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Fluorescence based live cell binding assays for the adenosine A_3 receptor – from High Content to High Throughput Screening

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Fluorescent ligands have emerged as reliable tools for the study of G protein coupled receptors. Recently, we have demonstrated that competitive equilibrium fluorescent binding assays can be performed in live cells using high content screening analysis, making multiplexing of such assays a possibility. . However, for the purposes of hit discovery and lead optimization a high throughput screening approach is highly desirable, providing that the assay can be modified to preserve the fidelity of the data. In the present study, we have used a high affinity fluorescent adenosine receptor antagonist, CA200645, to obtain binding affinity constants (pK_is) of competing ligands at the human adenosine-A_3 receptor. Results obtained from both a fluorescence microplate reader and a semi-automated high content screening assay were compared.

Binding assays were carried out in CHO-K1 cells expressing the human A_3AR at ~750fmol/mg protein. Cells were seeded into 96-well plates, and incubated with increasing concentrations of competing ligand (30 min, 37°C) followed by an incubation with CA200645 (25nM, 30 min). Fluorescent intensity in each well was initially read on a BMG Pherastar FS plate reader. Optimal focal height was determined automatically and total fluorescence intensity was assessed taking 81 reads per well (read time <3 min per plate). The same plates were subsequently analyzed using an MDC IX Ultra confocal plate reader (4 images per well), and the total fluorescent intensity of the images was used to quantify ligand binding (acquisition time of approximately 25 min per plate). Data were subsequently analyzed using Metamorph Software, with non-linear curve fitting performed in GraphPad Prism 5.

Competition binding experiments using CA200645 yielded a similar rank order of affinities for adenosine receptor antagonists when read on both the Pherastar and the IX Ultra (MRS1220> XAC> DPCPX). The pK_i values obtained showed a high degree of correlation between both platforms (pKi_{Ultra} *vs* $pKi_{Pherastar}$: MRS1220, 9.5 *vs* 9.0; XAC, 8.0 *vs* 8.0; DPCPX, 6.6 *vs* 6.5; R^2=0.99). Competitive binding assays for histamine-H_2, dopamine-D_1 and $beta_2$–adrenergic receptors using the Pherastar FS, as well as multiplexing them with luminescent measurements of cyclic AMP levels within the same assay, are under investigation. Taken together, our results strongly support the potential of the Pherastar FS plate reader as a high-throughput screening platform for competitive fluorescent binding assays for GPCRs.