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Development of a novel TR-FRET-based competition kinetic binding assay to assess the kinetics of unlabeled adenosine A2a receptor ligands

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Introduction

Adenosine A2a receptor (A2aR) agonists are important regulators of myocardial blood flow whilst antagonists regulate dopamine signalling and are used in Parkinson's disease. Existing data on adenosine A2aR agonists/antagonists show a range of dissociation and association rates reviewed in Guo et al.[1] The majority of these studies have been performed with radiolabelled compounds which are both expensive to synthesise and time consuming to characterise. We have developed a novel higher-throughput TR-FRET based competition kinetic method to investigate the kinetics of a series of adenosine A2 agonists/antagonists at the human A2aR under identical assay conditions in order that these parameters can be directly compared.

Method

The kinetic parameters of unlabeled compounds were assessed using a competition kinetic binding assay essentially as previously described by Klein-Herenbrink et al.[2] Specifically fluorescent CA200645 (100nM, final assay volume 40 µL in LABMED buffer (CisBio)) was incubated in the presence of unlabeled antagonist, and terbium-labelled CHO-A2a cell membranes (4 µg well^{-1}) in the presence of GppNHp (100µM) at room temperature. Assays were initiated by addition of membranes to a 384-well Optiplate plate and TR-FRET measurements were taken every 30 sec on a PHERAstar FS (BMG) using standard instrument settings. Specific binding, defined by ZM241385 (1 µM) was plotted against time and fitted to a competitive kinetic binding model (GraphPad Prism 7.0).

Results

The association and dissociation rates of fluorescent CA200645 were 1.93x10^{6} M^{-1} min^{-1} and 0.22 min^{-1} respectively, with a corresponding kinetic K_d of 166nM. The kinetics of competitive binding of 7 adenosine A2a ligands were examined and the results are summarised in Table 1.

Table 1. Kinetic parameters of A2aR ligands. Data are expressed as mean ± SEM (n ≥ 3). *Agonists are shown in italics.*

Compounds	***HTRF-derived equilibrium and kinetic parameters***			
	k_{on} (µM^{-1} min^{-1})	k_{off} (min^{-1})	*Kinetic K_d (nM)*	*Equilibrium K_i (nM)*
Theophylline	0.16 ± 0.10	0.42 ± 0.10	6240	5850
MRS1220	0.31 ± 0.06	0.27 ± 0.05	100	182
XAC	35.8 ± 17.0	0.28 ± 0.07	11	9
SCH58261	240 ± 116	0.40 ± 0.10	2	4

ZM241385	2140 ± 910	0.17 ± 0.03	0.14	0.33
CGS21680	2.45 ± 1.67	0.45 ± 0.12	811	695
NECA	0.57 ± 0.13	0.33 ±0.07	750	581

Conclusions

All the A2aR compounds tested displayed similar dissociation rates (<3-fold difference). The differences in receptor affinity observed appear driven almost entirely by differences in association rates, which varied by as much as 10,000-fold. The kinetic off-rates of the antagonists closely match existing literature values, whereas agonist off-rates are more rapid than previously reported,[1] potentially reflecting differences in assay temperature and the receptor states studied. The novel method described significantly increases assay throughput potentially allows the profiling of 100-1000s of unlabeled compounds and chemical fragments allowing the development of structure-kinetic relationships.

References

1. Guo D, et al., (2016). *Chemical Reviews* [Epub ahead of print].

2. Klein-Herenbrink C, *et al* (2016). *Nature Communications* 7, 10842.