (His-10) tag at the C-terminus was generated for baculovirus expression in *Sf*9 cells. The purified and solubilised human β2 adrenoceptor was captured via the His-10 tag on an NTA sensor chip. The β2 receptor was pre-incubated with a slow-off-rate agonist BI-167107 to create a control/reference target receptor (*i.e.* ligand binding site-blocked state).

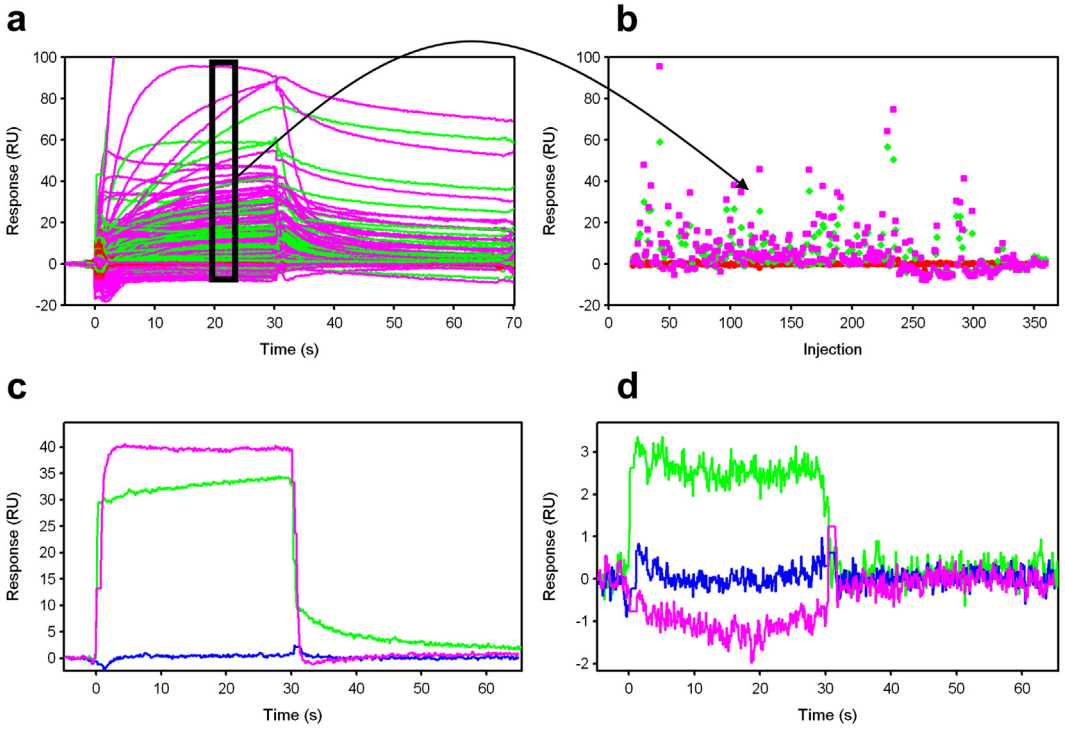


Fig. 8. Responses per injection measured at equilibrium for fragments binding β2 adrenergic receptor. (a) Overlay of sensorgrams of fragments binding to the active β2 adrenergic receptor (green), deactivated receptor (pink), blank surface (blue). (b) Datapoints collected from each binding sensorgram before the end of the injection plotted versus fragments. (c) and (d): example of binding sensorgrams collected for two fragment hits that were selected for further evaluation. The colour of each sensorgram corresponds to the surface used for data collection as described for figure a.

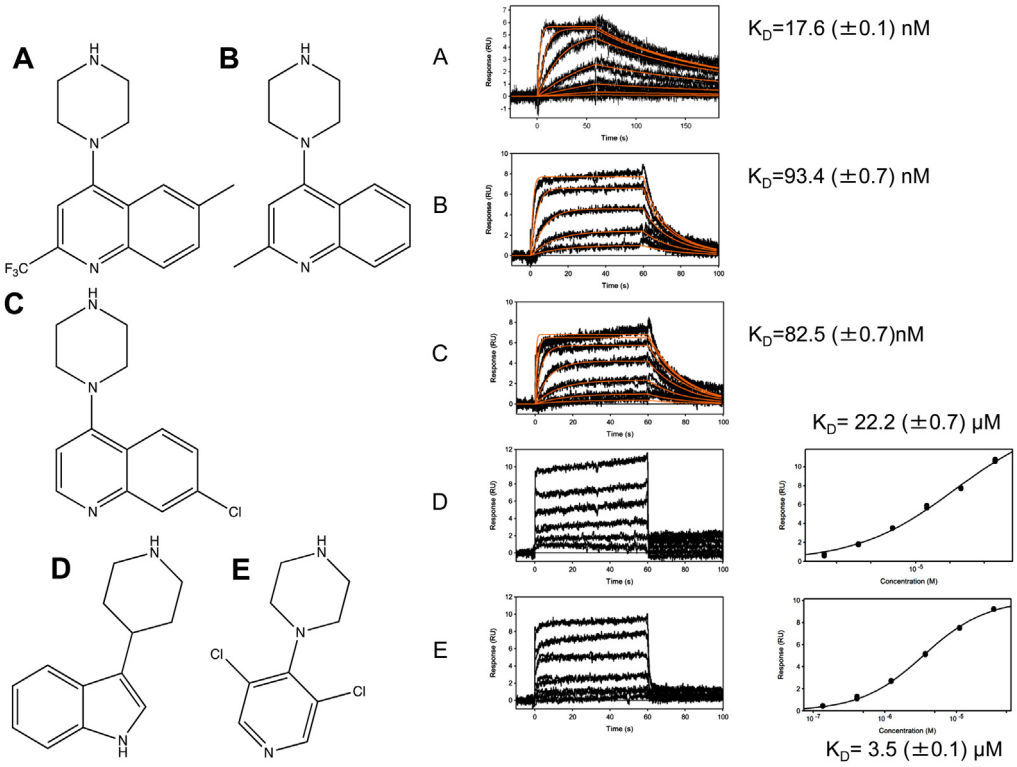


Fig. 9. β2 adrenergic receptor fragment hits. Chemical structures and SPR sensorgrams of Five β2 adrenergic receptor fragment hits (A to E) were confirmed with affinities ranging from K_D = 17 nM − 22 μM. Red lines represent kinetic fit. The right-side inserted graphs for fragments D and E represent equilibrium fits.

however one of the fragments (fragment A) showed around 10-fold selectivity towards β2AR. Furthermore, when the fragments were screened against a panel of 27 receptors, fragment A was shown to be relatively selective for β1AR. Interestingly, when tested in functional assays, all of the fragments inhibited isoproterenol-induced responses and none displayed agonistic activity, despite the assay being capable of detecting agonist binding, as demonstrated by fenoterol binding. Structure activity relationship (SAR) data was also generated for three of the fragments and a small number of fragment A analogues using the SPR assay. This revealed some chemical features responsible for the high-affinity of fragment A (K_D = 39 nM, LE = 0.45 kcal/mol/nonhydrogen-atom), which could be used to grow and optimise the fragment. This piece of work provides evidence that it is possible to utilise SPR for the fragment screening of wild-type GPCRs to identify high affinity, selective fragment starting points for drug discovery. It is also possible to further characterise binding of the fragments and generate SAR data in the absence of crystallographic information which, despite recent advances, is often lacking for GPCRs.

Generally, the limitations of measuring the kinetics of the rate of association and dissociation for fragments is more difficult than for measuring interactions with large analytes, such as proteins, due to the small signal observed for fragments. Additionally, fragments often have fast dissociation rates, which may be outside the limits of detection; although some fragments may also exhibit slower off-rates. On a modern instrument such as the Biacore T200 the limits of detection are an association rate of $1 \times 10^3 - 5 \times 10^7$ M^{-1} s^{-1} and a dissociation rate of $1 \times 10^{-5} - 1$ s^{-1}. To measure the kinetics of small molecules, a high stability of the surface is required with no drift of the baseline measurements. Zero baseline drift is usually difficult to achieve for membrane proteins, as capture via a His-tag is not 100% stable. Therefore, very slow off-rates less than 10^{-5} s^{-1} may not be accurate. For molecules with especially fast on-rates and slow off-rates, the on-rates are usually influenced by mass-transport due to the mechanics of flow cell configurations in SPR biosensors. To minimise mass transport effects, protein density needs to be maintained at a very low level and the flow rate increased to enable the molecule to be presented at the binding site faster than the on-rate. This can be problematic for GPCRs as it is not usual for 100% of the protein to be active, and initially it is difficult to estimate how much protein is required to achieve the appropriate immobilisation levels to obtain good quality data and minimal mass transport effects. Therefore, these parameters, such as immobilisation levels and flow rate, need to be optimised during assay development. However, the shape of the binding sensorgram observed for small molecules binding to the target can be informative as to whether there is possibility of mass transport or not. Usually mass transport influenced association does not have curvature in the data line a (*i.e.* it is a straight line) or even has sigmoidal shape if it is heavily mass transport limited.

8. Conclusion

In summary, surface plasmon resonance has emerged in recent years as one of the most popular and versatile methods for fragment screening. Practitioners have employed SPR biosensor interaction analysis to screen fragment libraries and to confirm the binding of bioactive compounds in fragment screening experiments. In addition to providing accurate information on the affinity of a molecular interaction, SPR analysis can also provide kinetic data, and when the experiments are designed correctly, thermodynamic data. One of the most exciting developments in fragment based drug discovery in recent years has been the use of SPR for fragment screening against membrane protein drug targets; such GPCRs. Although analysis of GPCR-ligand interactions using SPR has been against isolated receptors, the pharmacological measurements of affinity and kinetics for GPCR ligands are very close to those observed by cell based methods. Moreover, affinity and kinetics can be measured in the same experiment in label-free manner. The label-free methodology of SPR analysis also enables both orthosteric and allosteric ligands to be detected. Thus applying biophysical methods, such as SPR, to measure the binding of low affinity, low molecular weight fragment compounds to target proteins enables the execution of fragment-based drug discovery for GPCRs.

A number of SPR fragment screens against GPCRs have now been published and further confidential projects are being undertaken in industry. The two main approaches to SPR fragment screening, published thus far, are the use of thermostablised GPCRs and solubilised wild type receptors. Thermostabilisation of GPCRs reduces the complexity of assay development compared to using native receptors; however this is at the cost of losing some functional conformations. Recently, SPR fragment screens against tagged-native sequence GPCRs have been developed using a solubilisation methodology, which maintains the full range of receptor functionality. However, these examples represent only a few well-characterised GPCRs and the methods are not yet routinely applied to many other GPCR targets. The barriers to routine application of SPR to GPCRs are overexpression of functional GPCRs, purification of functional and stable receptors and immobilisation of a high density of functional receptors on the biosensor surface. However, if these assay development issues can be overcome; there is promising evidence that SPR and fragment screening will be an integral part of GPCR drug discovery in the future.

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