Peptidic and Non-Peptidic Ligand Activated GPCRs- Comparison of Assay Platforms

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Abstract

G-protein coupled receptors (GPCRs) are an important class of drug targets which are activated by a diverse series of both peptidic and non-peptidic ligands. During our studies to identify novel small molecule agonist / antagonists of GPCRs as potential new drugs, we have compared various assay platforms including homogeneous time resolved fluorescence (htrf). Our initial observations suggest that both agonists and antagonists tested across different assay platforms generate dissimilar EC50 and IC50 values. More specifically, we observed that GPCRs activated by peptidic ligands showed a decrease in sensitivity to ligand using the htrf platform relative to a standard calcium flux platform. This decrease in sensitivity to ligand was mirrored by the apparent IC50 values of antagonists as a decrease in potency. In contrast, a decrease in sensitivity was not observed for GPCRs activated by non-peptidic ligands studied. This observation highlights the need for careful selection of an assay dependent on the target GPCR and associated ligand.

Introduction

Paradigm Therapeutics is a company specialising in the identification of the therapeutic utility of novel drug targets identified from the sequencing of the human genome. This is achieved through 'knocking out' the orthologous gene in mice and determining its potential function. Paradigm then undertakes to develop assays to interesting targets and initiate drug discovery.

During our investigations into alternate assay platforms we observed a significant difference in the Ec50 values with certain peptidic liganded GPCRs. One such GPCR was investigated in more detail. This GPCR was chosen because we could look at several different signalling pathways to see if the shift in Ec50 was common to peptidic GPCRs or specific to the htrf assay platform. The shift was again observed with both htrf assays used. We did not observe this shift with a non-peptidic GPCR (M3 muscarinic receptor) in accord with the manufacturer's data.

Materials & Methods

Calcium 3 and Catch Point assay kits were obtained from Molecular Devices. Delfia GTPγS) assay kit was obtained from Perkin Elmer. IP1 and cAMP htrf assay kits were obtained from Cisbio. All of the kits were used according to the manufacturers protocols with the conditions indicated with the following data.

Proprietary Target GPRA

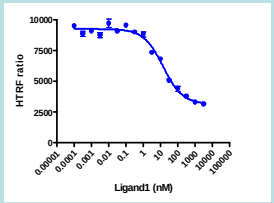

Fig.1 Response of HEK/GPRA cells to ligand1 measured by IP1 accumulation: Ec50 7.9nM. 25,000 cells/well

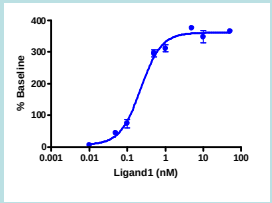

Fig. 2 Response of HEK/GPRA cells to ligand1 measured by calcium flux: Ec50 0.2nM. 30,000 cells/well

The response, of stably transfected HEK-293 cells with a human GPCR, to ligand1 (10mer peptide) was measured by Ca^{2+} flux and IP1 accumulation (Figs. 1 & 2 show representative data). This showed a 40 fold difference in the apparent Ec50 values. The Kd was determined in saturation binding experiments with purified membranes to be 0.88nM and Ki values of < 1nM (data not shown). The binding and Ca^{2+} flux data appear to correlate with published literature where as in contrast the IP1 htrf assay appeared to give an under-estimation of the Ec50 for the ligand to GPRA.

GnRH Receptor

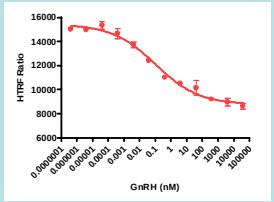

Fig. 3 Response of CHO/GnRHR cells to GnRH measured by IP1 accumulation: Ec50 0.13nM 25,000 cells / well

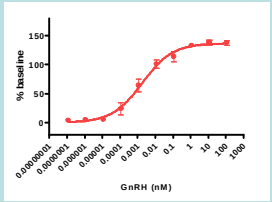

Fig. 4 Response of CHO/GnRH cells to GnRH measured by calcium flux: Ec50 0.002nM 20,000 cells / well

The response of stably transfected CHO-K1 cells with the rat GnRH receptor to GnRH was measured by calcium flux and IP1 accumulation (Figs. 3 & 4). Similarly there was a 65 fold decrease in Ec50 value with the htrf platform. This indicates that the affect was not cell line or GPCR specific.

Muscarinic M3 Receptor

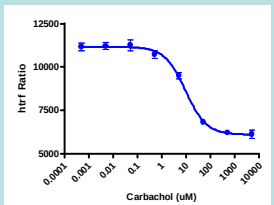

Fig. 5 Response of 1321N1 cells to carbachol measured by IP1 accumulation: Ec50 9uM 20,000 cells / well

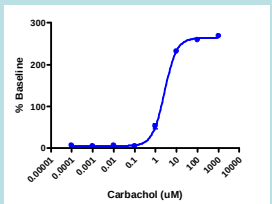

Fig. 6 Response of 1321N1 cells to carbachol measured by Calcium flux: Ec50 3uM 30,000 cells / well

The response of endogenously expressed muscarinic M3 receptor in 1321N1 cells to carbachol was also measured by calcium flux and IP1 accumulation (Figs. 5 & 6). Ec50 values were only three fold different with this non-peptide liganded GPCR.

Proprietary Target GPRB

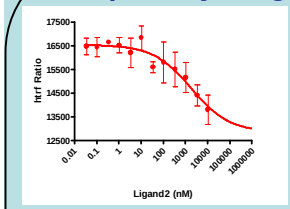

Fig. 7 Response of HEK/GPRB to ligand2 measured by IP1 accumulation 20,000 cells / well: Ec50 1800nM

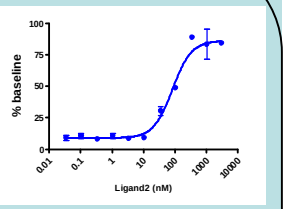

Fig. 8 Response of HEK/GPRB to ligand2 measured by calcium flux, 20,000 cells / well: Ec50 81nM

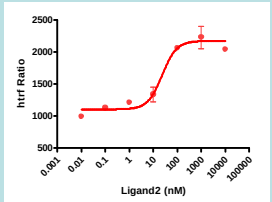

Fig. 9 Inhibition of 1μM forskolin induced cAMP accumulation by ligand2. Measured with cAMP htrf kit. Ec50 23nM

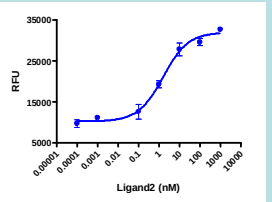

Fig. 10 Inhibition of 10μM forskolin induced cAMP accumulation by ligand2. Measured with Catchpoint cAMP kit. Ec50 2nM

GPRB was selected to further investigate if the shift could be due to the difference in assay technology (IP1 detection with antibodies v dye binding of calcium). The GPRB peptide ligand, (ligand2) is significantly different from ligand1 and GnRH (both 10 amino acids long). Ligand2 is composed of two chains (A: >20 amino acids, B: >20 amino acids) linked by inter-chain disulphide bridges. There is one intra-chain disulphide bridge on the B chain. GPRB is Gi linked and was stably expressed in CHO-K1 cells along with Gα16.

The apparent activity of ligand2 on human GPRB was compared in a number of different assays. There was good correlation with the literature using a Ca^{2+} flux assay (Fig 8). Measurement of adenylate cyclase inhibition using a Catchpoint assay (Fig. 10) correlated with GTPγS and radioligand binding (data not shown). A decrease in potency was observed with both the cAMP and IP1 htrf assays (Figs 7 & 9). This indicates that the differences in Ec50 values observed with the peptide liganded GPCRs may be intrinsic to the htrf technology (Summary in Table 1).

Table 1. Differences in Ec50 values htrf v other formats

Receptor	Ligand Type	Ca Flux (nM)	IP-1 (htrf) (nM)	Fold Difference
GPRA	Peptide	0.2	7.9	40
GnRH	Peptide	0.002	0.13	65
GPRB	Peptide	81	1800	22
M3	Non-peptide	3	9	3
		cAMP ELISA	cAMP (htrf)	
GPRB	Peptide	2	23	11

Conclusions

We observed a decrease in potency of several peptide ligands to GPCRs when determined using an IP1 htrf assay relative to a calcium flux assay. This decrease was not observed with a non-peptide liganded GPCR. Further experiments with a peptide GPCR in a cell line enabling the interrogation of Gi and Gα16 signalling pathways demonstrated a decrease in potency, shown by a rightward shift in the Ec50s, on both pathways when measured with htrf assay kits. It would appear that peptides to GPCRs appear less potent when assayed with htrf based technologies as compared to other techniques. Thus care must be exercised when choosing an assay platform for use on peptide liganded GPCRs.