IP-one, a HTRF homogeneous assay to monitor functional activation of G_q coupled receptors in a HTS format

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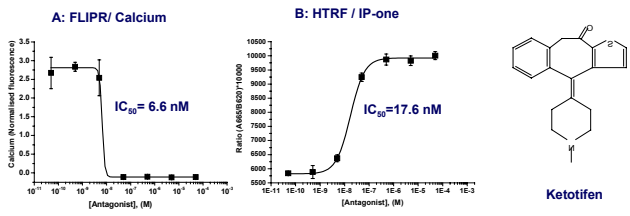
Summary

G protein-coupled receptors (GPCR) represents the largest group of proteins involved in signal transduction of messengers such as neurotransmitters, hormones, light, odorant principles, ions and more recently protons. Due to their location in the cell membrane and a high level of selectivity in agonist recognition, GPCRs are amongst the most successful therapeutic targets in diverse indications. Activation of GPCRs leads to the dissociation of heterotrimeric G proteins (Gα, Gβ and Gγ) and the subsequent activation of effector pathways. While several assay format were developed in the past to monitor second messengers in a high throughput fashion, however the inositol phosphate pathway suffered from a lack of such technologies. Recently, a homogeneous HTRF assay was developed (Trinquet et al. 2006) which measures IP1 the last component of the PIP2 degradation pathways using the homogenous time resolved fluorescence (HTRF) technology. We have undertaken a study at Novartis with the objective to validate this technology and to evaluate its usefulness in our discovery processes. Pharmacological and HTS data in addition to preliminary results in the 1536 well format are presented and show that this novel technology is suitable for HTS and secondary screening.

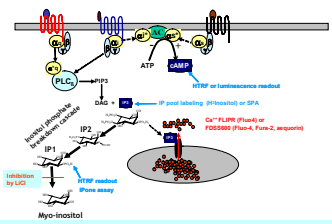
Antagonism of histamine effects



Compound	IC_{50} (nM) Ca^{++} (FLIPR) exp 1	exp 2	mean	IC_{50} (nM) IP1 (HTRF) exp 1)	exp 2	mean	$IC_{50,Ca++}$ / $IC_{50\ IP1}$
Cetirizine	925	1450	**1188**	86	167	**127**	9.38
Ketotifen	6.4	6.6	**6.5**	5.2	17.2	**11**	0.58
Astemizole	460	458	**459**	375	865	**620**	0.74
Loratadine	3910	2350	**3130**	635	939	**787**	3.98
Clemastine	48.5	24.4	**36**	15.7	46.3	**31**	1.18
Doxepin	13.0	33.6	**23**	7.5	13.4	**10**	2.23
Mirtazapine	12.4	7.9	**10**	7.0	12.4	**10**	1.05

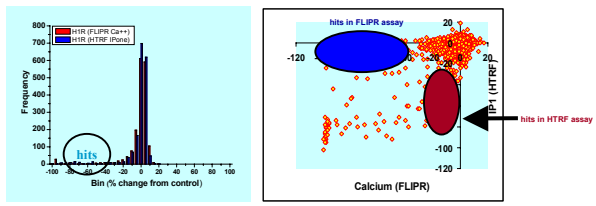
Cells were seeded in 384 well plates at 10,000 cell / well and 30,000 cells/well respectively for the calcium and the IP-one assays and were incubated for 24 hours. Several marketed anti-H1 histamine blockers were tested in the two assays against 100 nM histamine. Similar IC_{50}'s are obtained for most of the blockers except in one case, cetirizine, were the data are in favour of the IP-one assay.

G_q signalling pathways & inositol phosphate assays



G_q coupled receptor promote the activation of phospholipase C (PLC) enzyme leading to PIP2 breakdown into inositol tri, di and mono phosphates. IP3 opens a calcium channel located in the ER and leads to calcium efflux in the cytoplasm. Several assay formats exist to monitor all events in the GPCR activation cascade with a high throughput. While assays to monitor cAMP and calcium levels were developed the past 10 years and are now widely used in the pharmaceutical industry, current IP technologies, although useful are limited by their low throughput and safety issues and are hence not amenable to HTS. Moreover, IP3 cannot be easily used in an HTS bioassay due to its short lifetime. However, in the presence of lithium, IP1 accumulates and can be easily measured in an end-point assay

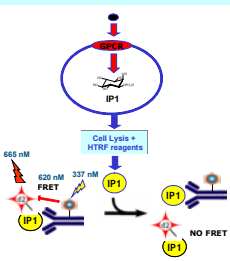
Pilot screen for HTS of H1R blockers



		Selection thresholds -40	-50	-60	-70
FLIPR	hit #	76	60	52	47
	rate (%)	4.3	3.4	3.0	2.7
IP1	hit #	82	71	55	49
	rate (%)	4.7	4.0	3.1	2.8

A compound set (1760 compounds) was tested at 10 µM using the two methods. In this screen, the calcium assay showed a better Z' value, but both assays were very robust $Z'_{IP\text{-}one}$ = 0.69 ± 0.07 and Z'_{FLIPR} = 0.88 ± 0.07 (n =5) were obtained during screening. The primary screening hit rates were similar in the two assays but were very high compared to other GPCR assays running on the FLIPR platform. Analysis of the data indicated that a large number of compounds are genuine hits and that some H1 pharmacophores were enriched in the compound plates that were selected. Moreover the H1R is known as a promiscuous target. Apart from the common hits in the two assays, 28 'FLIPR' hits inactive in IP-one and 34 hits in 'IP-one' inactive in FLIPR were identified with a -40% threshold.

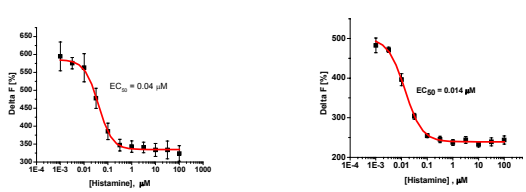
IP-one HTRF assay principle and methods



Assay description: The assay method is intended for the direct quantitative determination of myo-Inositol 1 phosphate (IP1),and has been optimized in order to measure IP1 directly on cultured cells. For this purpose a Eu Cryptate labelled monoclonal antibody specific for IP1 is used. The antibody is competing with both native IP1 produced by cells and IP1 coupled to the acceptor dye d2. The specific signal (i.e. energy transfer) is inversely proportional to the concentration of IP1 in the calibrator or in the cell lysate. As for all other HTRF assays, data reduction using the fluorescence ratio (665nm / 620 nm) eliminates possible photophysical interference by the usual medium conditions and colored compounds.

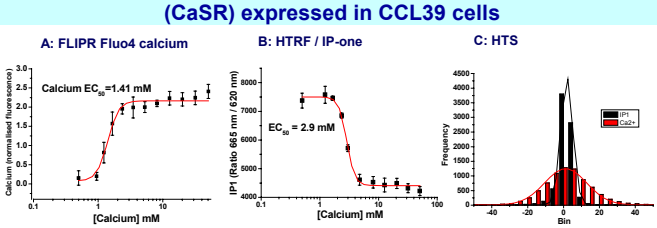
Experimental protocol: Cell are plated in tissue culture treated white microplates. Cells are then be incubated overnight at 37°C in a cell culture incubator. On the second day, following removal of the cell supernatant, cells are stimulated for 30 minutes by the addition of the compound diluted in the stimulation buffer provided in the kit in the presence of 50 mM LiCl. Both HTRF® conjugates (IP1-d2 and mab-anti-IP1) are dispensed into the wells. After one hour, plates are then read using the Viewlux imager. Assays in 384 well and 1536 well plates were running in 20 µl and 4 µl respectively.

Preliminary experiments using the 1536 well format



Preliminary experiments aiming at evaluating the IP-one 1536 well format were performed using CHOK1-H1R cells in 384 w plates (30,000 cells) and 1536 well plates (3000 cells/well) on the H1R system with the IP-one assay kit. Cells were recovering for 20 minutes in the assay buffer after cell addition. Similar EC_{50}'s for histamine were observed. The assay quality is still lower in the 1536 well format (Z' = 0.4 at 10 µM histamine) compared to the value in the 384 well format (Z' of 0.75).

Characterisation of the calcium sensing receptor (CaSR) expressed in CCL39 cells

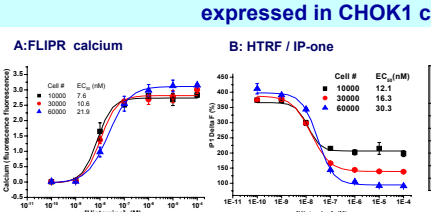


Low concentrations of calcium activate the CaSR. Increases in calcium mobilisation and IP1 levels occurred with a similar EC_{50}. A natural compound collection composed of 7744 compounds was screened using the two methods. Compounds were tested at 10 µM. Data with the IP-one generated higher quality results Z' values of 0.85 ± 0.05 and 0.56 ± 0.10 (n =22) were obtained during screening with the IP-one and the FLIPR screen respectively. FLIPR frequent hitters, i.e compounds scoring positive in all FLIPR screening campaigns mainly due to their auto-fluorescence at 488 nM or to their interaction with common downstream activation pathways were not detected in the IP one assay. The overall hit rate (threshold -30 % change) in the FLIPR assay (1.7 %) was higher than in the IP-one assay (0.21 %).

Conclusions

Our data indicate that IP1 is a useful and robust HTS assay. Some assay parameters could be optimised. In general similar data are obtained compared to those on the FLIPR platform with a slightly higher sensitivity in favour of calcium mobilization assays in the case of endogenous GPCR's. Pharmacological characterisation of two GPCR's studied so far led to similar data. Moreover, the IP-one assay can be used for secondary screening to exclude false positives and FLIPR specific hits. The assay (384 w format) quality was excellent in a productive screening set-up. Preliminary data with the 1536 format indicate that assay miniaturization is feasible but need optimisation. Further work will include the use of IP-one in secondary screening strategies in order to check its usefulness in the lead discovery process.

Characterisation of the recombinant histamine H1 receptor (H1R) expressed in CHOK1 cells



C: Summary of data

		EC_{50} (µM) Ca^{++} 1.26	0.5	IP1	n.
	cell number				
ATP	10,000	0.64 ± 0.10	0.43 ± 0.08	2.5 ; 9.8	2
	30,000	0.89 ± 0.16	0.73 ± 0.08	7.1 ± 4.6	3
	60,000	2.01 ± 0.01	1.19	5.8 ; 10	2
Histamine					
	10,000	0.009 ± 0.002	0.008 ± 0.001	0.013 ± 0.01	3
	30,000	0.020 ± 0.001	0.016 ± 0.003	0.024 ± 0.01	3
	60,000	0.022 ± 0.03	0.028	0.034 ± 0.01	2

Cells were seeded in 384 well plates at the densities indicated and were grown for 24 hours. Histamine evoked calcium mobilisation (A) and IP1 (B) with similar EC_{50}'s in both assays. Whereas increased signal amplitudes were obtained with high cell densities in the IP-one assay, no changes were detected in the calcium assay. (C): the FLIPR calcium assay can be run at two external calcium concentrations without major changes of the EC_{50}'s.

CHOK1 are known to express purinergic P2Y receptors than can be activated by ATP and its analogues. ATP stimulated calcium mobilisation and IP1 formation with however much lower EC_{50}'s in the calcium assay indicating that a certain level of receptor expression is necessary to obtain robust responses in the IP-one assay and that the calcium assay better amplifies receptor mediated signal.

E. Trinquet, M. Fink, H. Bazin, F. Grillet, F. Maurin, E. Bourrier, H. Ansanay, C. Leroy, A. Michaud, T. Durroux, D. Maurel, F. Malhaire, C. Goudet, J.P. Pin, M. Naval, O. Hernout, F. Chrétien, Y. Chapleur, G. Mathis D-myo-inositol 1-phosphate as a surrogate of D-myo-inositol 1,4,5-tris phosphate to monitor G protein-coupled receptor activation. Accepted to be published in Analytical Biochemistry in 2006.

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