Determination of Ligand Binding Affinities at the Human Adenosine A_1-Receptor using the Fluorescent Ligand Xanthine Amine Congener-BODIPY®630/650

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Introduction

Ligand binding affinities have historically been determined using a radioligand that competes for receptor binding against an unlabelled competitor. However, safety aspects associated with this method suggest the need for a safer alternative, which does not compromise the quality of data obtained. Fluorescence technology provides an equally quantitative and sensitive substitute for the determination of ligand binding affinities. We have developed a cell-based assay which uses the adenosine antagonist Xanthine Amine Congener (XAC) conjugated to the fluorophore BODIPY® 630/650 (BY630; Briddon *et al.,* 2004) to measure binding affinities at the human adenosine A_1-receptor. In conjunction, the 8200 Cellular Detection System from Applied Biosystems provides a unique system enabling the selective detection of cell bound fluorescence. The Cellular Detection System identifies defined events which enable a distinction between cell-bound fluorescence and fluorescence free in solution. In addition the system uses a red laser which is compatible with the BY630 fluorophore.

Methods

Experiments were performed on CHO cells stably expressing the human adenosine A_1-receptor (Briddon *et al.*, (2004). Cells were grown to semi-confluence in 384-well black view plates (Greiner) in DMEM/F12 medium supplemented with 10% foetal calf serum at 37°C, 5% CO_2/humidified air. Experiments were performed in HEPES buffered saline (HBS) supplemented with 0.1% BSA and 5mM HEPES. In saturation experiments non-specific binding was determined with 1μM XAC. Unlabelled competitors were pre-incubated on cells for 30 min at room temperature prior to the addition of the fluorescent XAC-BY630 antagonist. Incubations were continued for a further 20 minutes at room temperature. Prior to fixing all buffer was removed and the cells fixed in 4% paraformaldehyde for 10 minutes at room temperature. All paraformaldehyde was removed and the cells washed once in buffer before being resuspended in a final volume of 40μl/well. Cell associated fluorescence was determined using the 8200 Cellular Detection System (Applied Biosystems; Mellentin-Michelotti *et al.*, 1999).

Results

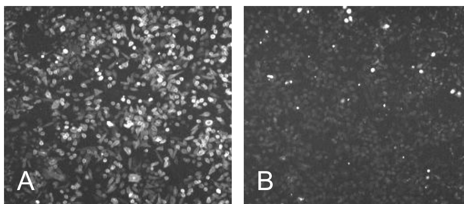


Figure 1: CHO cells stably expressing the human adenosine A_1-receptor were incubated with the fluorescent antagonist XAC-BY630 at a concentration of 40nM (A).
Binding of XAC-BY630 was displaced following a 30 min pre-incubation with 1μM XAC (B).

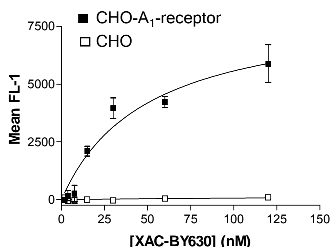
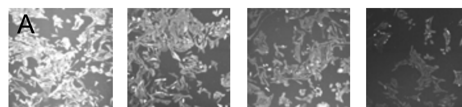


Figure 2: Specific binding of XAC-BY630 was determined in either native CHO cells or CHO cells stably expressing the human adenosine A1-receptor. Specific binding was taken as that displaceable by 1μM unlabelled XAC. Whole cell saturation binding studies on CHO-A1 cells using XAC-BY630 gave a KD value of 50.95 ± 0.81nM (n = 27). This value is similar to that obtained in membrane-based radioligand binding studies (Briddon et al., 2004).



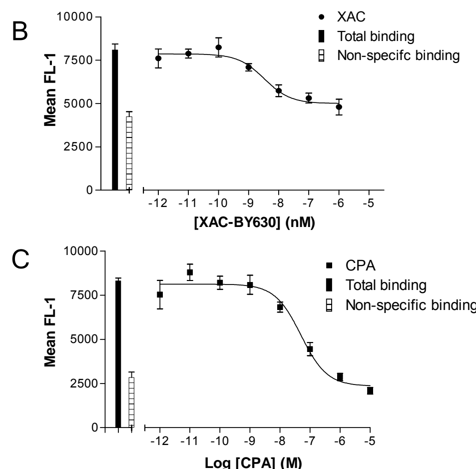


Figure 3. The non-selective adenosine antagonist XAC decreased A_1-receptor binding of 40nM XAC-BY630 in a dose dependent manner. Digital images of XAC-BY630 bound fluorescence on adherent CHO-A_1 cells shows displacement of the fluorescent ligand at four different concentrations of unlabelled XAC competitor (Figure 3A; 0.01, 1, 100 and 10,000nM). The digital images collected on the 8200 Cellular Detection System are converted to a numerical value which represents only cell-bound fluorescence (Figure 3B). Unlabelled XAC decreased XAC-BY630 binding to the A_1-receptor by 79.91 ± 5.61% (*n* = 5). A logK_i value for this event was calculated at −8.54 ± 0.02. The A_1-selective agonist CPA showed a higher affinity for the A_1-receptor (Figure 3C) reducing XAC-BY630 binding by 105.66 ± 3.51% (*n* = 7) and producing a logK_i value of −7.72 ± 0.14 (*n* = 7). These values are similar to that quoted in radioligand binding studies (Baker *et al.*, 2006).

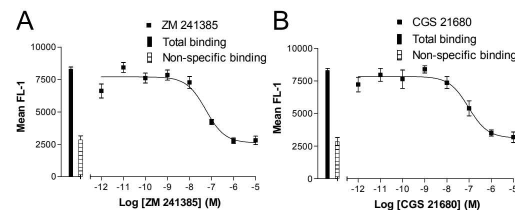


Figure 4. The adenosine A_{2a}-receptor selective antagonist ZM 241385 and selective agonist CGS 21680 had a lower affinity for the A_1-receptor and displaced 40nM XAC-BY630 in CHO-A_1 cells by 93.44 ± 4.54% (*n* = 7;Figure 4a) and 86.56 ± 3.21% (*n* = 3; Figure 4b) respectively. LogK_i values determined from this displacement equaled -7.46 ± 0.06 (*n* = 7) and -7.36 ± 0.31 (*n* = 3) for ZM 241385 and CGS 21680 respectively. Total and non-specific binding were determined in the presence of 1μM XAC.

Table 1: LogKi values for known adenosine ligands were determined through the competitive displacement of 40nM XAC-BY630. The logKi values determined were comparative to those determined through radioligand binding studies (Refs).

Ligand	Radioligand	FMAT	n
CPA	-8.64	-7.72 ± 0.14	7
NECA	-7.85	-7.65 ± 0.09	4
CGS 21680	-6.54	-7.36 ± 0.31	3
DPCPX	-8.43	-9.74 ± 0.06	3
XAC	-7.50	-8.54 ± 0.02	5
ZM 241385	-6.59	-7.46 ± 0.06	7
CGS 15943	-8.37	-9.49 ± 0.11	3
SCH 58261	-6.54	-8.57 ± 0.08	3
Aminophylline		-6.38 ± 0.06	8
8-Phenyltheophylline		-7.92 ± 0.02	3
Theophylline	-5.17	-7.09 ± 0.06	3

Discussion

These data indicate that ligand binding assays based on fluorescence technology provide a sensitive and quantitative alternative approach for the determination of ligand binding affinities while providing a safer alternative to radioligands.

References

Baker, J.G., *et al.* (2006) J. Pharmacol. Exp. Ther. 320, 218-228.
Briddon, S.J., *et al.* (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 4673-4678.
Klotz, K.N. (2000) Naunyn. Schmiedebergs Arch. Pharmacol. 362, 382-391.
Mellentin-Michelotti, J., *et al.* (1999) Anal. Biochem. 272, 182-190.

Acknowledgements

The XAC-BY630 ligand, as described in Briddon et al. 2004, and used in this study is commercially available from CellAura Technologies as cat no. A-633-AN.