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Development of a label-free biosensor method for the identification of sticky compounds which disturb GPCR-assays

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The aim of this project was to develop a SPR based assay for pre-screening of low molecular weight (LMW) drug compounds, to enable filtering away disturbing compounds when interacting with drugs. The interaction between 47 LMW compounds and biological ligands were investigated using the instrument Biacore™, which is based on SPR-technology.

Keywords

Surface plasmon resonance (SPR), biosensor, Biacore™, G-protein-coupled receptors (GPCRs), Low molecular weight (LMW) compounds, C-C chemokine receptor type 5 (CCR5), acid-sensing ion channel 1a (ASIC1a), Thrombin, Carbonic Anhydrase II (CA II), P38α MAP kinase.

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Utvecklandet av en biosensor metod för att detektera substanser som orsakar problem vid läkemedelsframställning.

Hamno Mohammed Kader

Populärvetenskaplig sammanfattning

Människan består av många olika celltyper. På varje cellyta sitter olika mottagarmolekyler (receptorer) som har till uppgift att ta emot signaler. Dessa receptorer består av proteiner som styr cellens funktion. Proteinerna bestämmer exempelvis vad som tas emot och passerar ut och in genom cellen. När man blir sjuk beror det oftast på att dessa proteiner inte fungerar rätt.

Idag tar det ungefär tio till femton år att forska fram ett läkemedel från idé till färdig produkt. Ett tidigt stadium av läkemedelsforskning är att identifiera ett specifikt protein som är kopplat till en viss sjukdom, därefter letar man vidare efter molekyler som kan blockera eller förstärka proteinets signaler. Som läkemedelsforskare letar man oftast efter kemiska molekyler, hormoner eller antikroppar som kan tänkas påverka och fungera mot proteinet. Det är viktigt att redan i ett tidigt stadium vid framställning av läkemedel få reda på vilka kemiska substanser som binder till det önskade proteinet och hur selektiv bindningen är.

Det finns en hel del tekniker för att identifiera potentiella läkemedel och proteiner i ett tidigt stadium. I denna studie användes instrumentet Biacore, som är baserat på tekniken Surface Plasmon Resonance (SPR). Normalt sett fungerar tekniken genom att man först binder en läkemedelsubstans på en sensoryta, därefter injicera en anti-läkemedelssubstans över ytan som kan binda till läkemedlet och detekteras. Efteråt kan man injicera en så kallad regenereringslösning över ytan som tvättar bort anti-läkemedelssubstansen, och därefter injicera en annan anti-läkemedelssubstans över samma yta och fortsätta studera bindning och detektion. Bindnings- samt regenereringsförhållanden är unika för varje läkemedel som används, vilket kan vara ett tidskrävande och besvärligt steg, dessutom finns det stor risk att förstöra läkemedlet eller att man får en sämre eller helt utebliven detektion.

Membranproteiner som sitter på eller i cellmembranet medverkar dels i signalering och agerar som en transportör till värdcellen, därmed utgör de ett av de mest attraktiva forskningsområdena för läkemedelsdesign och -utveckling. I det här examensarbetet har interaktionen av 47 olika små molekyler från ett läkemedelsbibliotek studerats med membranproteiner, övriga proteiner och liposomer (fetter) med Biacore™ teknik. Målet med studien var att utveckla en SPR baserad metod för små molekyler från ett läkemedelsbibliotek, för att i ett tidigt stadium identifiera ospecifika bindare och filtrera bort substanser som kan vara störande där mål proteinet återanvänds.

Den metod som presenteras i rapporten kan användas för enkel och effektiv identifiering och eliminering av ”problematiska” substanser i ett tidigt stadium av läkemedelsforskning.

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Uppsala universitet, juni 2013

Development of a label-free biosensor method for the identification of sticky compounds which disturb GPCR-assays

Abstract

Integral membrane proteins are one of the main targets in drug discovery, largely to their function in signalling and transporting in living cells. Such proteins need to be surrounded by a lipid bilayer to remain active, and therefore they are difficult to study. Furthermore, compounds that reactive towards proteins and/or membranes can easily deactivate membrane proteins and this will raise difficulty to interpret screening results.

It is widely known that early estimates about the binding properties of drug candidates are important in the drug discovery process. Surface plasmon resonance (SPR) biosensors have become a standard tool for characterizing interactions between a great variety of biomolecules and it offers a unique opportunity to study binding activity of integral membrane proteins such as G-protein-coupled receptors (GPCRs), in real time and with minimal sample preparation. The strength and limitation of this technology is that the protein is reused, which gives very low protein consumption and also a great sensitivity for non-specific binders.

The aim of this project is to develop a SPR based assay for pre-screening of low molecular weight (LMW) compounds libraries, to enable filtering away disturbing compounds. The interaction between 47 LMW compounds and immobilized ligands were investigated using the instrument Biacore™ which is based on SPR-technology. The LMW compounds were screened at a single concentration and allowed to interact separately with membrane proteins, dummy proteins and liposomes. When the binding signal to different immobilized ligands of the LMW compounds were analyzed, in general, three distinct groups could be identified: a) potential binders, b) non-binders and c) potential non-specific binders. The potential binders were further characterized using dose response based on affinity screening against two membrane proteins. When optimized assay conditions were used, the study of the interaction of LMW´s with the membrane proteins could be performed without problems. However, the optimized assay conditions together with the pre-screening approach have the potential to be used as a membrane protein assay and a screening tool for the characterization of "problem" compounds in SPR-based assays.

Keywords: surface plasmon resonance (SPR), biosensor, Biacore™, G-protein-coupled receptors (GPCRs), Low molecular weight (LMW) compounds, C-C chemokine receptor type 5 (CCR5), acid-sensing ion channel 1a (ASIC1a), Thrombin, Carbonic Anhydrase II (CA II), P38α MAP kinase.

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1.0 Introduction

Integral membrane proteins are embedded in a lipid bilayer. Such proteins have major roles as transporters, channels and receptors for many drugs, ions, and also large molecules such as proteins, RNA and DNA. Integral membrane proteins are widely investigated and are one of the most interesting targets for drug discovery. Since the membrane proteins are attached to biological membranes, they need to be surrounded by a lipid bilayer to remain active and therefore are difficult to study [18].

G protein-coupled receptors (GPCRs) are one of the largest family of membrane cell surface receptors. The GPCRs have a wide range of physiological functions and respond to a diversity of extracellular moieties, ions, lipids, hormones and glycoproteins. GPCRs are therefore very interesting target for therapeutic treatment. The structural conformation of GPCR is defined by seven trans-membrane α-helices, which makes the receptors hydrophobic and therefore a lipid environment is needed to conserve the native conformation [15]. Normally, GPCRs are expressed at a very low level in the cells, which complicates the study of this class of membrane proteins. The results from traditional cell-based assay techniques using fluorescent or radio-labelled ligands are often difficult to interpret due to many false positives [16].

Surface plasmon resonance (SPR), which measures binding as changes in refractive index on a chip surface, has become a widely known biosensor technology tool for characterizing protein interactions [14]. One of the emerging applications for SPR is to study membrane-associated receptors. Biacore™ systems offer fast and highly sensitive interaction analysis in real time. No labelled reagents or further purification of receptors is needed, if capturing is used for the attachment of the receptors on the surface. Initially, Karlson and Löfås [7] illustrated that immobilization of a purified receptor onto the sensor surface followed by reconstruction of membrane environment using lipid/detergent-mixed micelles resulted in active receptor. Since this approach, further work has been carried out on how biosensor may be more routinely used to study membrane-associated receptors, and to maintain structural and functional activity [10,13 & 15].

When screening of small molecules for selection of drug candidates, the membrane proteins can be easily deactivated by compounds that are protein or membrane reactive, which raise a difficulty to interpret the screen result [6]. Here we aim to develop a SPR method for screening of low molecular weight (LMW) compounds for the identification of sticky LMW compounds. The target protein is often re-used in Biacore™ assays and therefore identification and elimination of sticky compounds from libraries is an important task.

The first target used in this work was the C-C chemokine receptor type 5 (CCR5), which play an important role in HIV infection [10]. The receptor was engineered with a C-terminal peptide tag called C9, and could be captured selectively on 1D4 antibody surface [12]. Biacore™ T200 instrument was used to screen 47 LMW compounds against CCR5.

To further characterize the 47 LMW compounds the binding to another membrane protein (ASIC1a-see below), two types of liposome (POPC and POPC/POPS) and three other proteins (Thrombin, P38α MAP kinase and Carbonic anhydrase II) were studied.

The membrane protein Acid-Sensing Ion Channel 1a (ASIC1a) is a splice variant of the ASIC family which functions as neuronal cationic channel activated by extra cellular protons. ASIC1a is expressed in the central nervous system and are potential drug targets for a wide range of diseases [17]. It is known that animal toxins can inhibit ASICs channel function, and also the psalmotoxin, which is used here as control sample binds specifically the ASIC1a channels with high affinity [2]. In this work we used a construct of His-tagged ASIC1a to be able to capture this membrane protein on an anti-His antibody.

Thrombin is an enzyme produced in the blood and plays an important role in the blood clotting process. This enzyme is formed from pro-thrombin that facilitates blood clotting by reacting with fibrinogen to form fibrin. [9]

P38 mitogen-activated protein (p38 MAP) kinases are a class of mitogen-activated protein kinases, which are responsible for stress stimuli and are also involved in cell differentiation, apoptosis and autophagy. P38α MAP kinase is participating in signaling cascade, controlling cellular responses to cytokine and stress in mammalian. [8]

Carbonic anhydrase II (CA II) catalyzes the reaction of carbon dioxide and water to form bicarbonate and protons (or vice versa). One function of the enzyme in animals is to catalyze carbon dioxide and bicarbonate to maintain acid-base balance in blood and other tissues [1 & 5].

By comparing the binding pattern to all these targets, we were able to classify the compounds either to be potentially sticky or being specific binders/non-binders for each target. Finally, we carried out an affinity screen to CCR5 and ASIC1a to confirm screen results in terms of dose-response curves and, if possible, try to estimate the affinity.

1.1 SPR

Surface plasmon resonance (SPR) phenomena occur between two media with different refractive index [5]. When light energy (photon) strikes a metal film (plasmon), it interacts with delocalized electrons in the plasmon, which reduces the reflected light intensity.

When photons strike the metallic film it creates an electromagnetic wave field, called evanescent. Usually photons will not pass through this field, but photons at a certain angle will pass through the field and excite the surface plasmons on the adsorbed side of the metallic film. Every time this phenomenon occurs, one photon will lose energy and produce a dip in reflected light at that specific angle (Figure 1). The change in reflection angle is dependent on the refractive index of the adsorb compound.

The protein binding measurement in Biacore™ occurs when protein is immobilized on the sensor surface. The immobilized protein will give a change of refractive index, while the running buffer which is used as blank from the start have a refractive index used as a baseline. The difference between refractive index from the immobilized protein and the running buffer could be converted into mass and thickness of adsorbate on the sensor surface. The precise angle of incidence of photons is usually determined by several factors. In Biacore™ the angle is determined by the backside of the metal film, on which target molecules are immobilized in a flow cell and addressed as ligands (Biacore™ terminology). A flow of mobile phase of the

second interaction partners' addressed as analyte will run along the flow cell with immobilized ligand. When the analyte binds to the immobilized ligand, the native refractive index changes, which gives a change in SPR angle, this is monitored in real time by detecting changes of intensity in the reflected light and also plotted as a sensorgram. The change in SPR signal is directly proportional the change in mass on the sensor chip surface, so the mass being immobilized can be interpreted roughly in terms of stoichiometry of the interaction, as well as dissociation and association rates [12].

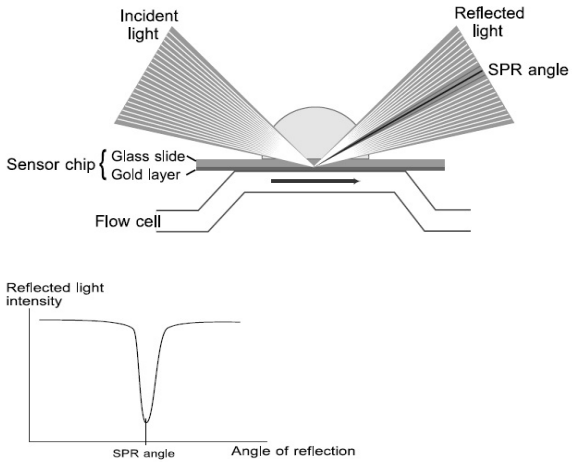

Figure 1: An overview of the SPR detection principle. The incident light will cause a change of refractive index when light is reflected due to the change of mass that occurs on the sensor surface. Figure taken from [5], with permission.

1.2 The sensor surface

The binding between two or more interaction partners occurs on the surface of the sensor chip. In the terminology for Biacore™, ligand is the interaction partner attached to a matrix on the surface. The other interaction partner is the analyte, which is passed in buffer flow over the immobilized ligand through a microfluidic system (here similar terminology used as affinity chromatography).

The metal sensor chip consists of a thin gold layer attached to a glass surface. The gold layer is coated with a dextran providing a matrix for immobilization of the ligand and an environment where interaction studies will occur (Figure 2). The gold layer with the covered dextran matrix is very stable and can be used in many extreme chemical environments [4].

There are three different approaches to attach biomolecules to the sensor surface. Depending on the properties of the molecule, it can either be attached covalently, by high affinity capture or by hydrophobic adsorption (Figure 3) [5].

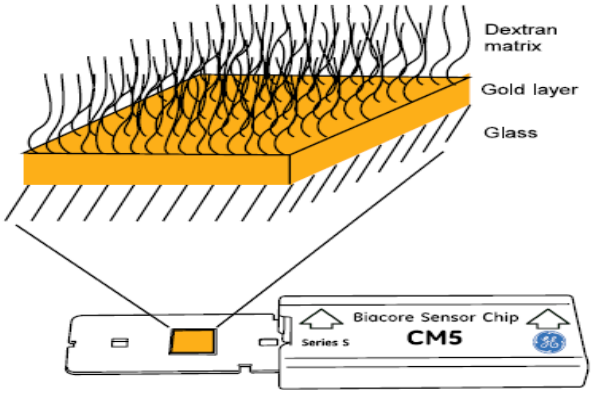

Figure 2: A scheme of the sensor surface chip used in Biacore ™. Figure taken from [4], with permission.

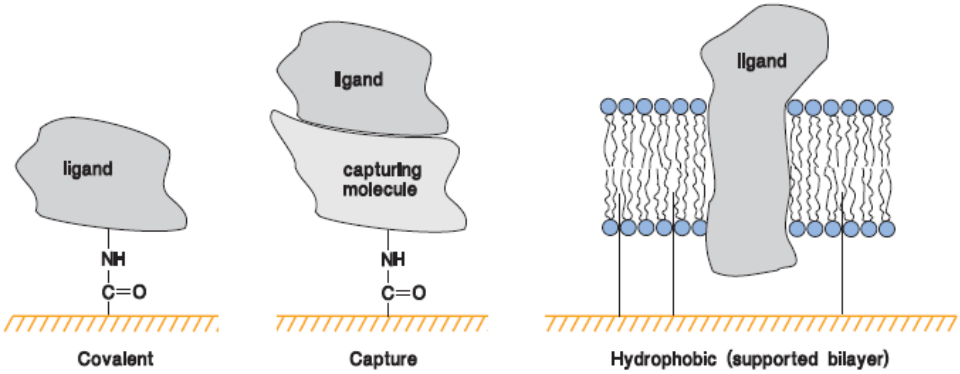

Figure 3: Demonstration of the various possible ways to attach biomolecules to the sensor surface. Figure taken from [5], with permission.

1.3 The microfluidic system

In Biacore™, the interaction occurs on the gold covered side of the sensor chip, opposite direction where the light is reflected. Through integrated microfluidic cartridge (IFC) on the chip, the samples are delivered very precisely to the sensor surface (Figure 4). The IFC used in Biacore™ T200 has four flow cells, which makes it possible to use various combinations of assay set-up [4 & 5].

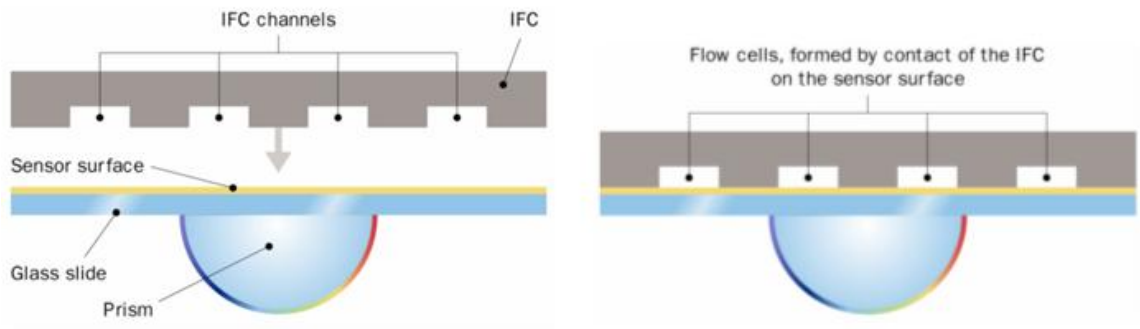

Figure 4: Cross-section of IFC channels connected to the sensor surface. Samples are delivered through the flow cells, which are formed when the integrated microfluidic cartridge is pressed against the sensor surface. Figure taken from the homepage, with permission.
"http://www.biacore.com/lifesciences/technology/introduction/Flow_cells/index.html"

1.4 The Sensorgram

The interaction includes the association and dissociation phases. The association occurs when the analyte binds to the immobilized ligand. This can be monitored in real time. The dissociation phase occurs after the analyte injection, when pure running buffer flows over the sensor surface, the analyte will dissociate from the ligand and a dissociation curve of analyte/ligand complex will be monitored on the screen (Figure 5).

The monitoring for dissociation- and association phase in Biacore™ technology is based on a measurement of changes in refractive index (RI) that is proportional to the changes in density on the sensor surface. A sensorgram presents a graph where the density on the sensor surface is plotted against the time. The association will be noticed as a rise of density and the dissociation as a decrease of density.

The principle of measurement allows the use of sample in crude environments, such as cell culture supernatants. Even though this is possible, it is known that if sample environment differs from the running buffer it will give rise to a bulk refractive index (RI). The bulk of refractive index will not affect the binding of analyte to the ligand, but to minimize the bulk shifts, it is recommended that the samples should be diluted in the running buffer. One feature that can be used in Biacore™ technology is to subtract the bulk contribution by using one of the flow cells as reference while another flow cell is immobilized with ligand and used as an active flow cell on the same sensor chip. The reference will be then subtracted from the active and will provide a reference-subtracted sensorgram [5].

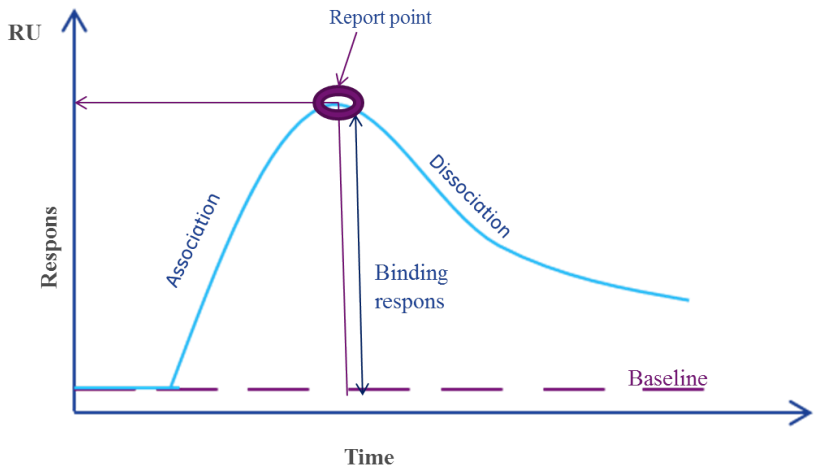

Figure 5: Illustration of a typical sensorgram**.** The sensorgram reveals all the interaction data in real time.

1.5 Immobilization

Sensor chips used in the Biacore™ system is a glass slide covered with a thin layer of gold, with a matrix of carboxylmethylated dextran covalently attached to a self-assembled monolayer of alkanethiols. A wide range of biomolecules can be immobilized covalently on the dextran matrix by using different well-defined chemistries. Therefore, depending on the properties of the molecule, the choice of immobilization method can vary. Immobilization procedure occurs when one of the interaction partners, the ligand, is to be attached to the dextran matrix on the gold layer. There are two major immobilization techniques, covalent coupling and capturing (high affinity or hydrophobic) [5].

Covalent coupling is when the ligand is attached to the matrix by a covalent link. Amine coupling chemistry is the most widely used covalently approach for attaching biomolecules to the sensor surface. Other covalently immobilization techniques on the surface includes thiol-, ligand and aldehyde coupling.

The pH of coupling buffer has a major role for immobilization. Since the carboxy methylated dextran matrix has a negative charge at a pH value 3.5, the charge of the molecule to be attached to the matrix should be positive to be attracted to the surface. Moreover, it is critical that the pH buffer used for immobilization does not damage the molecule. Usually a pH scouting experiment is carried out on the Biacore™ instrument before immobilization procedure, to obtain a suitable pH for immobilization of the ligand of interest [5].

High affinity capturing is when a molecule with high affinity to the ligand is covalently attached to the matrix. The ligand is then captured on this molecule.

Hydrophobic capturing is possible since the sensor chip surface can be modified with a derivate of lipophilic alkanes, which makes it possible to use hydrophobic interactions to capture the ligand.

1.6 Immobilization level

The immobilization level of the ligand will determine the binding capacity of the surface. In Biacore™ experiments the term maximum response (Rmax) is used for determination of the maximum binding capacity on the surface. The theoretical Rmax value can be calculated according to the formula:

Rmax = (analyte MW/ligand MW) x immobilized amount x stoichiometric ratio

Usually a theoretically calculated Rmax is higher than the experimentally measure Rmax for the same interaction. The reason could be that the concentration of ligand is too low, the ligand is not fully active, or it could be a steric hindrance.

Depending on the type of experiment and analysis that is carried out, the requirement of binding capacity may vary. It is often more useful to have a low Rmax for kinetic analysis, while a higher Rmax is more beneficial for concentration measurement [4].

1.7 Amine coupling

The chemistry used in amine coupling is to create a covalent link between the matrix and the free amino groups on the ligand. The coupling is achieved by first introducing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to activate the matrix surface with reactive esters (Figure 6). When ligand flows over the matrix surface, the ester groups will spontaneously react with the amino groups or other nucleophilic groups on the ligand to form an amide bond and attach the ligand covalently to the dextran. After injection of the ligand, the remaining ester groups are deactivated by a flow of ethanolamine-HCl over the sensor surface [5].

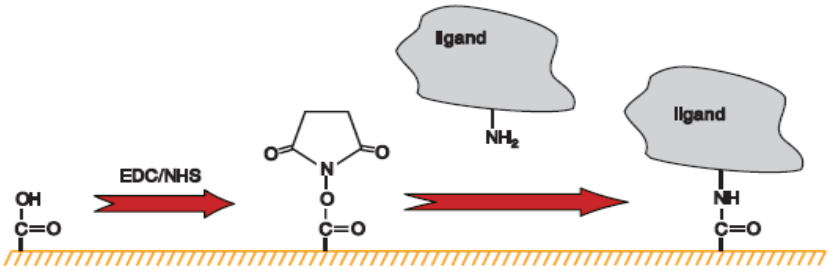

Figure 6: Amine coupling. A mixture of EDC/NHS is presented on the surface and reactive esters are produced. The esters will react with the amino groups of the ligand and introduce a covalent bond between matrix and the ligand. Figure taken from [5], with permission.

1.8 Capture coupling

Capturing mechanism is used when a ligand cannot be immobilized on the sensor surface directly or when it is a more convenient approach. Usually the ligand is tagged and can be captured on capturing molecule. Additionally, it is important that the ligand is attached to the capturing molecule with high affinity so the binding is stable during each analyse cycle. Generally, regeneration of the surface is carried out at the end of each cycle, the ligand on the capturing molecule together with any bound analyte is removed from the surface, and new fresh ligand is again injected in a new cycle [5].

Some standards of capturing approaches are streptavidin-avidin/biotin capture, antibody-based capture, or capture of based on other tagged proteins. However, a capturing mechanism could be used for any ligand capturing molecule pair binding to each other with high affinity.

1.9 Hydrophobic attachment

Hydrophobic capturing can be achieved on sensor chip L1, this surface carries hydrophobic linkers on the dextran matrix that can insert into liposomes and attach them to the surface. The L1 chip surface is coated with carboxymethyl dextran with modified lipophilic structures.

Liposomes that are used for adsorption should be prepared in running buffer and according to the standard preparation of liposome techniques. Usually the procedure for attaching liposome to the sensor surfaces includes a washing of the sensor surface with detergent, liposome injection, reduce the loosely bound liposome and stabilize the surface [5].

1.10 Regeneration

Regeneration is a procedure to remove the bound analyte from the ligand on the sensor chip surface. After each immobilization the ligand should be stably linked to the matrix, during the interaction and when the analyte is supposed to bind to the ligand and then be removed after the analysis. Theoretically this will give identical condition for each cycle analysis.

By using regeneration solution the link between the ligand and analyte can be demolished and the analyte can be washed away from the sensor chip surface, without affecting the ligand. The regeneration can be helpful to reduce time for interaction analysis in cases when the analyte dissociates slowly from the ligand [4 & 5]

2.0 Materials and Methods

2.1 Equipment, assay temperature and reagents

The instrument used for this study was Biacore™ T200 together with the Biacore™ T200 Evaluation Software and the Biacore™ T200 control software, both of version 2.0. The sensor chip type CM5-, CM4 - and L1 (series S) were used in this work together with the amine coupling kit, (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

All covalent immobilizations on sensor chip surface were carried out at 25 ˚C. During the screens the temperature of analysis and in sample compartment were either both set to 25 ˚C, or to 20 ˚C and 10˚C respectively, depending on experimentation (see table 2).

Amine coupling kit:
N-Hydroxysuccinimide (NHS)
N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC)
1 M ethanolamine hydrochloride pH 8.5 (ethanolamine-HCL)
BIAdesorb solution 1
BIAdesorb solution 2
BIAnormalization solution

Immobilization buffers:
10mM acetate buffer pH 4.0, 4.5, 5.0, 5.5

Regeneration solutions
10 mM glycine-HCL pH 1.5, 2.0, 2.5, 3.0
50 mM NaOH

Buffers and reagents:
0.2 M Phosphate buffer, 27 mM KCl and 1,37 M NaCl (PBS) (GE bioscience, Switzerland)
0.2 M Phosphate buffer, 27 mM KCl and 1,37 M NaCl, 0.5 % surfactant P20 (PBS-P+) (GE Healtchcare bioscience AB, Sweden)
0.1 M Hepes, 1.5 M NaCl, 0.5 % surfactant P20 (HBS-P+)-(GE bioscience, Switzerland)
Trizma (TRIS) (pH 7.4) (Sigma Aldrich, USA)
Hepes (GE bioscience, Switzerland)
Surfactant P20 (GE Healthcare bioscience AB, Sweden)

Moreover, those reagents were also used:
n-octyl β-D-Gluco pyranoside (DDM)
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)
Dimethyl sulfoxide (DMSO)
All those solutions above are bought from Sigma Aldrich (Alabaster, USA).

The ligands used in the investigation were:

Liposomes:
1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)
1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (POPS)

In case of liposomes a 1-mL extruder were used to get uniform liposomes.
The equipment and the liposomes were bought from Avanti polar lipids Inc. (Alabaster, USA)

Proteins:
Carbonic anhydrase II (CA II) and thrombin were bought from Sigma Aldrich (Alabaster, USA) and the P38 mitogen-activated protein kinase were purchased from Millipore (UK).
The RHO 1D4 Antibody (antibody to Rhodopsin) was bought from Invitrogen life technology (Netherland) and anti Histidine antibody was from GE healthcare bioscience AB (Sweden).

Membrane proteins:
C-C- chemokine receptor type5 (CCR5)
Acid-sensing Ion Channel 1a (ASIC1a)

The membrane proteins were gift from Dr. S. Huber, F. Hoffmann-La Roche Ltd., Basel, Switzerland.

The control samples lactulose, propranolol, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA), benzenosulfonamid and psalmotoxin were bought from Sigma Aldrich (Alabaster, USA). The P38 mitogen-activated protein kinase inhibitors SB 203580 and 202190 were obtained from Upstate Biotechnology.

Low molecular weight compounds library were obtained from Dr. S. Huber, F. Hoffmann-La Roche Ltd., Basel, Switzerland. The library contained 47 LMW compounds and a reference compound (positive control) for CCR5. The structural formula was unknown to us, we obtained only the molecular weight and some limited physical data for these compounds from Rosche (see table 1).

Table 1: Physical data for the 47 LMW compound.

GE Label of LMW compounds	pKa (1)	pKa (2)	Partition constant (Log P)	Molecular weight (MW)
1	10,61	-	3,913	315,715
2	5,76	-	4,688	468,187
3	9,78	4,24	2,605	312,339
4	9,78	4,42	2,524	324,374
5	10,33	3,7	2,562	308,375
6	-	-	3,168	346,808
7	-	-	3,276	346,808
8	-	-	3,417	312,791
9	-	-	3,42	312,791
10	-	-	3,044	342,389
11	-	-	3,293	308,372
12	-	-	2,394	318,391
13	-	-	3,146	313,828
14	7,81	9,58	3,253	515,537
15	4,36	-	3,164	306,384
16	3,01	-	2,639	313,351
17	-	-	2,081	314,339
18	4,54	-	3,124	306,337
19	-		2,264	305,353
20	-	-	3,614	335,81
21	8,56	-	0,885	491,628
22	8,45	-	0,04	587,181
23	8,45	-	0,022	611,139
24	8,45	-	-0,007	552,115
25	8,6	-	0,616	543,63
26	8,6	-	0,152	505,659
27	8,45	-	-0,187	625,166
28	8,55	-	2,77	571,754
29	8,81	-	1,107	557,657
30	10,78	8,4	0,988	589,123
31	2,51	8,4	0,846	647,159
32	3,22	8,45	0,321	647,159
33	10,78	8,56	1,012	508,634
34	9,24	-	3,596	511,746
35	8,56	-	0,841	554,678
36	8,87	-	2,282	553,787
37	8,03	-	5,578	559,673
38	8,87	-	2,253	538,772
39	11,11	7,73	3,428	509,711
40	8,45	-	1,079	638,252
41	8,6	-	0,696	493,623
42	8,28	-	0,879	618,174
43	4,24	8,44	0,156	629,154
44	10,47	8,28	0,036	-
45	8,45	-	1,323	689,243
46	8,56	-	0,167	506,643
47	9,32	8,66	0,93	547,043

The table contains the information obtained about the low molecular weight compounds pKa carried out twice for some of the compounds, partition coefficient LogP between water and lipid, and the Molecular weight (MW).

2.2 Preparation of liposomes and mixed micelles

The used lipids were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and a combination of POPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (POPS). To obtain thin lipid film layers, the round flask containing chloroform solution of lipids was rotated under a stream of nitrogen gas to evaporate. This process was followed by drying of lipids overnight in vacuum. The dried lipids were re-suspended in a buffer containing 50 mM HEPES, 150 mM NaCl (pH 7.0). In order to obtain larger liposomes, three cycles of freezing (-60 ºC for 30 minutes)/thawing (on a shaker at room temperature about 30 minutes) were carried out. To obtain uniform liposomes, the lipid solution was extruded through a polycarbonate filter of 100 nm. The lipid suspension was passed through the 100 nm filter at least 19 times using 1-mL extruder (Avanti polar lipids).

To obtain mixed micelles the liposomes was handled according to a previous work by David G.Myszka and his collages [11] i.e., one ml of the 5 mM liposome solution were mixed with 500 µl of 20 % DDM and 500 µl of 20 % CHAPS, vigorously vortexed for 10 seconds and equilibrated for 30 minutes at room temperature.

2.3 Solubilization of CCR5 and ASIC1a

Solubilization procedure was carried out carefully by thawing the cell pellet slowly on ice. The cell pellet was from cf2Th cells expressing CCR5 receptor (stimulated with 4 mM NaButyrate) and usually stored at -57˚C. After thawing, the cells was resuspended with 900 µl of solubilization buffer consisted of 20 mM Tris, 100 mM $(NH_4)SO_4$, 10% glycerol and 2 tablets per 50 ml buffer of EDTA-free protease inhibitor cocktail tablets (Roche diagnostics Scandinavia AB) and the pH of this buffer was 7.0. The solubilization was performed ~1 h at 4 ˚C with a tabletop rotor at 5 rpm. After solubilization the cell debris that was centrifuged at 4 ˚C for 15 minutes at 16000 rpm. The supernatant contained the solubilized receptor that was used for immobilization of the receptor on the Biacore ™ sensor chip.

2.4 Immobilization of liposomes and proteins

The chip used for liposome immobilization was sensor chip L1 series S (GE Healthcare Bio-Science AB, Uppsala, Sweden). 0.5 mM POPC and 1.5 mM POPC/POPS were immobilized on flow cell two and four, respectively, the flow cell one and three being used as unmodified references.

For the protein immobilizations, a sensor chip CM5 series S was used, and immobilization was carried out at a temperature of 25 ºC [4]. Here, an amine coupling chemistry was used to covalently attach the proteins to the sensor surface. For the HSA, CA II and thrombin the concentration of 30 µg/ml was injected for 10 min at a flow rate 10 µl/min in 10 mM sodium acetate (pH 5.0).

The same procedure of covalent coupling as for the proteins was carried out for the antibodies. The antibody 1D4 was diluted in the coupling buffer 10 mM sodium acetate pH 4.5 to the concentration of 20 µM and the anti-HIS antibody was diluted in 10 mM sodium acetate pH 5.0 to a final concentration of 50 µM. The immobilization buffer used here was HBS-P+.

P38α MAP kinase was immobilized in the presence of 10 μM inhibitor, SB 203530, using similar conditions as above but in 10 mM sodium acetate (pH 5.5). After immobilization, p38α MAP kinase was deactivated with two injections of EDC/NHS and ethanolamine for 2.5 min each. The immobilization buffers for each ligand are presented in table 2.

2.5 Immobilization via capturing mechanism

Membrane proteins CCR5 and ASIC1a were captured on the sensor surface using 1D4 and anti-histidine antibodies, respectively. To confirm the activity of the membrane proteins, the LMW reference compound and psalmotoxin were used against CCR5 and ASIC1a, respectively.

In this investigation two different sensor chips were used in parallel, sensor chip type CM4 and CM 5 of series S. CM4 sensor chip has lower level of carboxylmethylation on the surface than CM5, which can make it more appropriate to avoid non-specific binding.

2.6 Screening procedure

Measurement for screening of binding- and stability level was carried out in the Biacore™ T200 instrument. The sample compartment temperature was either set to 10 or 25 ˚C and the temperature of interaction analysis to 20 ˚C. Table 2 shows the information about running conditions for each screening. In this table, the immobilization buffer, running buffer, ligand, control sample and temperature is presented. For each screen all the LMW compounds used as analyte were diluted to a final concentration of 100 μM with 3 % dimethyl sulfoxide (DMSO).

An affinity screen of selected LMW hits of CCR5 and ASIC1a was performed using concentration series from 6-100 μM. Here, the same buffer conditions and temperature settings were used as during the screen of all the compounds against CCR5 and ASIC1a (shown in table 2).

Table 2: The model system used for each running experiment.

Ligand	Immobilization	Coupling Buffer	Immbolization buffer	Assay Running Buffer	Control Sample	Analyze and Sample compartment temperature ˚C
POPC/POPS	Lipophilic	-	PBS	50mM PBS + 3% DMSO (pH 6,5)	Propanolol + Lactulose -	#1 = 25 #2 = 20 & 10
POPC	Lipophilic	-	PBS	50mM PBS + 3% DMSO (pH 6,5)	Propanolol + Lactulose -	#1 = 25 #2 = 20 & 10
POPC/POPS	Lipophilic	-	50 mM Hepes,0,15 M NaCl + mixed micelles (pH 7.4)	50 mM Hepes, 0,15 M NaCl + 0.058 % mixed micelles (POPC/POPS) + 3 % DMSO, DDM,(HS) (pH 7.0)	Propanolol + Lactulose -	#1 = 20 & 10
POPC	Lipophilic	-	50 mM Hepes,0,15 M NaCl + mixed micelles (pH 7.4)	50 mM Hepes,0,15 M NaCl + 0.058 % mixed micelles (POPC/POPS) + 3 % DMSO, DDM,(HS) (pH 7.0)	Propanolol + Lactulose -	#1 = 20 & 10
Carbonic Anhydrase II	Amine coupled	Acetate pH 5.0	PBS-P+	PBS-P+ + 3% DMSO	Benzenosulfonamid	#1 = 25 #2 = 20 & 10
p38α MAP kinase	Amine coupled protected	Acetate pH 5.5	HBS-P+	50 mM Tris,150 mM NaCl, 10 mm $MgCl_2$ + 3 % DMSO + 0,05% p20	SB202190 + SB203580 +	25
Thrombin	Amine coupled	Acetate pH 5.0	HBS-P+	50 mM Tris,150 mM NaCl, 10 mm $MgCl_2$ + 3 % DMSO + 0,05%, p20	DAPA	25
C-C chemokine receptor type 5 (CCR5) Receptor	Amine coupled CCR5 Capture on (1D4)	Acetate pH 4.5	HBS-P+	50mM Hepes, 0,15 M NaCl, 3 % DMSO + 1 % mixed micelles	2D7+ Reference Compound (CCR5) +	20 & 10
Acid-sensing Ion Channel (ASIC protein)	Amine coupled ASIC caputure with anti-His kit	Acetate pH 5.0	HBS-P+	50mM Hepes, 0,15 M NaCl, 3 % DMSO + 1 % mixed micelles	psalmotoxin	20 & 10

The analyze- and sample compartment temperature is either 25 ˚C respectively or set to 20˚C for analyzing, and 10 ˚C for sample compartment temperature depending on number of running experiment.

2.7 Solvent correction with DMSO

Small variation in the amounts of high refractive index solvent such as dimethyl sulfoxide (DMSO) can result in large shifts in refractive index during injection [3]. These small variations in bulk signal can have huge impact when LMW compounds are studied and the analyte responses are low. Therefore it is important to perform a solvent correction with DMSO to compensate such a variation in bulk signal between the samples. Eight solutions with increasing concentrations of DMSO (2.5% - 3.8 %) were prepared according to standard procedure [12]. The solvent correction responses for DMSO were obtained at the reference surface, which covered a range of response from -500 to +1000 RU relative to the baseline. The solvent correction was performed using the evaluation software Biacore™ T200. For each experiment the bulk responses were kept within the range of DMSO correction curves to achieve high-quality data.

2.8 Low molecular weight compounds and control samples

The molecular weight and some biophysical data of LMW compound are given in table 1. All the compounds were dissolved to a final concentration of 10 mM in 100% dimethyl sulfoxide (DMSO). For the binding analysis the compounds were diluted first to 300 µM with DMSO-free buffer and then further diluted to a final concentration of 100 µM with running buffer containing 3 % DMSO.

Since various proteins and liposomes are immobilized, it is important to have control samples, which provide information about the activity of immobilized surfaces. Negative (running buffer or non-binding compound) and positive (ligand-dependent) controls were used in each experiment (See table 2).

2.9 Data Evaluation

All the responses were solvent-corrected to eliminate DMSO bulk effects. The evaluation of data was done by studying the sensorgram shape and binding level using the Biacore™ T200 evaluation software. Furthermore, the binding level was first determined by calculating the theoretical maximal response (Rmax) and using a positive control. The classification of compounds was made by dividing them into three different categories: non-binders, potential binders, and non-specific binders.

The evaluation procedure for protein screen was done by molecular weight adjustment, adjustment for controls (positive and negative) and a cut off setting of 6 standard deviation of the negative control sample.

2.9.1 Affinity models

The dissociation equilibrium constant (K_D) is describing the affinity between two biomolecules. The evaluation software from Biacore™ offers three models for calculation of steady state affinity. The affinity is most often calculated from the formula:

$$Steady\ state\ Affinity = C * \frac{Rmax}{C+KD} + offset \quad (1)$$

Here, C is the concentration and Rmax is the maximum Response.

The second model calculates steady state affinity with a constant Rmax:

$$Steady\ state\ affinity\ with\ constant\ Rmax = C * \frac{Rmax(constant)}{C+KD} + offset \quad (2)$$

In this formula Rmax is provided by the user as a constant obtained from maximal binding response of a known binder (usually positive control sample).

The third and last model calculates steady state affinity with constant Rmax (provided by the used, as above) and with two binding sites. The calculation of affinity differs from the second model by assuming two binding sites (of higher and lower affinity). The formula used for calculation is:

$$Steady\ state\ affinity\ Rmax\ (multi\ site) = C * \frac{Rmax}{C+KD} + C * \frac{Rmax2}{C+KD2} + offset \quad (3)$$

Here, K_D, K_{D2} and R_{max2} are fitted parameters.

3.0 Result

3.1 Immobilization

The immobilization strategies used for this study were (i) covalent amine coupling (ii) affinity capture on antibody and (ii) hydrophobic coupling. Immobilization of proteins and liposomes was carried out as described in Materials and Methods section.

The membrane proteins where captured on an antibody using a single capture injection directly from the cell extract. The membrane protein CCR5 was captured upon the antibody 1D4 while the ASIC1a was captured on an anti-His antibody. The captured level of membrane protein on the antibodies is demonstrated in the sensorgrams in figure 7. The red curves are showing the responses on reference surfaces while the green curves present the responses on the active surfaces, with the antibodies immobilized. The captured level of CCR5 on the 1D4 antibody surface was approximately ≈ 2600 RU and the level of ASIC1a captured on anti-His antibody surface was approx. 2900 RU. The corresponding responses on reference surfaces were 90 RU and 120 RU, respectively (Figure 7). The buffer used here consisted of 50 mM Hepes (pH 7.0), 0.15 M NaCl, 0.05% DDM, 0.05% Chaps, 0.01% CHS, 2.5μM POPC.

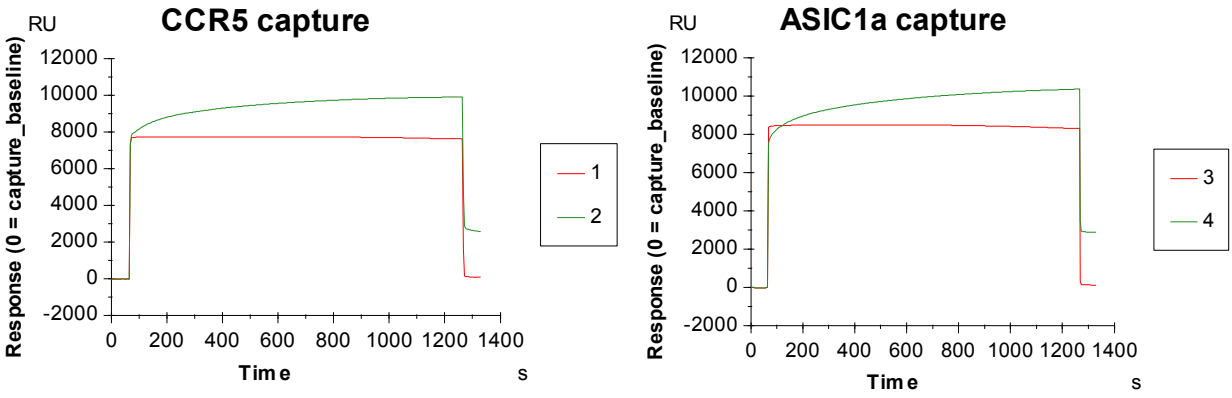

Figure 7: Capture level of membrane proteins. The green curve in the sensorgrams shows the capture level of CCR5 (left panel) and ASIC1a (right panel) from cell extract on 1D4 and anti-His antibodies, respectively. The red curves represent the binding to reference surfaces (unmodified dextran).

The immobilization of liposomes was carried out on L1 sensor chip. The result of immobilization is shown in figure 8. The liposome is first injected (3 min) followed by an extra wash of flow system with regeneration solution. The amounts of POPC and POPC/POPS immobilized on the surfaces were approx. 8000 RU and 6000 RU, respectively (Figure 8). The buffer used under liposome capture consisted of 50 mM PBS and 3% DMSO (pH 6,5).

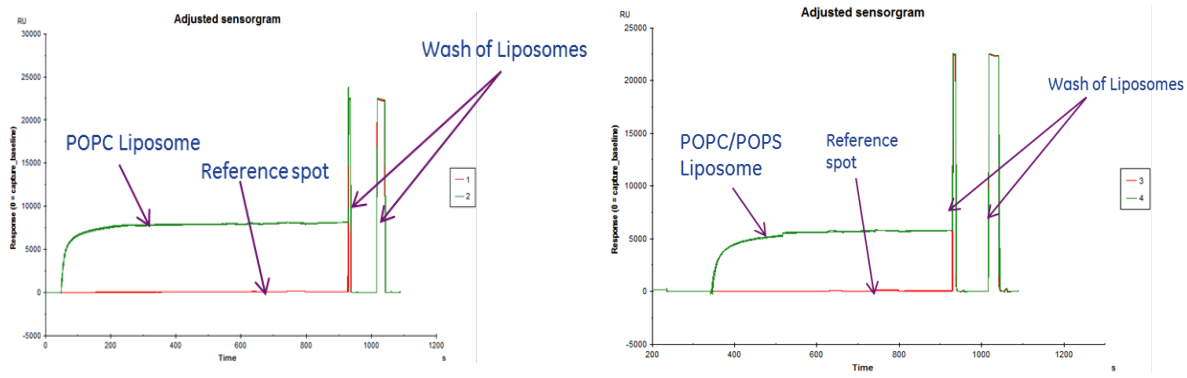

Figure 8: Sensorgrams for liposome immobilizations. The right and left hand sensorgrams show the injections of POPC and POPC/POPS, respectively.

A new injection of liposomes was performed in each cycle (meaning that each compound was analyzed on freshly prepared liposome surface). Immobilization of proteins was carried out according to the steps described in material and method. The amounts of immobilized proteins are shown in table 3. Here, the experimental and theoretical Rmax is presented for each ligand.

Table 3: Proteins used as ligands.

Ligand	MW [Da]	Immobilization	Amount immobilized (RU)	Control samples	MW [DA]	Experimental Rmax	Theorietical Rmax
Carbon Anhydrase II	29000	Amine coupled	9627,6	Benzenosulfonamide	157,19	22,5	52,18
Thrombin	36000	Amine coupled	7568,1	DAPA	502,67	100	105,67
p38 MAP kinase	41293	Amine coupled protected	8920,8	SB202190	331,35	30,45	71,58
p38 MAP kinase	41293	Amine coupled protected	8920,8	SB203580	377,43	27,4	81,54

The information about proteins used as ligands to be immobilized, the molecular weight of the ligand and control sample, immobilization approach, typical immobilization level, the inhibitor used as control sample for each ligand and the experimental, and theoretical Rmax.

3.2 Surface activity

The preservation of the membrane protein activity after immobilization procedure is important for reliable subsequent binding analysis. Therefore, a surface performance experiment on both membrane proteins was carried out in order to study their activity over time. The reference compound for CCR5 was diluted in a concentration series of 3-50 µM and was injected repeatedly over the same CCR5 surface each third hour during 18 hours. The surface activity of ASIC1a was studied similarly for about 24 h. The positive control, psalmotoxin, was used in a concentration series of 0.6-50 nM and injected repeatedly over the same ASIC1a surface every fourth hour. The binding level report point used for performance testing over time is depicted in sensorgrams in figures 9 and 10 (left side) using a green dot. The sensorgrams and plot reveals similar binding level during 18 and 24 hours runs, which suggest that CCR5 and ASIC1a, respectively, are fully active during this time. The responses in the binding level plot (right) are not blank subtracted, that is why they are higher than in sensorgram windows. However, the kinetic constants calculated for the first interaction analysis between reference compound and CCR5, deviate slightly from the constants measured later by having higher association and dissociation rate constants, which results nevertheless in similar affini-

ty of about 1 µM (Figure 9). The kinetic constants of the interaction between psalmotoxin and ASIC1a could not be measured because association rate constant, k_a, is outside the limit that can be measured by the instrument. However, the apparent affinity calculated from the ratio k_d/k_a, was similar, about 11-12 nM, for all measurements except the first one.

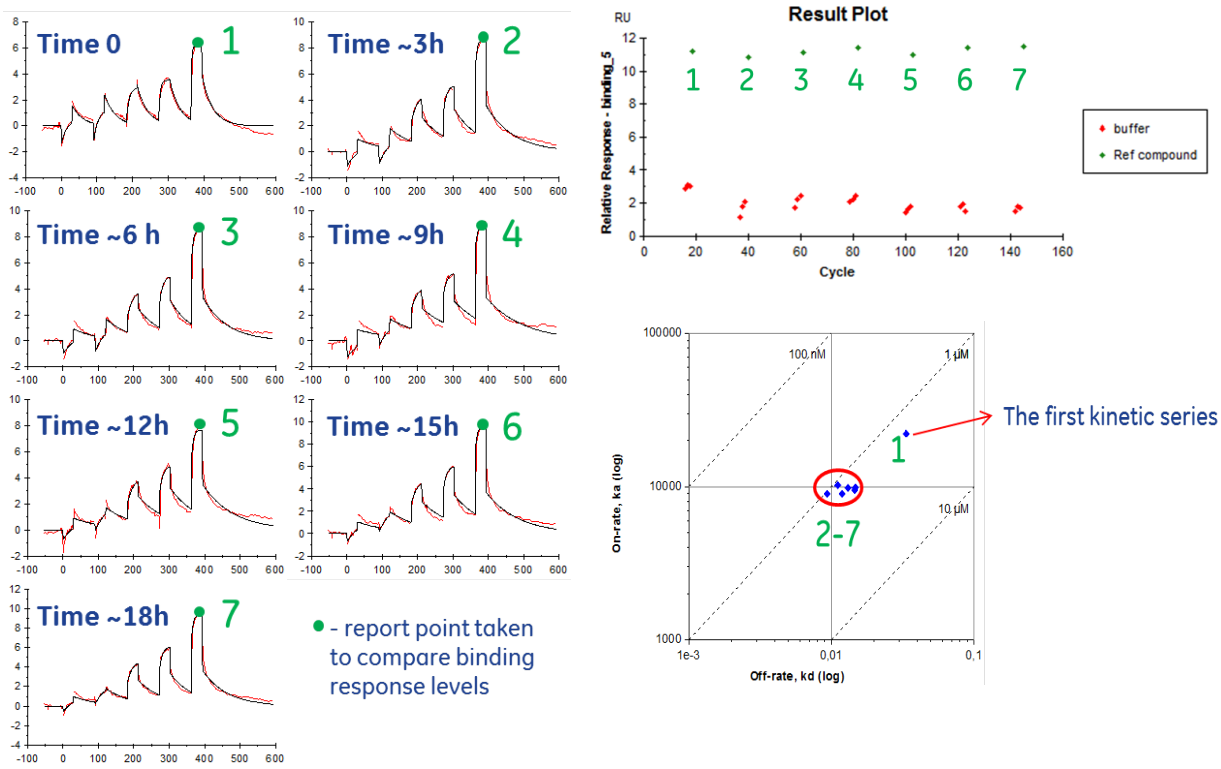

Figure 9: Sensorgrams, plot of binding level and kinetic map of reference compound for CCR5. The left panel presents the sensorgrams corresponding to concentration series of reference compound (3-50 µM) injected each third hour over CCR5 surface. Report points, shown in green, were taken to compare binding response level over time. On right top, the plot of binding level for every third hour is presented. On bottom right side, a kinetic map shows an overview of binding kinetic constants for each time point.

For both membrane proteins, the affinity to an interaction partner measured at the beginning of the activity study was deviating from the affinities to the same interaction partner measured later. One hypothesis on this behavior is that membrane protein could have different conformation, probably heterogeneous, before the binding of an interaction partner. A possible way to avoid such deviation is to have a startup cycles with control samples instead of buffer only.

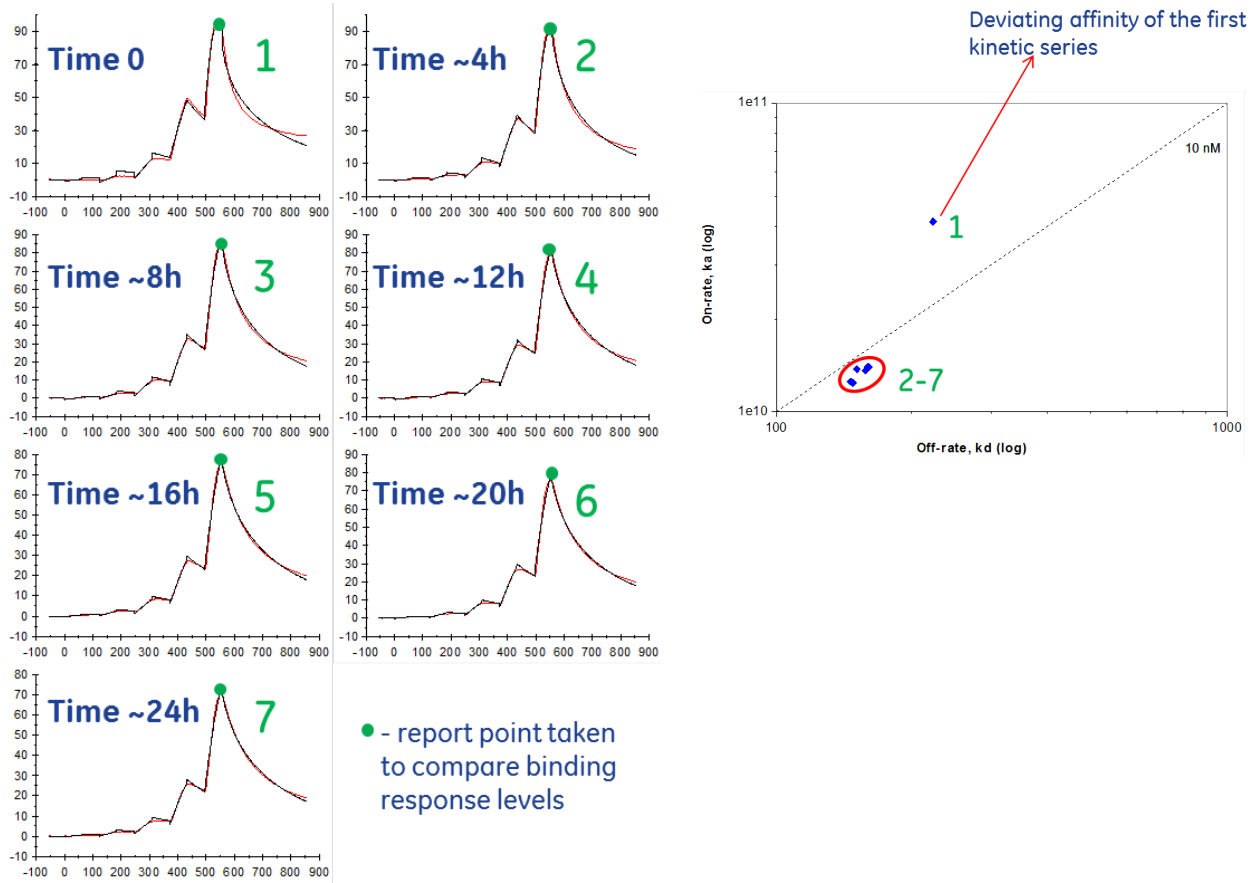

Figure 10: Sensorgram and kinetic map of reference compound for ASIC1a**.** The left panel presents the sensorgrams corresponding to concentration series of reference compound (0.6-50 nM) injected every third hour over ASIC1a surface. Report points, shown in green, were taken to compare binding response level over time (see Figure 11). The left panel shows a kinetic map for all kinetic series from the different time points.

The binding level of control sample for ASIC1a is decreasing slightly over time. The decreasing response level of control samples can be handled in the Biacore™ evaluation software by using "adjustment for controls". In figure 11 the adjustment for control principle is shown: the decreasing responses of psalmotoxin are adjusted to the same binding level using psalmotoxin and buffer as positive and negative controls, respectively

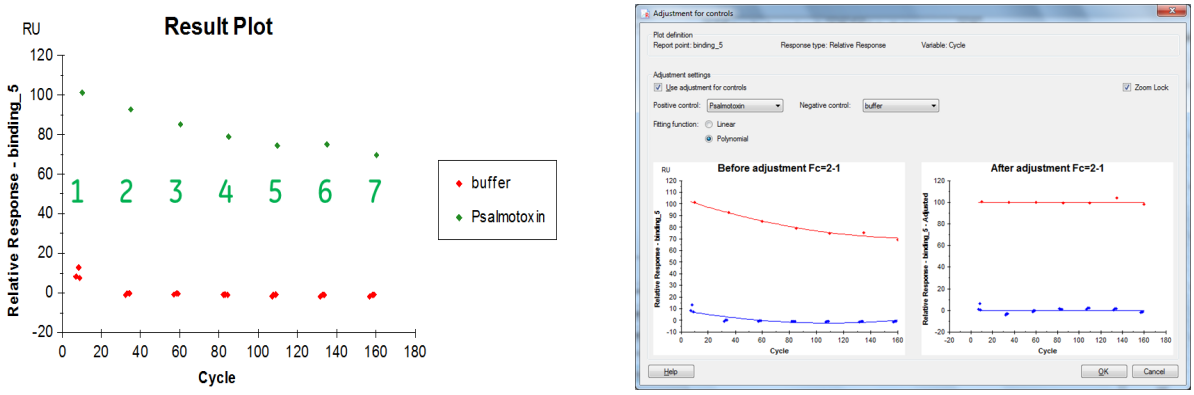

Figure 11: Adjustment for controls. The plot of psalmotoxin and buffer binding levels is presented on the left panel. By the application "adjustment for controls" in the evaluation software of Biacore™**,** the binding level of psalmotoxin and buffer can be adjusted to 100 and 0, respectively, shown on the right panel.

The activity of membrane proteins was further measured during 40 hours (Figure 12). The plot reveals a very stable and active surface when using a single injection of membrane protein on the antibody and tested against the 47 compounds on two different sensor chip types (CM 5 and CM4 series S, in two instruments), and during two consecutive screens. There is a good correlation of responses on CM4 and CM5 sensor surfaces for the analyzed compounds (Figure 12, right panel). The binding responses for the compounds on the CM4 chip are slightly lower than on the CM5 chip, which is most likely due to lower capture level of membrane protein on CM4 chip.

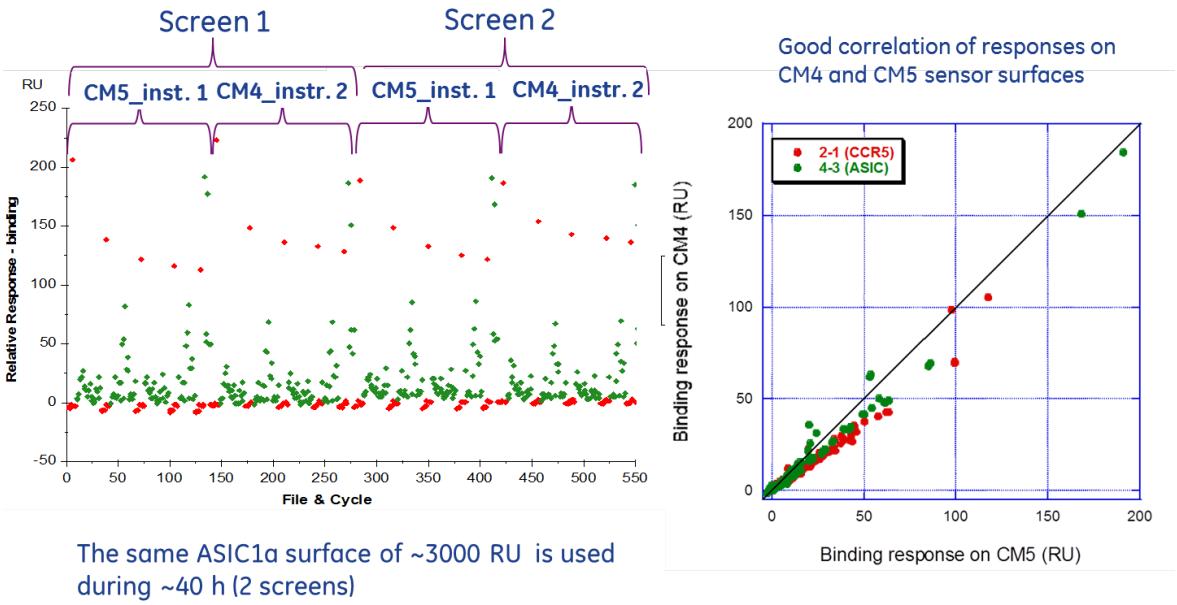

Figure 12: Binding responses of CCR5 and ASIC1a**.** Plots showing good correlation of binding responses, obtained on CCR5 and ASIC1a surfaces on CM 4 and CM5 surfaces.

Interestingly, when a second screen on the same ASIC1a surface starts after several hours in buffer flow (standby), the response level of psalmotoxin increases to the same level as in the beginning of the first screen. This phenomenon was observed on both CM5 and CM4 sensor surfaces.

Surface performance for liposomes was tested during about 24 hours. Figures 13 and 14 are showing the activity of liposome on the sensor chip surface during 24 hours, identified by the injection of the control sample propanolol. The result here shows an active surface during 20 hours, with similar binding levels of propanolol.

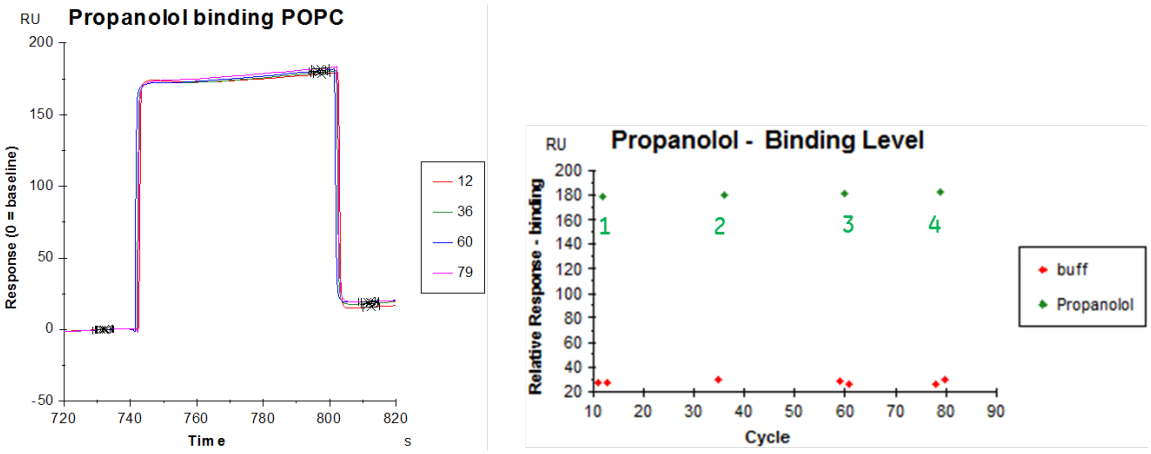

Figure 13: Binding response propranolol. Left panel: Sensorgram for each injection cycle of propranolol. Time between injections of propanolol is 7 hours in the first three cycles and 6 hours between the third and last cycle. Right panel: Similar binding response of each propranolol injection.

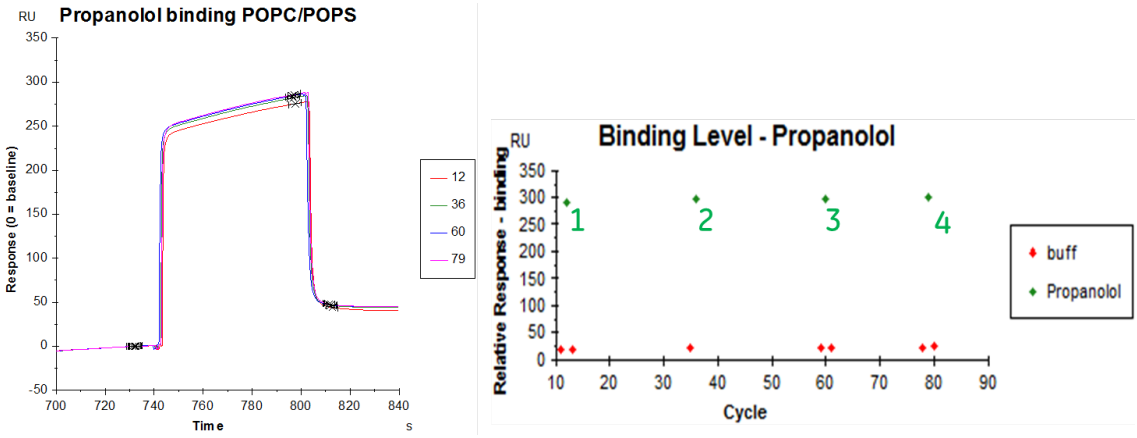

Figure 14: Binding response of propranolol over POPC/POPS. Left panel: Sensorgrams of propranolol injection for each cycle over immobilized POPC/POPS surface. It is 7 hours between the first three cycles and 6 hours between the third and the last cycle. Right panel: Demonstrates similar binding level of propranolol after each injection cycle.

3.3 Screen results

Screen assay was used to identify the compounds having high, medium, or low absorption, or being sticky on the used ligand and to examine a binding behavior. We didn't know the structures of this particular set of LMW compounds – proprietary information of Hoffman La Roche.

3.3.1 Membrane protein screen

In those screens only one concentration (100 µM) of each compounds was injected over the immobilized surface. The evaluation of screen was carried out by studying the binding level of 47 LMW compounds to the ligands and the shape of the sensorgrams. Evaluation steps included adjustment for control samples and cut off settings. For binding response levels, a cut off was set for each screen, as a value of 6 standard deviation (SD) of negative control samples, to differentiate between potential binders and non-binders. Stability responses (on dissociation phase) allowed to quickly identify slowly dissociating, potentially sticky com-

pounds. Molecular weight adjustment was not performed in these screens since the positive control of ASIC1a, Psalmotoxin, has about 10 times higher molecular weight than screened compounds and buffer was used as negative control.

In the CCR5 screen, the binding response level of the reference compound was almost identical during the entire ~16 hours experiment (Figure 15).

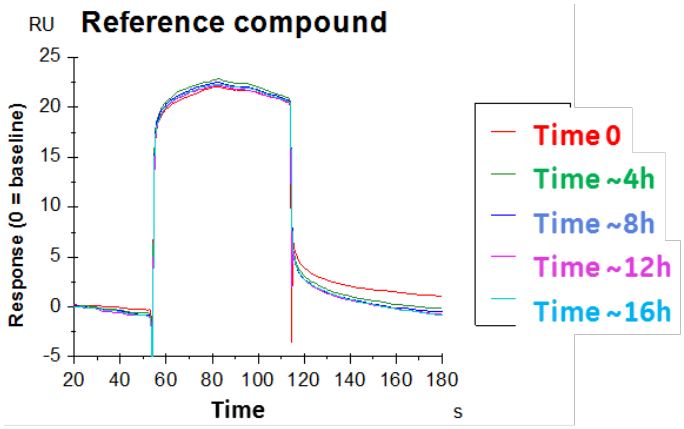

Figure 15: Active CCR5 during 16 hours. Overlay of sensorgrams of the control sample for CCR5 showing similar binding level during 16 hours.

Figure 16 shows the results of a 47 compound screen against CCR5 run in duplicate. Two binders were identified as slowly dissociating by studying the report point "stability" and by visual examination of sensorgram shape.

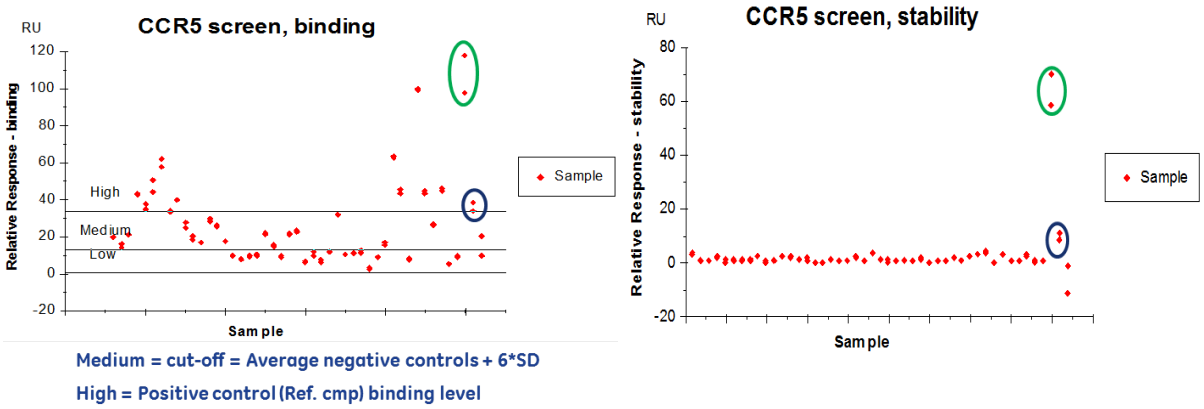

Figure 16: Binding and stability against CCR5. The binding and stability report points for CCR5 plotted against compounds, in duplicate. The green and blue circles identify the compounds that have a slow dissociation.

Compound 21 and 34, marked with circles in figure 16, are potentially non-specific and slowly dissociating compounds. The sensorgrams for those two compounds are shown in figure 17.

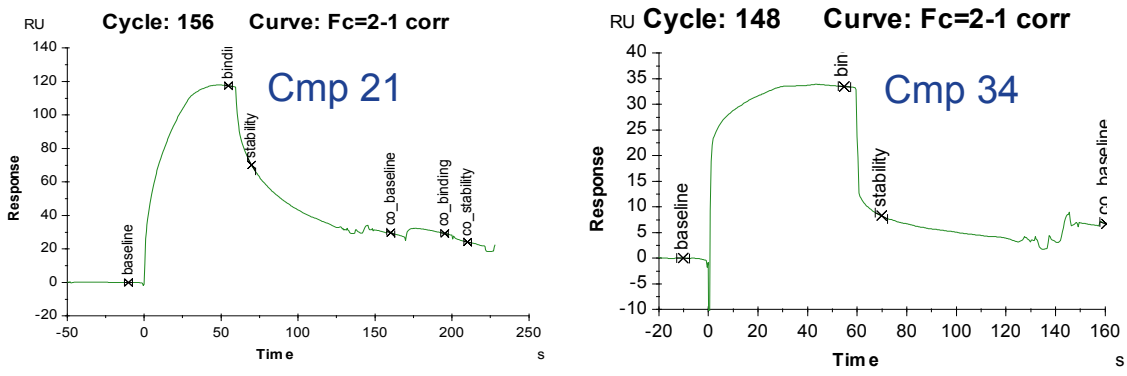

Figure 17: Sensorgram for two potentially sticky compounds to CCR5 surface.

In ASIC1a screen, the binding responses of the control sample decreased over time, and were corrected using "adjustment controls" tool in Biacore™ T200 Evaluation Software. However, good repeatability of the duplicate responses for the 47 compounds indicates reliable assay, even if the responses of control sample were decreasing. Figure 18 demonstrates ASIC1a screen in terms of cut off setting to allow identification of non-binders, potential binders and potentially sticky compounds. The slowly dissociating compounds are marked with green (compound 21), blue (compound 37) and pink (compound 34) circle. In figure 19, the sensorgrams for these three compounds are shown, which reveals their slow dissociating rate.

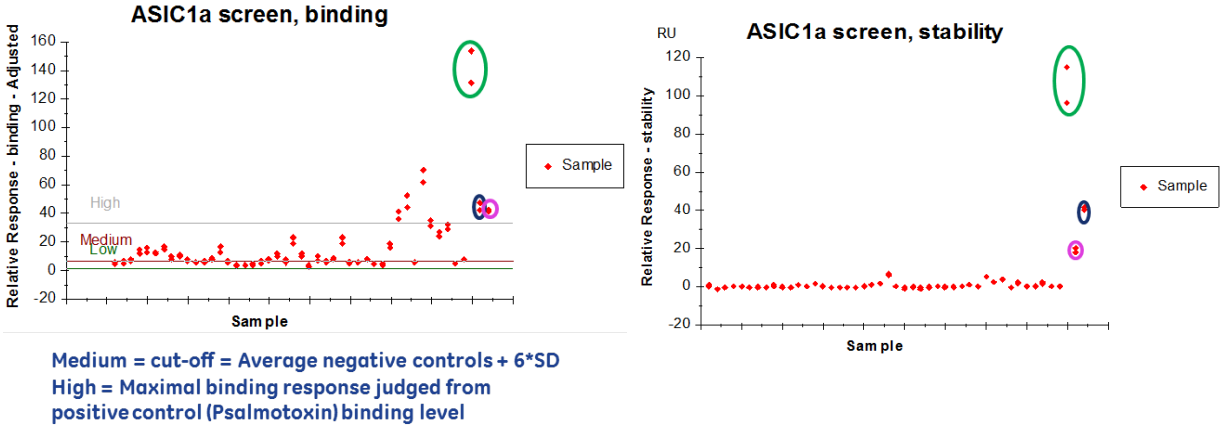

Figure 18: Binding and stability against ASIC1a. The binding and stability report points of the compounds are plotted against CCR5 are shown in left and right panel, respectively, in duplicate. The green, pink and blue circles identify the compounds that show slow dissociation.

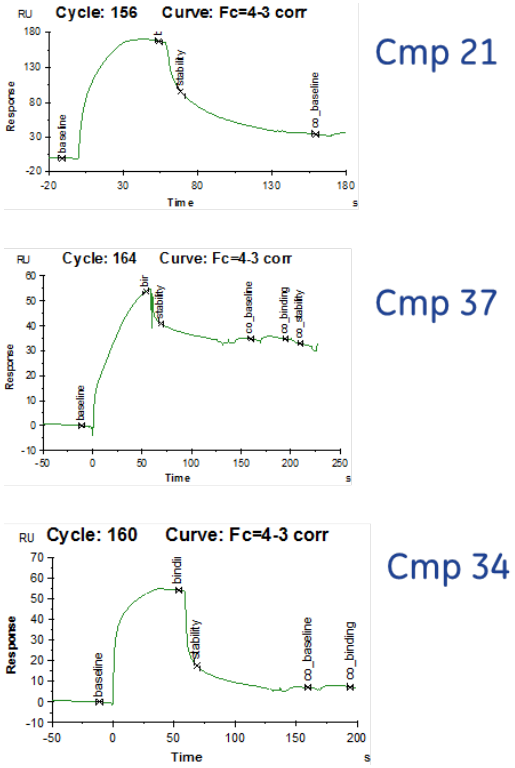

Figure 19: Three compounds with a slow dissociation rate. Compound 21,37 and 34 identified as slowly dissociating and potentially sticky compounds on the ASIC1a surface.

The potential binders selected in the screens against membrane proteins, CCR5 and ASIC1a, were further analyzed by affinity screen (see table 4).

Table 4: The compounds selected as potential binders and non-specific binders of CCR5 and ASIC1a.

CCR5 and ASIC1a Screen	
Potential binders selected to affinity screen	**Common non-specific binders of CCR5 and ASIC1a**
1, 2, 3, 4, 5 ,6, 8, 9, 1 0, 12, 11, 13, 14, 15, 20, 22, 24, 25, 30, 38, 43, 44, 45	7, 21, 34, 37, 39, 40, 42

3.3.2 Liposome screen

The 47 compounds were screened against liposomes, since liposome vesicles can be used as a transporter of the drugs [5]. In this screen, two types of liposome vesicles, POPC and POPC/POPS were used. Freshly made liposomes were captured on an L1 sensor chip surface before each compound injection. The compounds were divided into groups using cut off lines described in figure 20.

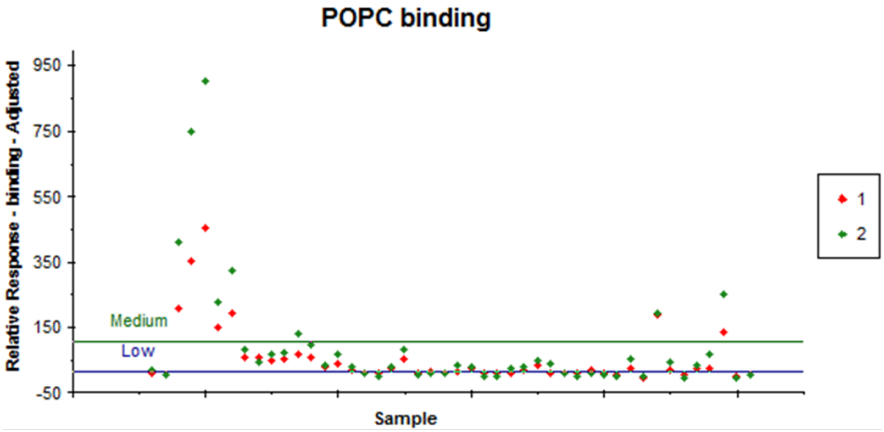

Figure 20: The binding level of compounds in a screen against POPC. The experiment is performed in duplicate marked as 1 (red) and 2 (green) points.

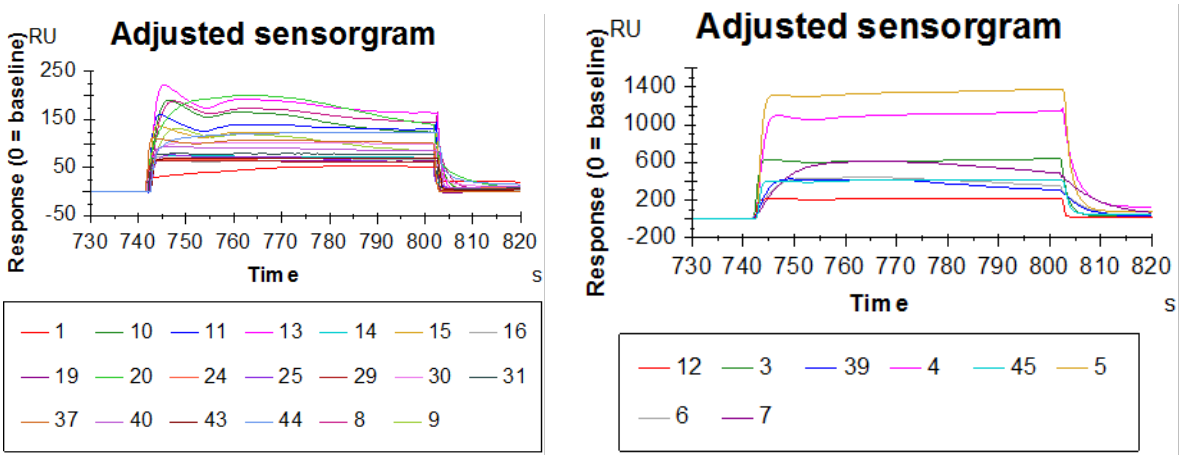

Figure 21: Potential binders. Left: Sensorgram for all the compounds below propranolol binding level and above the cut off, Right: The sensorgrams for the compounds that are binding above the propranolol binding level. The numbers indicate the compound name.

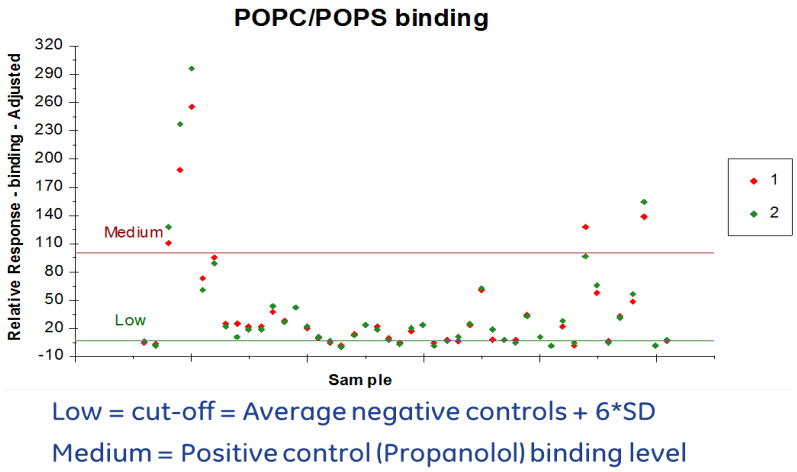

Figure 22: The binding level of compounds in a screen against POPC/POPS. The experiment is performed in duplicate marked as 1 (red) and 2 (green) points.

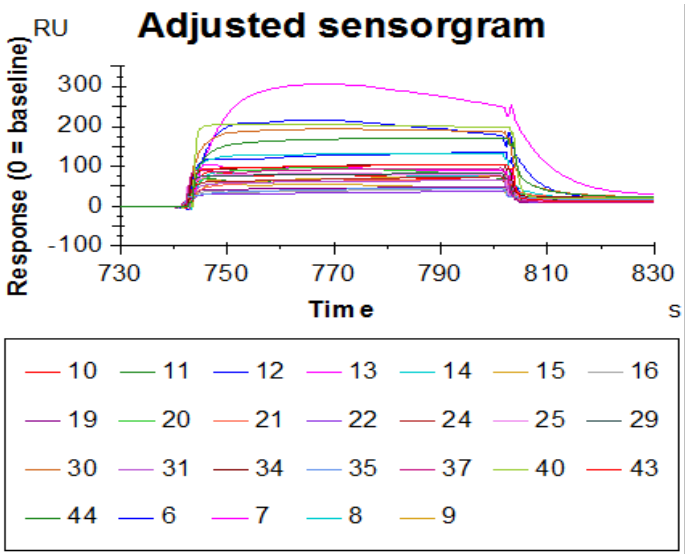

Figure 23: Binding level above propranolol. Sensorgrams for the compounds that are binding above the propranolol binding level. The numbers indicate the compound name.

A correlation was found between the responses on POPC and POPC/POPS liposome surfaces (figure 24). The fact that the correlation is not perfect indicates that the use of two different liposome surfaces may give additional information. The pattern in the graph suggests that the correlation might be composed by 2 different lines (Fig 25a). That type of separation into lines composed by acidic and neutral compounds have been seen previously in a work by Frostell *et al*. [3] (figure 25b).

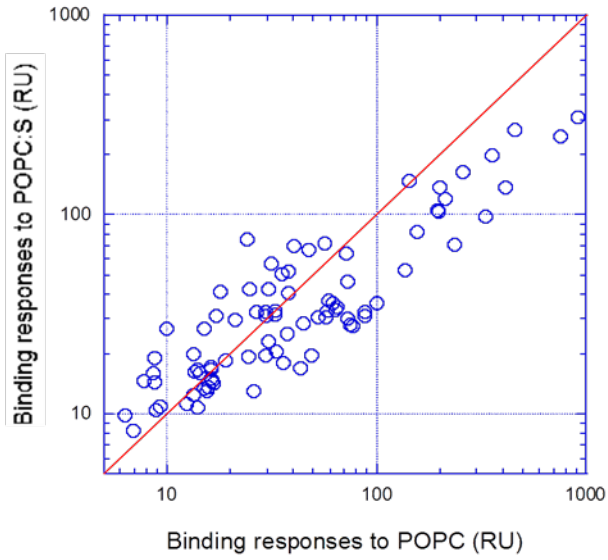

Figure 24: Correlation of binding responses. The correlation of binding responses on POPC and POPC/POPS surfaces shows that many compounds is giving similar responses on both type of liposomes.

a) b)

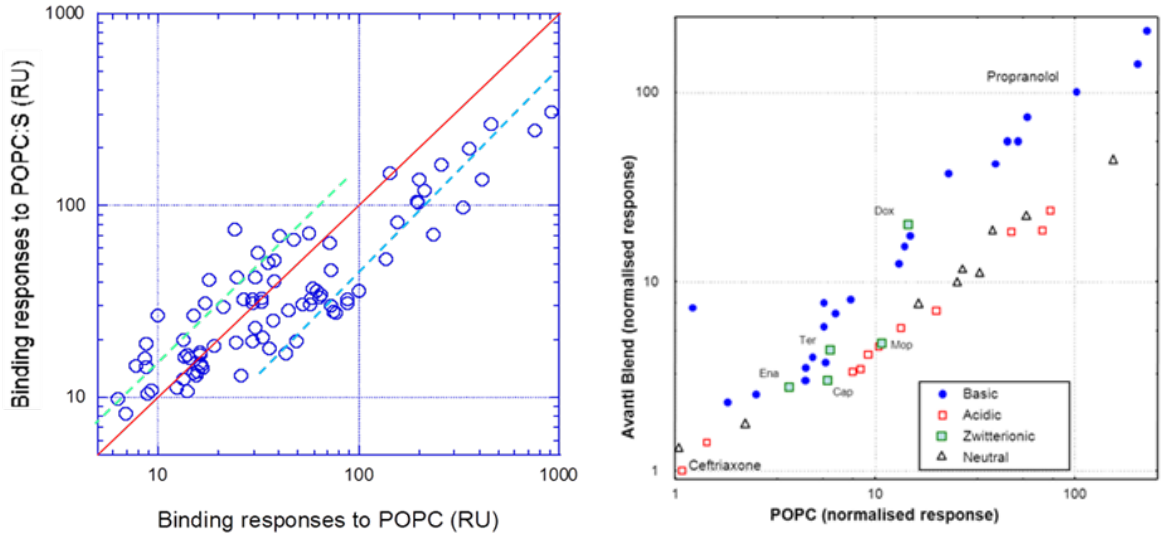

Figure 25: Correlation pattern**.** a) The correlation pattern indicates that the compounds might follow two lines, b) a figure from the work of Frostell *et al.* [3], with permission.

3.3.3 Screens on protein surfaces

We investigated whether the 47 LMW compounds have potential to bind to various type of protein surfaces and how stable they are on those surfaces. This study, together with liposome screens, was done as an attempt to develop a method for identification of sticky compounds. Evaluation steps included molecular weight and control samples adjustment, and cut off setting.

In screen against thrombin, the control sample DAPA was used as a positive control to assess the surface activity and calculate the threshold for potential binders. The binding and stability report points of the 47 compounds interacting with immobilized thrombin, run in duplicate, are presented in figure 26 and 27 together with sensorgrams for potentially sticky (as they are slowly dissociating) compounds.

In order to compare binding responses from duplicate runs on two instruments (marked as 1 and 2 in figure 26), where the ligand (thrombin) was immobilized to slightly different levels, the data was normalized according to the evaluation steps described above (chapter 2.9 Data Evaluation).

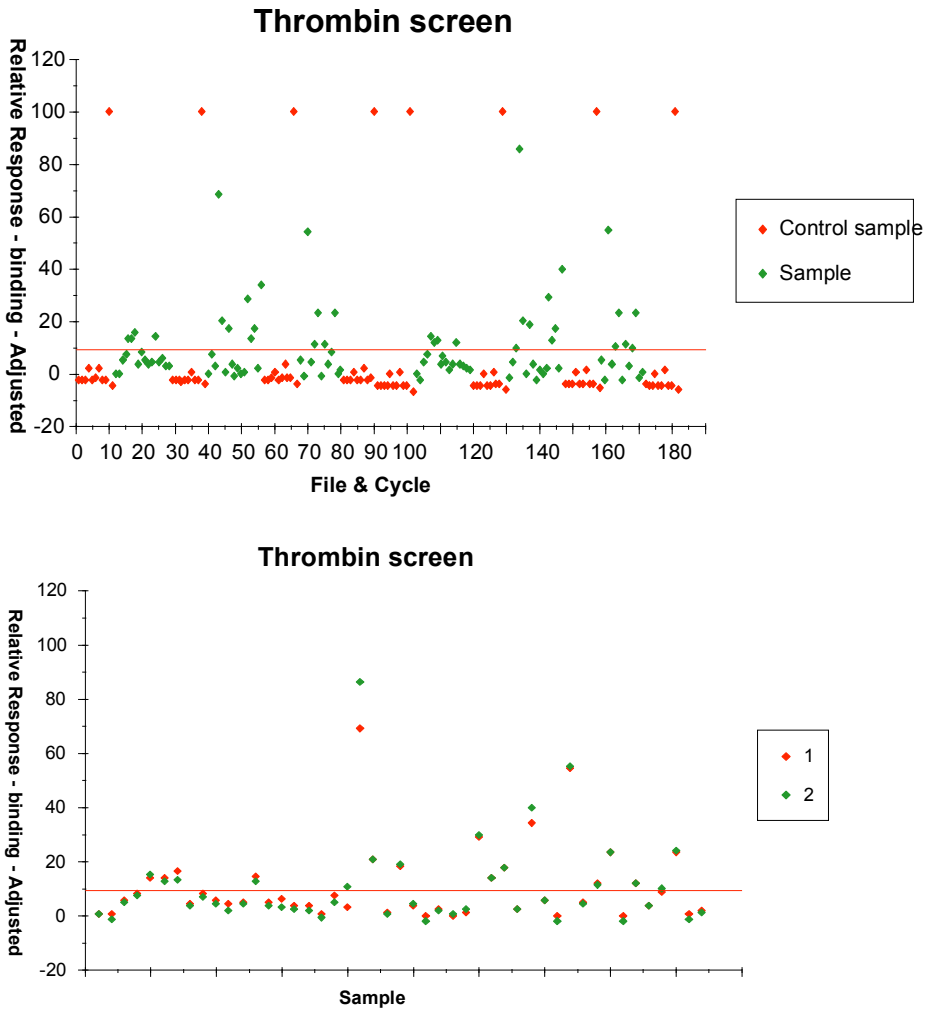

Figure 26: Binding response against thrombin. **Top plot:** binding response of compounds using duplicate view: screen 1 (in red) and screen 2 (in green). **Down plot:** binding response of compounds using sample type view, control samples (in red) and samples (in green). Positive control: DAPA, negative control: SB203580, cut off setting: 6*SD of SB203580

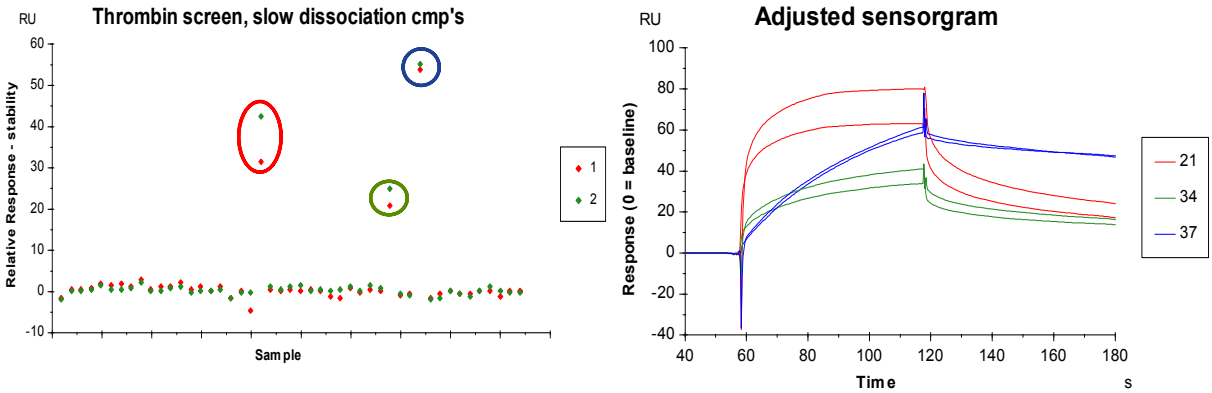

Figure 27: Stability response against thrombin. **Left panel:** stability plot of compounds using duplicate view: screen 1 (in red) and screen 2 (in green). Slow dissociation compounds are in circles. **Right panel:** Sensorgram of the slow dissociating compounds, curve colored after compound number (curve color corresponds to circle color in the stability plot).

The evaluation of screens against p38α MAP kinase and carbonic anhydrase II were performed similarly as for thrombin (see figures 28- 32 for results). Good reproducibility of responses in duplicate assays on two instruments and surfaces was observed for all protein screens.

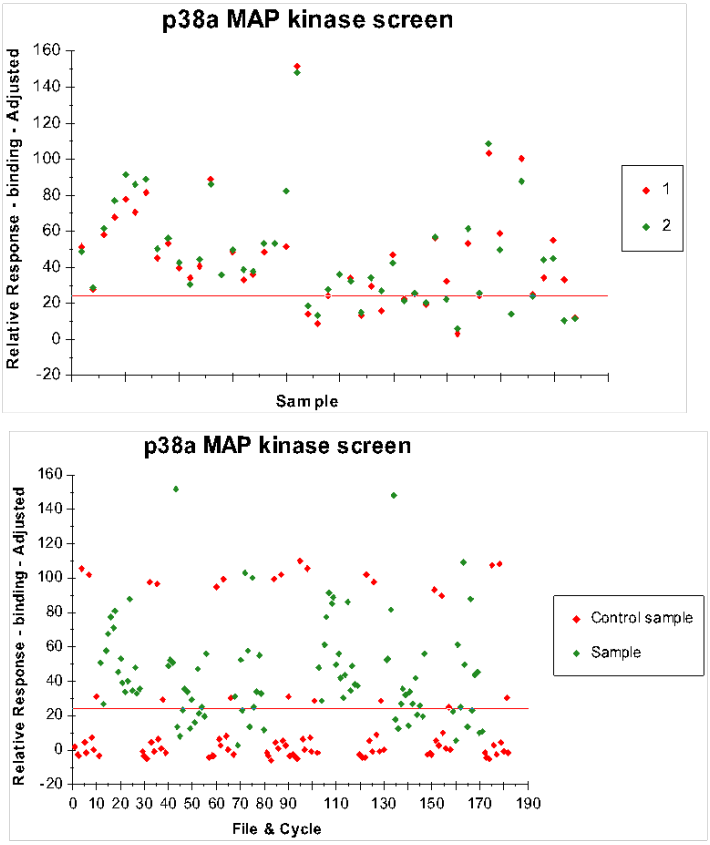

Figure 28: Binding response against p38α MAP kinase. **Top plot:** binding response of compounds against p38α MAP kinase in duplicate view: screen 1 (in red) and screen 2 (in green). **Down plot:** Binding response in sample type view: control samples (in red) and samples (in green). Positive control: SB202190, negative control: running buffer, cut off setting: 6*SD of negative controls.

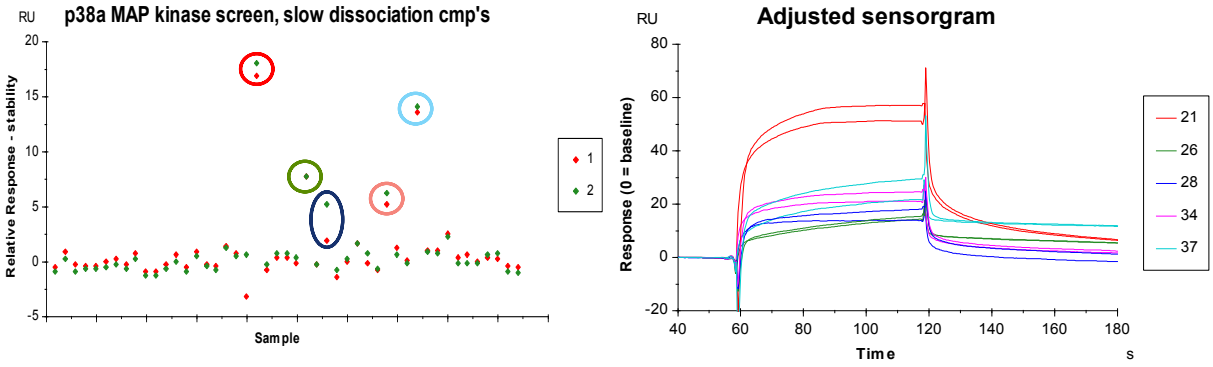

Figure 29: Stability response against p38α MAP kinase. **Left panel:** stability plot of compound against p38α kinase MAP, duplicate view: screen 1 (in red) and screen 2 (in green). Slow dissociation compounds are in circles. **Right panel:** Sensorgram of the slow dissociating compounds, curve colored after compound number (curve color corresponds to circle color in the stability plot).

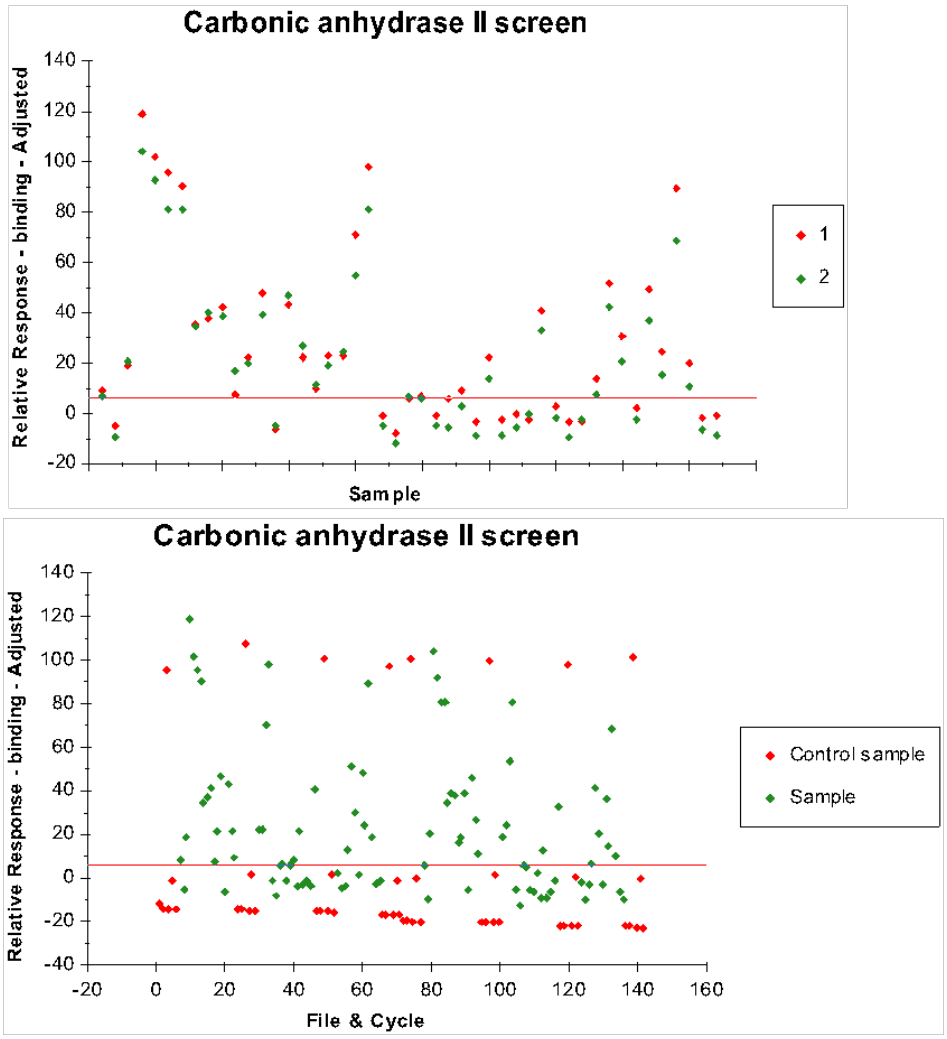

Figure 31: Binding response against Carbonic anhydrase II. **Top plot:** binding response of compounds against carbon anhydrase II in duplicate view: screen 1 (in red) and screen 2 (in green). **Down plot:** Binding response in sample type view: control samples (in red) and samples (in green). Positive control: Benzenosulfonamide, negative control: Warfarin, cut off setting: 6*SD of Warfarin.

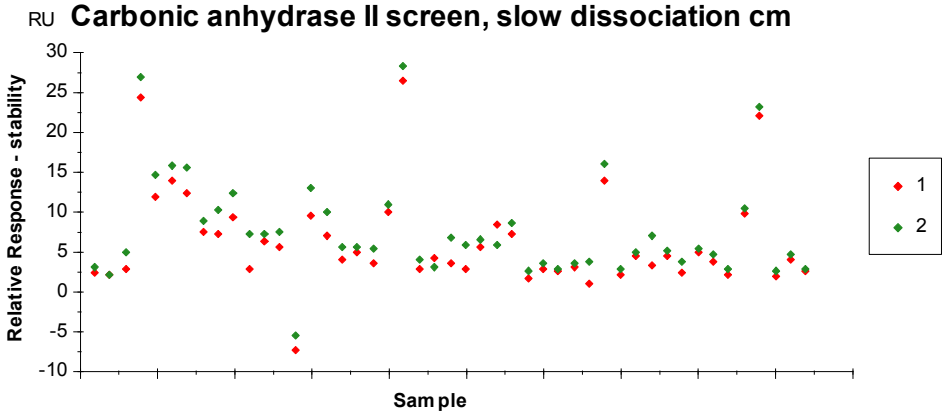

Figure 32: Stability response against Carbonic anhydrase II. Stability plot of compounds against carbonic anhydrase II, duplicate view: screen 1 (in red) and screen 2 (in green). Large number of potentially sticky compound was observed in this screen.

3.4 Sticky compounds

Figure 33 shows sensorgrams for compounds that most likely stick to the protein surfaces as they are slowly dissociating. Three compounds (21, 34 and 37) are dissociating slowly from all protein surfaces, and are also binding to unmodified dextran surface (figure 34). This indicates that those compounds are generally sticky and have the potential to bind to different types of surfaces. Compound 37 binds even to the L1 sensor surface (figure 34).

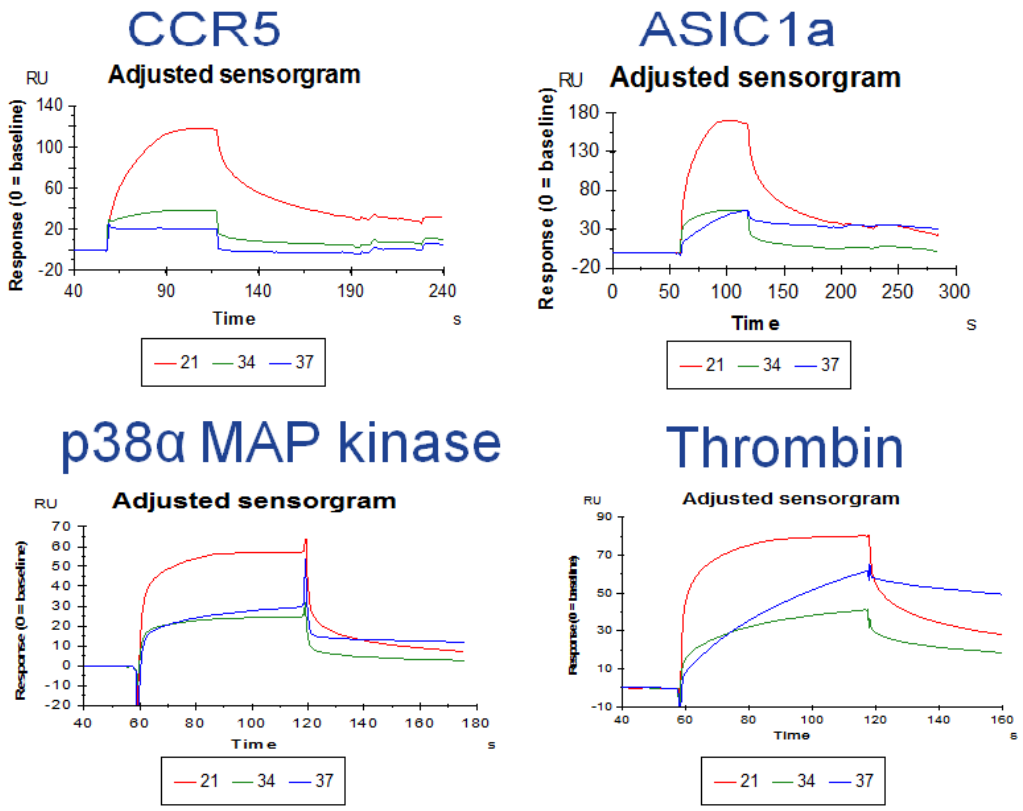

Figure 33: Sensorgrams for compounds that slowly dissociates on protein surfaces.

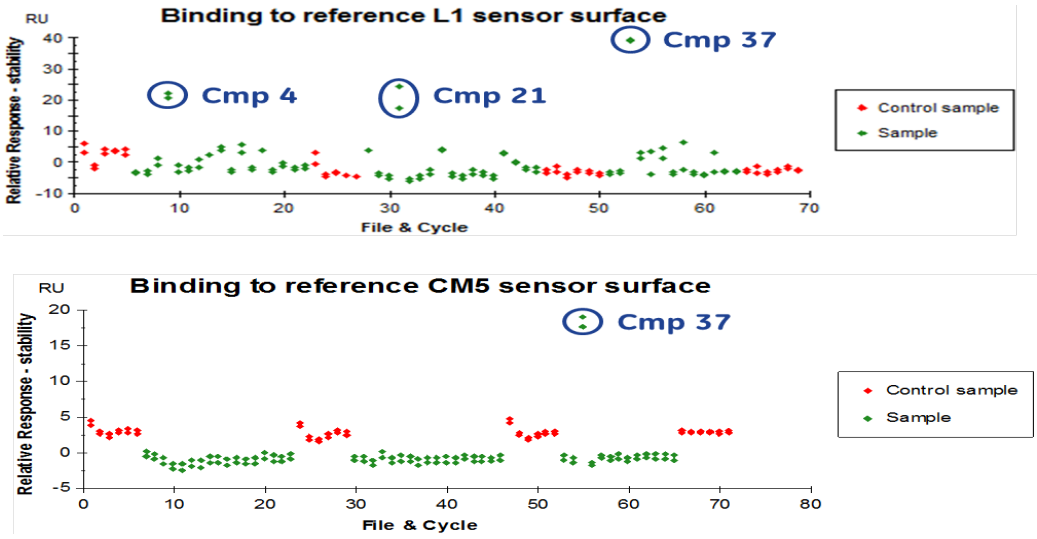

Figure 34: Binding responses of 47 compounds on reference surfaces. Top: Sensor chip L1 Series S. Bottom: Sensor chip CM5 series S. Binding compound are indicated with circles.

The results of all screens are summarized in table 5. By using the following symbols (-) non-binder, (+) potential binder, and (++) potentially non-specific or sticky binder on protein surface or binding to higher level than propanolol on liposome surface. As shown in table 3, the activity of CAII and p38α MAP kinase was less than 50%, as indicated from the theoretical Rmax of control samples. Moreover, the screen results from CA II showed that the majority of compounds are slowly dissociating, which, together with low activity, suggests that this protein may be partly denatured.

Table 5: Summary of the screens of 47 compounds against various types of ligand.

	Membrane Proteins		Liposomes		Proteins		
sample	CCR5	ASIC1a	POPC	POPC/POPS	Thrombin	p38α MAP Kinase	CA II
1	+	+	+	-	-	+	+
2	-	+	-	-	-	+	+
3	+	+	++	++	-	+	+
4	-	+	++	++	-	+	+
5	+	+	++	++	+	+	+
6	+	+	++	+	+	+	+
7	++	-	++	+	+	+	+
8	+	+	+	+	-	+	+
9	+	-	+	+	-	+	+
10	+	-	+	+	-	+	+
11	+	-	+	+	-	+	+
12	-	-	++	+	-	+	+
13	+	-	+	+	+	+	+
14	+	-	+	+	-	+	+
15	+	+	+	+	-	+	+
16	-	-	+	+	-	+	+
17	-	-	-	-	-	+	+
18	-	-	-	-	-	+	+
19	-	-	+	+	-	+	+
20	+	+	+	+	+	+	+
21	++	++	-	+	++	++	+
22	+	+	-	+	+	-	+
23	-	-	-	-	-	-	+
24	+	+	+	+	+	+	+
25	+	+	+	+	-	+	+
26	-	-	-	-	-	++	+
27	-	-	-	-	-	-	+
28	-	-	-	-	-	++	+
29	-	-	+	+	-	+	+
30	+	+	+	+	+	+	+
31	-	-	+	+	+	-	+
32	-	-	-	-	+	+	+
33	-	-	-	-	-	-	+
34	++	++	-	+	++	++	+
35	-	-	-	+	-	+	+
36	-	-	-	-	-	-	+
37	++	++	+	+	++	++	+
38	+	+	-	-	-	+	+
39	-	-	++	++	+	+	+
40	++	++	+	+	+	+	+
41	-	-	-	-	-	-	+
42	++	++	-	-	+	+	+
43	+	+	+	+	-	+	+
44	+	+	+	+	+	+	+
45	+	+	++	++	+	+	+
46	-	-	-	-	-	+	+
47	-	-	-	-	-	-	+
Non-Binder (-)			Binder (+)			Slow dissociation/ Sticky (++)	

4.0 Affinity Screen

The compounds identified as potential binders of ASIC1a and CCR5 were selected for affinity screen (see table 4). To assess the affinity of the compounds that were potential binders of the membrane proteins, the concentration series, 6-100 µM, of these compounds were injected over ASIC1a and CCR5 surfaces. However, in case the K_D values are higher than about 50 µM (a half value of the highest concentration used), the affinity cannot be accurately determined and models with constant Rmax are used (see chapter 2.9.1) to estimate the affinities to the best of ability. Figures 35 and 36 present the graphs and sensorgrams, and tables 6 and 7 show the calculated parameters together with model used, for interaction of tested compounds with CCR5 and ASIC1a, respectively.

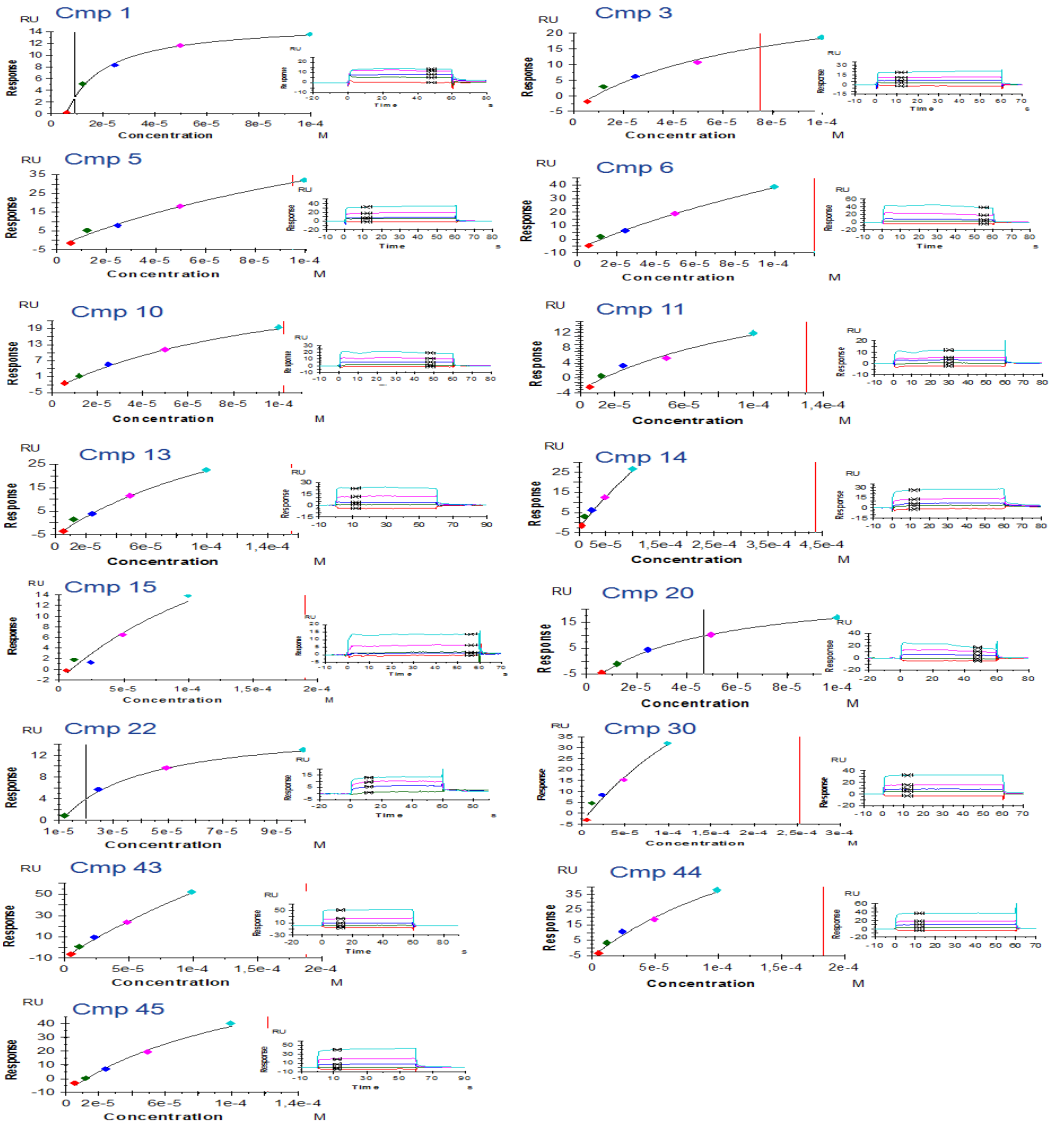

Figure 35: Affinity plot against CCR5. Overview of affinity plot and sensorgrams for compounds that are potential binders to CCR5. Each plot shows steady state responses plotted against concentration fitted to steady state model (indicated in table 6). Inserts represent corresponding sensorgrams with report point used for evaluation marked in black.

Table 6: Affinity screen data for CCR5.

Sample	CCR5_KD (M)	model	Rmax fit	Rmax theor	Chi² (RU²)
1	9,12E-06	SSA	25,7	41,48	0,19
3	7,46E-05	SSA	39	41,03	1,91
5	9,50E-05	SSA_MS	45,1	40,51	7,37
6	1,20E-04	SSA_MS	51,1	45,56	5,69
10	1,02E-04	SSA	46,8	44,98	0,09
11	1,30E-04	SSA	33,7	40,51	1,41
13	1,55E-04	SSA	70,9	41,23	1,71
14	4,37E-04	SSA	153,5	67,73	2,20
15	1,91E-04	SSA_cRmax	43,2	40,25	1,85
20	4,66E-05	SSA	37,5	44,12	0,05
22	1,97E-05	SSA	26,7	77,14	0,08
30	2,53E-04	SSA	125	77,40	7,45
43	1,88E-04	SSA_MS	95,1	82,65	4,06
44	1,83E-04	SSA	120,9	82,26	4,26
45	1,22E-04	SSA_cRmax	105,6	90,55	4,17

SSA - Steady State Affinity **SSA_MS - Steady State Affinity Constant Rmax (Multi Site)**

SSA_cRmax - Steady State Affinity Constant Rmax

The dissociation equilibrium constant, K_D, type of steady state model used for calculation, Rmax fitted according to experimental data, Rmax theoretical, and goodness of fit, Ch2, are presented for compounds examined in affinity screen against CCR5.

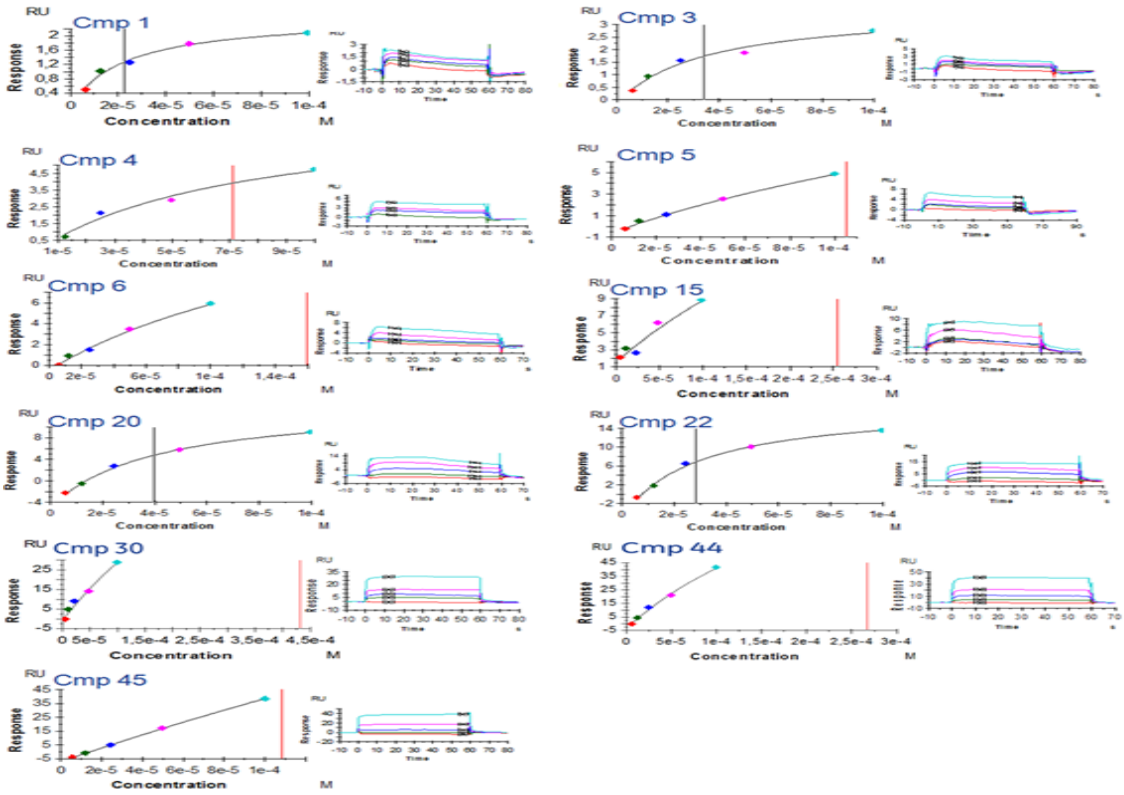

Figure 36: Affinity plota against ASIC1a. Each plot shows steady state responses plotted against concentration, for potential ASIC1a binders, and fitted to steady state model (indicated in table 7). Insets represent corresponding sensorgrams with report point used for evaluation marked in black.

Table 7: Affinity screen data for ASIC1a.

Sample	ASIC1a_KD (M)	model	Rmax fit	Rmax theor	Chi² (RU²)
1	2,25E-05	SSA	2,6	5,8	0,01
3	3,39E-05	SSA	3,8	5,7	0,04
4	7,15E-05	SSA	8,8	5,9	0,20
5	1,05E-04	SSA_MS	5,4	5,7	0,05
6	1,47E-04	SSA_MS	6,1	6,4	0,11
15	4,33E-04	SSA	6,3	8,5	1,67
20	3,99E-05	SSA	19,7	9,3	0,04
22	2,84E-05	SSA	24,2	16,3	0,15
30	3,81E-06	SSA_MS	157,5	16,3	4,08
44	6,93E-06	SSA_MS	162,4	17,4	1,78
45	1,08E-04	SSA_MS	6,8	19,1	0,00
SSA - Steady State Affinity SSA_MS - Steady State Affinity Constant Rmax (Multi Site)					
SSA_cRmax - Steady State Affinity Constant Rmax					

The dissociation equilibrium constant, K_D, type of steady state model used for calculation, Rmax fitted according to experimental data, Rmax theoretical, and goodness of fit, Ch2, are presented for compounds examined in affinity screen against ASIC1a.

5.0 Discussion

The aim of this project was to develop SPR-assay for the pre-screening of LMW-compounds to identify potentially harmful sticky binders. The sticky compounds may disturb membrane protein surfaces thus preventing the binding of subsequent compounds. However, in the optimized assay, the sticky compounds that bind either to sensor surfaces or to protein surfaces, appear not to destroy membrane proteins, as the binding capacity remains similar or follows a smooth decay, which can easily be compensated by using control samples and the "adjustment for controls" functionality of the evaluation software. The membrane proteins could be captured directly from the cell extract, using single injection only, and the resulting surfaces could be used for screen for as long as 40 hours. Deviating sensorgram shape and affinity of the control sample injected in the first cycle suggests that a binding sample rather than buffer should be used in start-up cycles.A single capture injection gives an advantage of reproducibility of sample injection and makes it possible to avoid problems connected to different capture levels.

We tested the binding behavior of all the 47 compounds provided by Roche against three different proteins and two liposome surfaces to examine the binding pattern. Irregularly shaped sensorgrams, with apparent dissociation phase and a higher than calculated Rmax binding level, could indicate non-specific binding. We found that 3 compounds (21, 34 and 37) were sticky to all tested protein surfaces and should be thereby easily identified in a pre-screen. Compounds 4, 21 and 37 bound also to L1 surface and compound 37 to dextran surface. Table 8 shows the binding characteristics of compounds, selected for affinity screen, against proteins, liposomes and sensor surfaces. The majority of compounds bind to liposomes and carbonic anhydrase II (see chapter 3.4). Compounds 3, 4, 5, 6, 7, 12, 39 and 45 have higher binding response to liposomes than the positive control, propanolol, and do not fully dissociate from liposome surfaces as indicated from "stability" report point. Interestingly, the com-

pounds identified as sticky on protein surfaces are behaving well (binders or no binders) on the liposome surfaces (table 5).

Table 8: Summary of compounds tested for affinity screen.

	Membrane Proteins				Liposomes		Proteins			Sensor Surfaces	
Sample	CCR5_KD (M)	CCR5_model	ASIC1a_KD (M)	ASIC1a_model	POPC	POPC:S	Thrombin	CAII	p38alpha	L1 chip	CM5 chip
1	9,12E-06	SSA	2,25E-05	SSA	+	-	-	-	-	-	-
3	7,46E-05	SSA	3,39E-05	SSA	++	++	-	-	-	-	-
4	-	-	7,15E-05	SSA	++	++	-	++	-	+	-
5	9,50E-05	SSA_MS	1,05E-04	SSA_MS	++	++	-	++	-	-	-
6	1,20E-04	SSA_MS	1,47E-04	SSA_MS	++	+	-	++	-	-	-
10	1,02E-04	SSA	-	-	+	+	-	++	-	-	-
11	1,30E-04	SSA	-	-	+	+	-	-	-	-	-
13	1,55E-04	SSA	-	-	+	+	-	+	-	-	-
14	4,37E-04	SSA	-	-	+	+	-	-	-	-	-
15	1,91E-04	SSA_cRmax	4,33E-04	SSA	+	+	-	++	-	-	-
20	4,66E-05	SSA	3,99E-05	SSA	+	+	-	++	-	-	-
22	1,97E-05	SSA	2,84E-05	SSA	-	+	+	-	-	-	-
30	2,53E-04	SSA	3,81E-06	SSA_MS	+	+	+	+	-	-	-
43	1,88E-04	SSA_MS	-	-	+	+	-	++	-	-	-
44	1,83E-04	SSA	6,93E-06	SSA_MS	+	+	+	++	-	-	-
45	1,22E-04	SSA_cRmax	1,08E-04	SSA_MS	++	++	+	+	-	-	-

SSA - Steady State Affinity **SSA_MS** - Steady State Affinity Constant Rmax (Multi Site)

+ binding minus: no binding detected at 100 µM

++ higher binding level than propanolol on Liposome surface or slow dissociation on Protein surface

Compounds tested for affinity screen against the membrane proteins CCR5 and ASIC1a, the used model for calculation of K_D, together with binding properties of the compounds to liposomes, proteins, and sensor chip surfaces.

None of the potential membrane proteins binders, selected for affinity screen, show a binding to p38α MAP kinase and to dextran surface, and only few to thrombin and one to L1 surface, thus indicating that most of the identified binders are specific to CCR5 and ASIC1a. On the other hand, all of CCR5 binders, except compound 4, appear to bind also to ASIC1a. According to our results, four compounds (10, 11, 13 and 14) and one compound (cmp 4) are most likely unique binders of CCR5 and ASIC1a, respectively. In affinity screen, the data has only 5 experimental points, with the highest concentration being 100 μM. For reliable determination of affinity constant from steady state models, the highest concentration of compound should ideally be at least 2 times higher than affinity constant. These conditions are only fulfilled for few compounds. The affinity of remaining compounds to membrane proteins are only estimated using different variants of steady state model with constant (pre-determined) Rmax (described in materials and methods). The selection of the model was based on fitted and theoretical Rmax and chi^2 (goodness of fit), and it was also taken into consideration that if the fit is forced to theoretical Rmax and only 5 experimental points are available, then a misleading fitted K_D (sometimes underestimated=better affinity) can be obtained. Estimated affinity values range from about 4 to 400 μM. For accurate affinity determination, however, the measurements should be repeated using adequate concentration range and the higher number of concentrations.

Based on our results, screen against membrane proteins can be performed without any prescreen procedure. It is, however, essential to keep bulk variation within solvent correction range. The careful sample preparation is then very important since bulk, if not corrected, can

be misinterpreted. In addition, plot tools of T200 software version 2.0 are useful to compensate for activity decrease of the surfaces, to perform molecular weight adjustment and to set cut off. Nevertheless, using pre-screen against optional proteins can give additional information about the stickiness and specificity of binding to a membrane protein. Our work shows that the biosensor assays are convenient tools for screen and characterization of membrane-associated proteins.

6.0 Acknowledgements

This project was collaboration between Dr Walter Huber and Dr Sylwia Huber at Hoffmann La Roche in Basel and supported by GE Healthcare Bio-science AB. I would like to express my deep and respectful gratitude for those who made the project possible. First and foremost, I would like to thank my supervisor Ewa Pol for her valuable guidance, support and advice throughout the whole project. Her willingness to motivate me contributed a lot to this project. She encouraged and inspired me greatly during the whole project. Besides, I would also like to thank my topic examiner Markku Hämäläinen for revising my work and providing with insight full comments. Also, I would like to take this opportunity to thank all the co-workers at GE Healthcare who had assisted me during the project.

From the University I would like to thank my examiner Lars-Göran Josefsson, for his consideration and support during the course. I would also like to thank my opponents Ara-Shant Migoyan and Anders Bankefors for their proofreading.

I would like to acknowledge my family for their support, especially my parents, Adnan and Perwen, who are truly behind every success and achievement in my life.

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