Mass spectrometry-based detection of ligand binding to membrane-embedded receptors.

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Introduction

Affinity and kinetics of compound binding to a protein of interest (POI) are fundamental parameters that guide any drug discovery effort. Their characterisation is, however, often hindered by technical challenges such as lack of active, purified POI that could be used in biophysical assays. This is particularly true for membrane proteins, including plasma membrane-localised receptors, which are notoriously difficult to purify in an active, correctly folded state. Binding of radioactively labelled ligands to POIs present in membrane fractions derived from cells that express them, overcomes this problem and has been widely used in receptor-ligand interaction studies[1,2,3]. However, environmental concerns, high cost, time-line considerations and the need of generating a radioactive derivative of a ligand limit the utility of this approach. For these reasons, non-radioactive, label-free approaches have been investigated as a substitute for radioactivity-based ligand detection. Advances in mass spectrometry allow it to be used for detection of picomolar concentrations of compounds and as such has recently been successfully used in receptor-ligand interaction studies[3,4,5]. Driven by published data[3], our current work on ligand binding to adenosine A2A receptor validates the utility of this approach to monitoring receptor-ligand interactions both at steady-state and in a kinetic mode. Our results also further exemplify the vast expertise in receptor-oriented drug discovery of Sygnature Discovery.

Experimental approach

receptor-ligand incubation
(overexpressed adenosine A2A receptor in HEK293 membranes + ZM-241,385)

↓

separation of unbound ligand
(96-well filter plate, wash and vacuum filtration)

↓

elution of bound ligand with 50% MeOH

↓

quantification of ligand by LC-MS
(based on a standard curve)

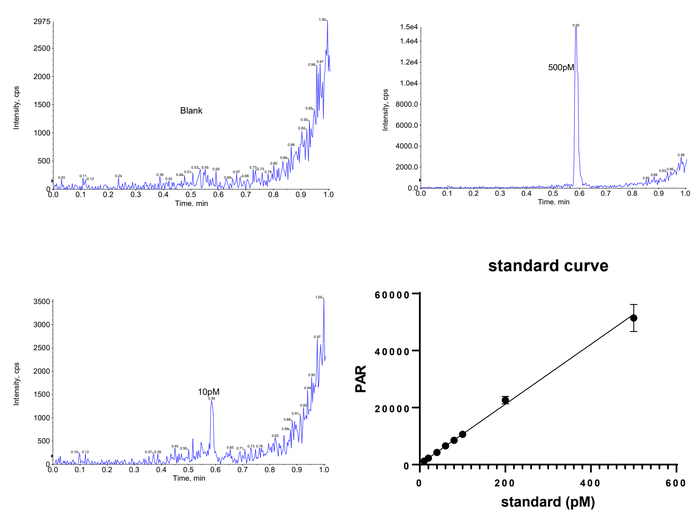

Figure 1: Outline of the experimental strategy. Protocol flow-chart and representative mass spectrometry chromatograms, which exemplify elution profiles for the indicated ZM-241,385 concentrations, are shown. Mass spectrometry chromatograms were then used to calculate Peak Area Response (PAR) for standard curve generation.

Concentrations of ZM-241,385 as low as ~10 pM can be reproducibly detected using mass spectrometry indicating that the methodology is highly sensitive and can be used in receptor-ligand interaction studies.

Saturation binding

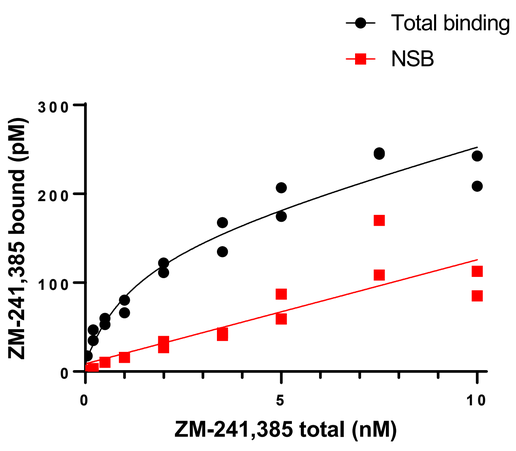

Figure 2: Steady-state binding of ZM-241,385 to adenosine A2A receptor. Increasing concentrations of ZM-241,385 were incubated with adenosine A2A receptor membranes for 3h at 4ºC, unbound ligand was removed by vacuum filtration and filter plates were extensively washed with ice-cold assay buffer. Plates were dried, bound ligand eluted and quantified by mass spectrometry. Non-specific binding (NSB) was measured in the presence of 100 µM competitor, NECA. Shown is a representative graph from n=3 biological replicates.

Mass spectrometry-based detection of ZM-241,385 binding to adenosine A2A receptor is highly reproducible and the resulting mean K_D of 1.16 nM (n=3 biological replicates) is consistent with published data[3]. Non-specific binding is below 50% of total binding allowing for reliable calculation of specific binding.

Competition binding

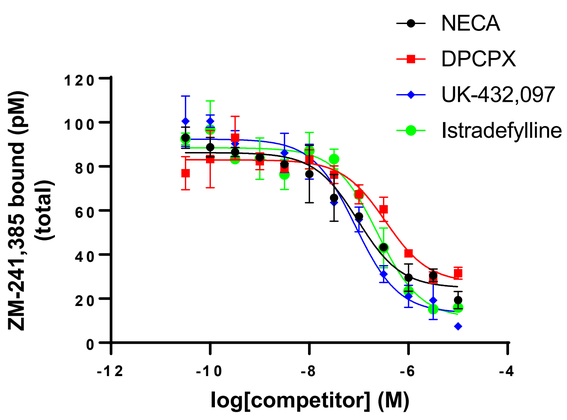

Figure 3: ZM-241,385 as a marker ligand in competition binding studies. ZM-241,385 at 1 nM concentration was incubated with adenosine A2A receptor membranes for 3h at 4ºC in the presence of increasing concentrations of the indicated competitor ligands. Unbound ligands were removed by vacuum filtration and filter plates were extensively washed with ice-cold buffer. ZM-241,385 was then eluted and quantified by mass spectrometry. Shown is a representative graph from n=3 biological replicates.

Competition binding - summary

	N1	N2	N3	Mean (± SEM)
NECA	74.5 nM	51 nM	60 nM	62 ± 7 nM
DPCPX	293 nM	185 nM	279 nM	252 ± 34 nM
UK-432,097	61 nM	46 nM	44 nM	50 ± 5 nM
Istradefylline	116 nM	127 nM	188 nM	144 ± 23 nM

Table 1: Summary of competition binding data. K_i values for adenosine A2A receptor binding by the indicated ligands were calculated from competition binding experiments (see Figure 3) using ZM-241,385 as a marker. Values for each biological replicate and mean (± SEM) are shown.

K_i values of the indicated ligands for adenosine A2A receptor binding obtained at Sygnature Discovery are in close agreement with the ones published elsewhere[3,6], and show that mass spectrometry-based detection and radioligand binding yield comparable results.

Kinetics of ligand binding

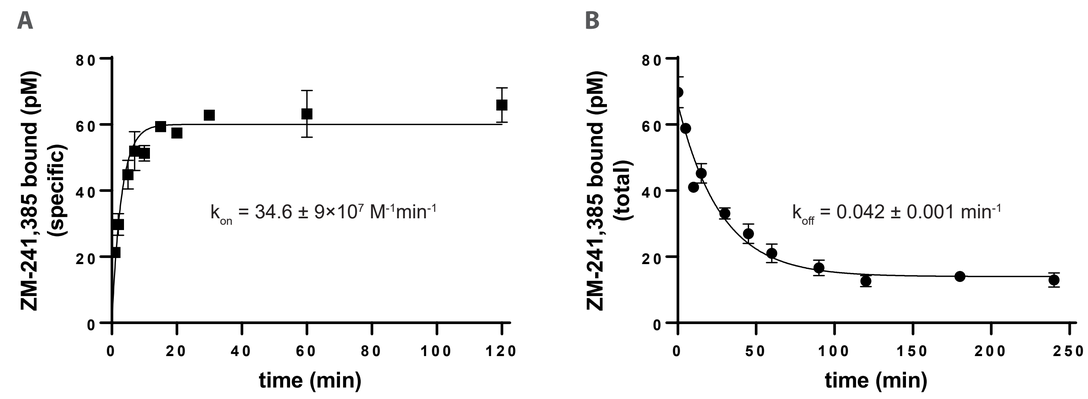

Figure 4: Kinetics of ZM-241,385 binding to adenosine A2A receptor. A) Association of 0.5 nM ZM-241,385 with adenosine A2A receptor was monitored over time. **B)** Following a 60-min association of 0.5 nM ZM-241,385 with adenosine A2A receptor, ligand dissociation was initiated by the addition of 100 µM NECA and bound ZM-241,385 was quantified at the indicated time points. Kinetics parameters obtained for ZM-241,385 binding to adenosine A2A receptor using mass spectrometry-based detection of unlabelled ligand are also shown (mean ± SEM from n=3 biological replicates).

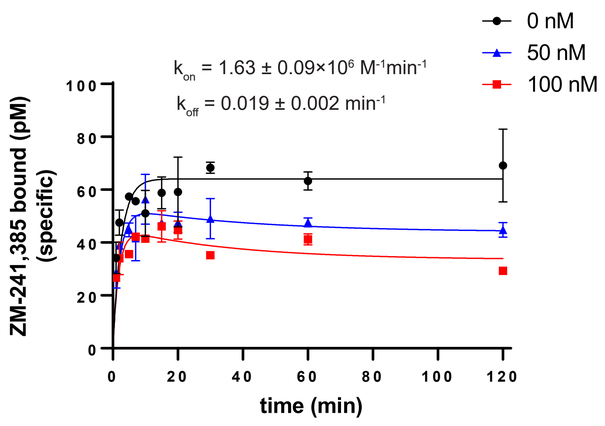

Figure 5: Kinetics of competitive binding. Association kinetics of 1 nM ZM-241,385 with adenosine A2A receptor was determined in the presence of the indicated concentrations of a competing ligand, UK-432,097, and was used to calculate the kinetics parameters of UK-432,097 binding to adenosine A2A receptor. ZM-241,385 binding in the presence of 100 µM NECA was used to quantify non-specific binding. Representative graph and calculated kinetics parameters (mean ± SEM from n=2 biological replicates) are shown.

Mass spectrometry-based detection of unlabelled ligands is suitable for kinetics analysis of receptor-ligand interaction and yields high-quality, reproducible data.

Summary

Being able to monitor ligand binding to membrane-embedded receptors is crucial to advance drug discovery campaigns in areas such as GPCR signalling. While traditionally radioligand binding assays have been used for this purpose[1,2], preparation of a radiolabelled ligand derivative is expensive and time-consuming. Here, we have reproduced published data[3] and validated the potential of mass spectrometry to monitor receptor-ligand interactions. Our results are robust, highly reproducible and illustrate the capability and expertise that Sygnature Discovery can offer to its clients.

References

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