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Novartis Institutes for BioMedical Research

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SBS Sept. 2005

A new HTRF inositol phosphate assay to monitor G_q coupled GPCRs responses

— **Comparative study calcium mobilisation / IP1**

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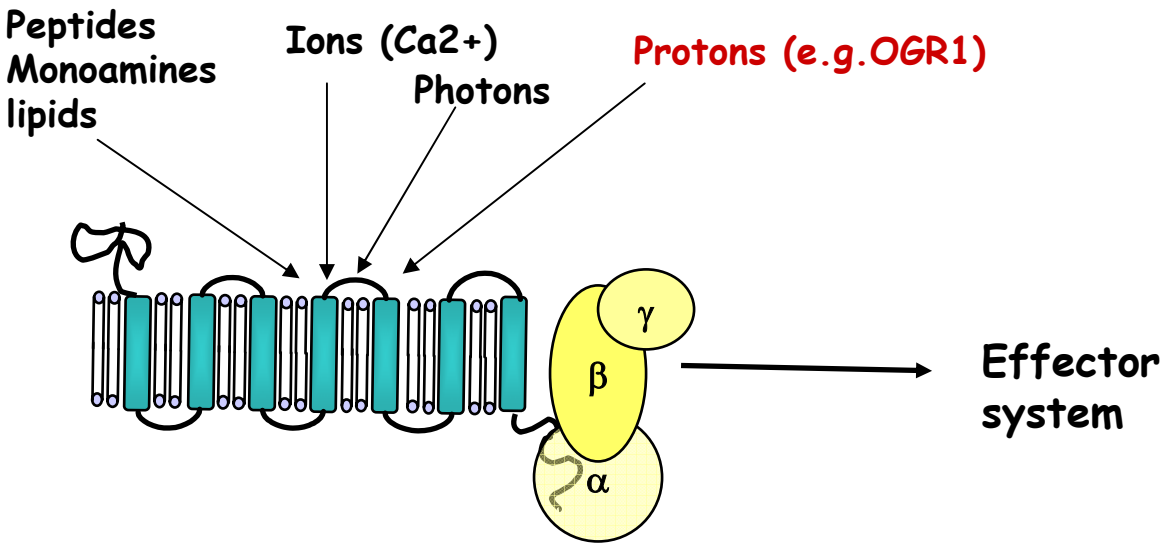
GPCRs: A universal communication system



Signal Transduction mechanisms

- cGMP PDE,
- Adenylate cyclase (cAMP) & Protein kinase A
- Phospholipase C , IP3 , calcium & Protein kinase C,
- MAP kinase pathways,
- Ion channel channels conductance

Need for a HTS assay format for IP formation

— Several assay formats exist to monitor all events in the GPCR activation cascade with a high throughput.

— While such 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 are limited by their low throughput and safety issues.

— Need of assays amenable to HTS or MTS in the lead discovery process.

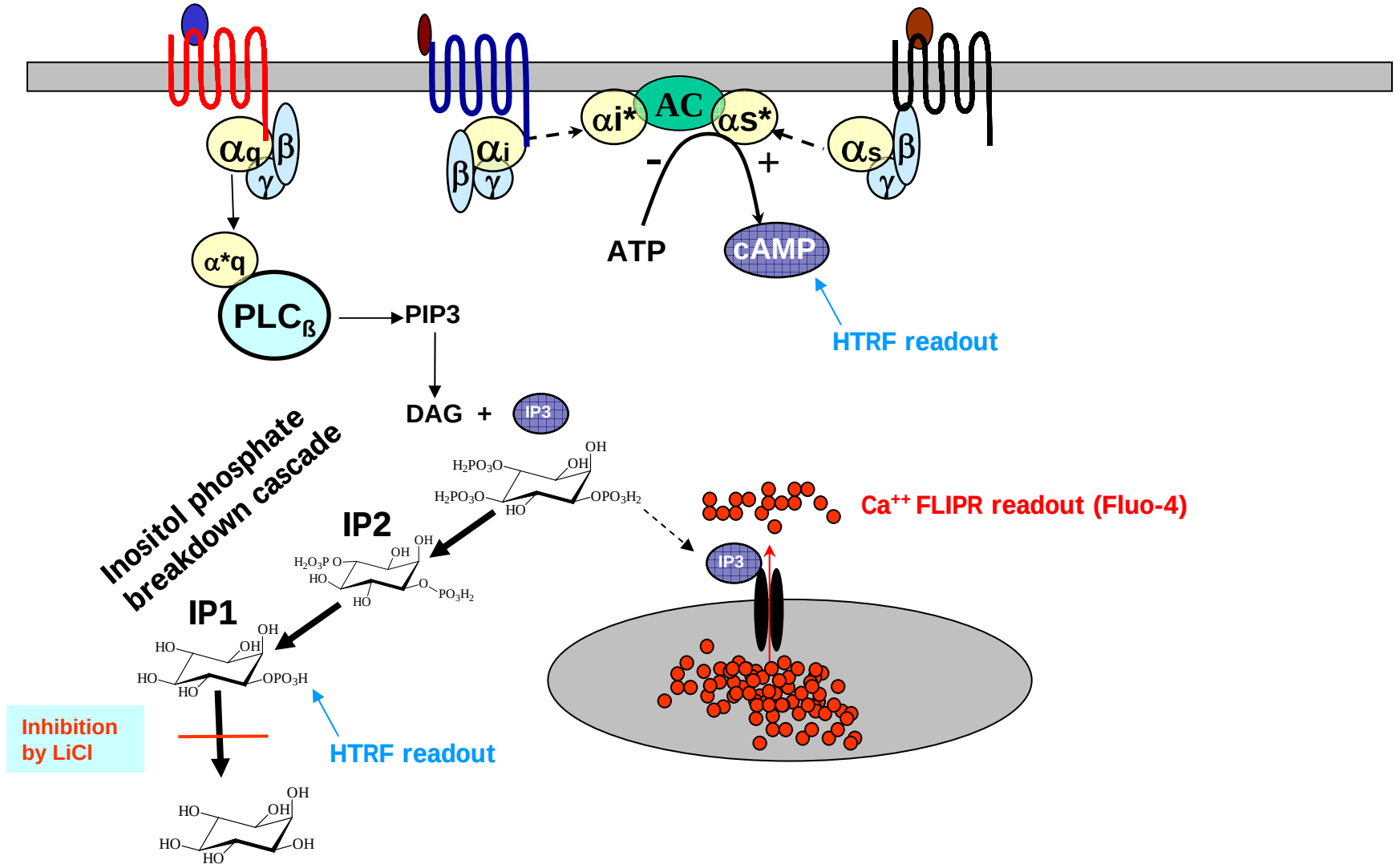
— Recently, a homogeneous HTRF assay was developed by Cisbio which measures IP1 the last component of the PIP2 degradation pathways.

— A study was initiated at Novartis with the objective to validate this novel assay format and to evaluate its usefulness in our discovery processes.

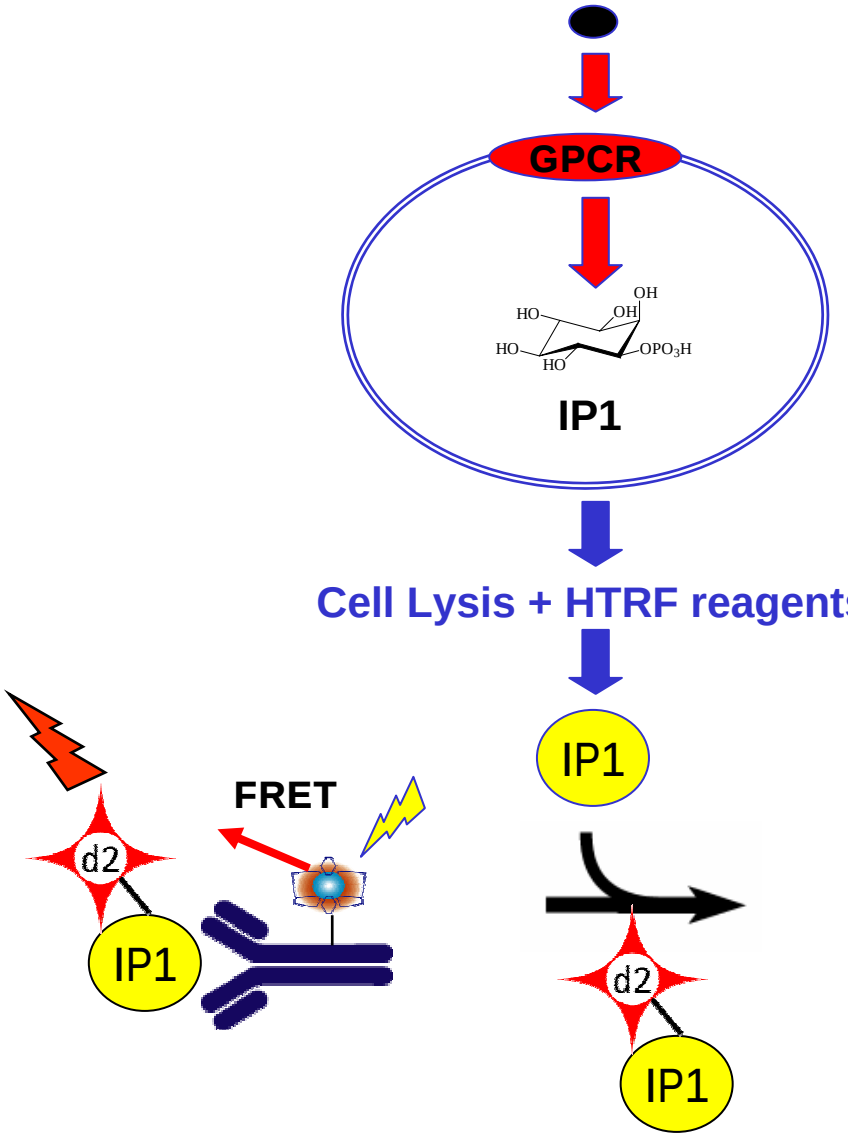
Study description

- The assay was assessed in various cell systems
  - HEK293 and CHOK1 cell expressing native GPCRs
  - CCL39 cell expressing the recombinant human parathyroid calcium sensing receptor (HupCaR).
- Pharmacological characterization of GPCRs with agonists and antagonists.
- In addition, Novartis libraries are tested in the HupCaR calcium mobilization assay using the FLIPR technology and the HTRF IP1 accumulation assay using an imaging reader (Viewlux)
- **Aim**: Comparison of hit rates obtained in the two assays and the suitability of the IP1 assay as a primary or secondary assay for GPCR screening.

Gq signaling pathways



IP-one assay principle



based on unique and proprietary reagents

- A fluorescent analog of IP1
- A monoclonal antibody against IP1

Methods: HTRF & Calcium assays

HTRF / IP1

Cell seeding

— 10 000 cells/well/50 ul media in a white tissue culture treated 384 MTP

— 24 h incubation at 37 °C and 5 % CO_2

Cell stimulation

— Cell media removal

— Cells stimulation with agonists / compounds

— diluted in HBS Buffer with (LiCL 50 mM) for 30 min

— addition of HTRF reagents and incubation for 1 h

HTRF Reading:

— read plate in Viewlux at 665 and 620 nm

FLIPR Fluo4 calcium

Cell seeding

- 10 000 cells/well/50 ul media in a black tissue
- culture treated 384 MTP with clear bottom.
- 24 h incubation at 37 °C and 5 % CO2

Cell loading:

- After removing cell media, load cells with Fluo4
- incubate 1 h at 37°C and 5 % CO2

Cell washing:

- Remove remaining Fluo4 by washing plates

Cell stimulation & Calcium Reading

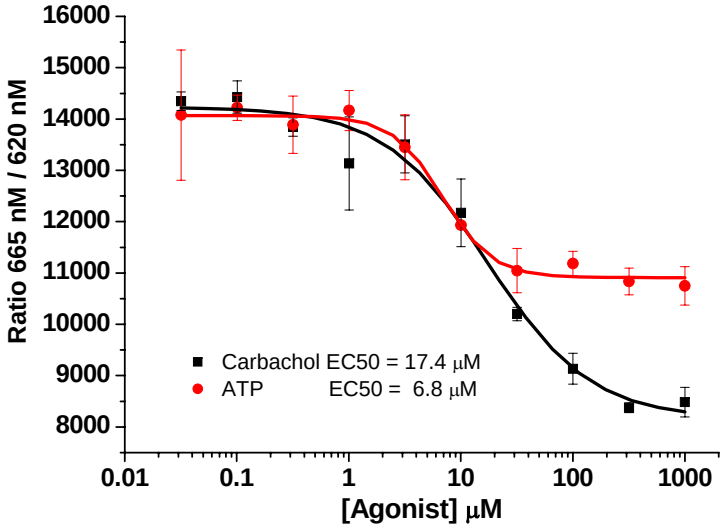
- Read plate in FLIPR at 525 nm during compound injection

Endogenous P2YR and M3R in HEK293 cells

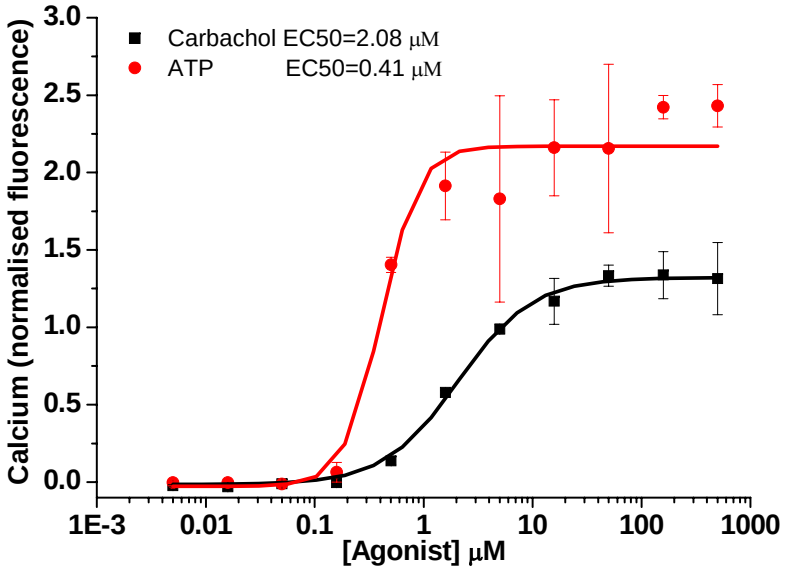
Endogenous Muscarinic M3R and P2YR in HEK293 cells

Agonist effects

HTRF / IP1
60,000 cells, 24 h



FLIPR Fluo4 calcium
10,000 cells, 24h

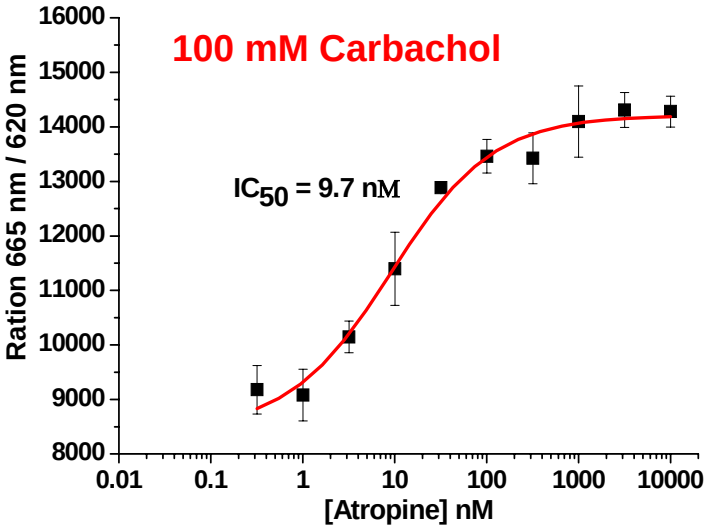


- Carbachol and ATP are more potent in the calcium assay
- Curve shift factors 8 and 16 for carbachol and ATP
- Differences between efficacies in the two assay systems are also reflected.
- Calcium mobilisation assay is more sensitive (amplification step) in particular with low receptor expression (endogenous receptors)

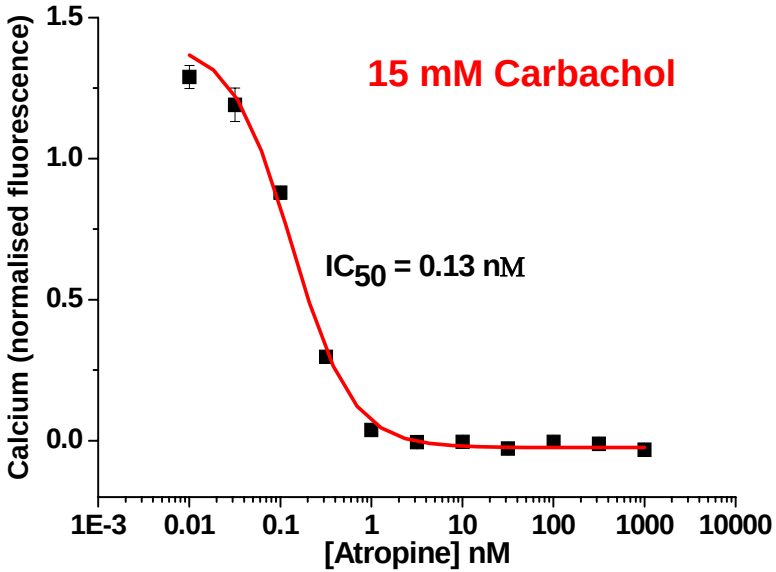
Endogenous Muscarinic M3R in HEK293 cells

Antagonist effects

HTRF / IP1



FLIPR Fluo4 calcium



- Atropine potency higher in calcium assay (75 fold)
- Reflects differences of agonist concentrations and receptor number used

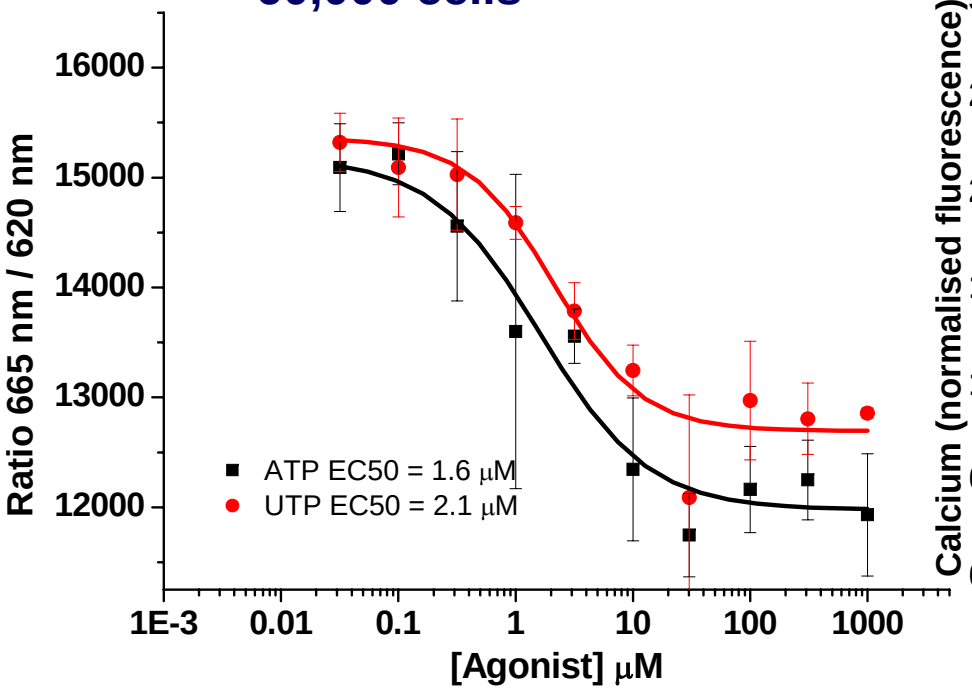
  Carbachol 100 µM in IP1 / 15 µM in Calcium

  60,000 cells in IP1 / 10,000 in calcium

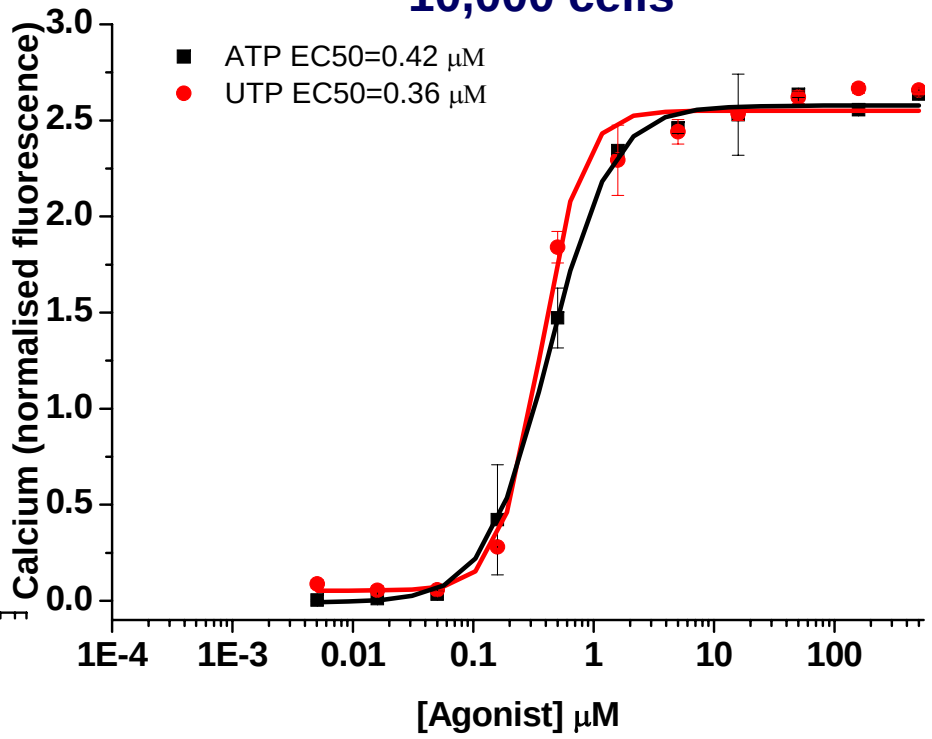
Conclusion: No major difference in antagonist potency expected

Endogenous P2Y receptors in CHOK1 cells: Agonist effects

HTRF / IP1, 60,000 cells

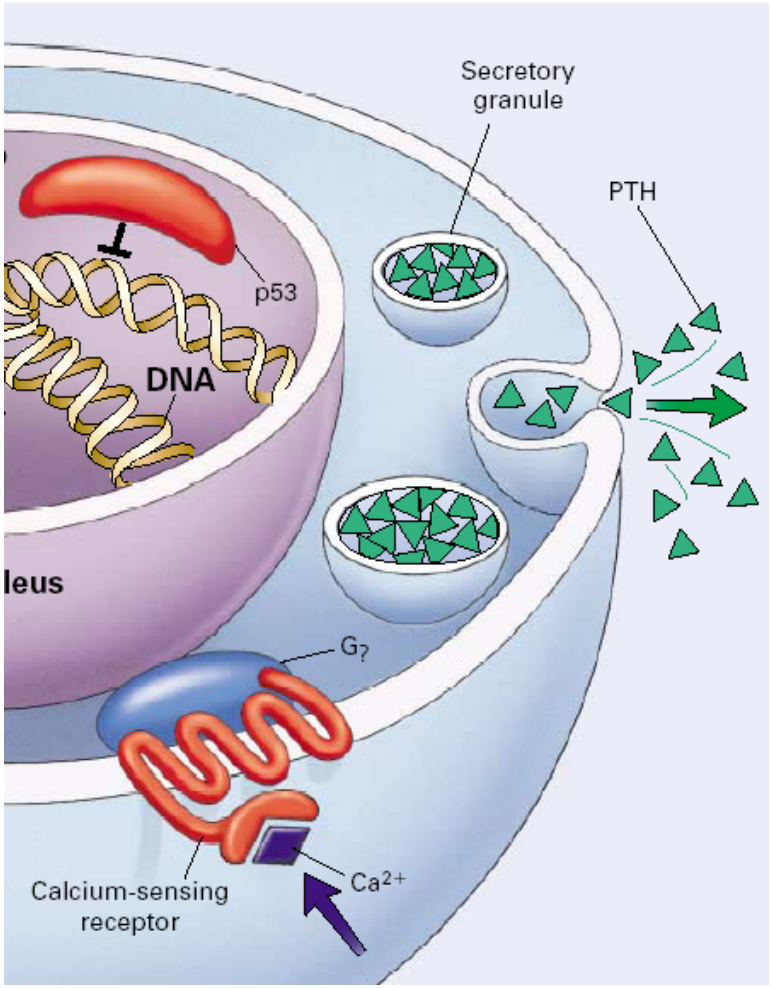


FLIPR Fluo4 calcium, 10,000 cells



Recombinant human calcium sensing receptor (HupCaR) in CCL39 cells

The parathyroid calcium sensing receptor



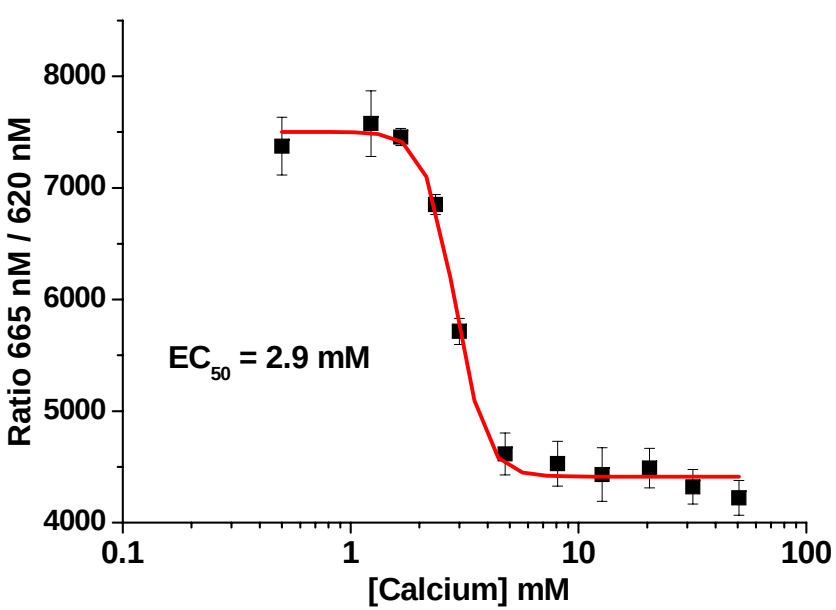
- A Gq coupled receptor highly expressed

In the parathyroid gland

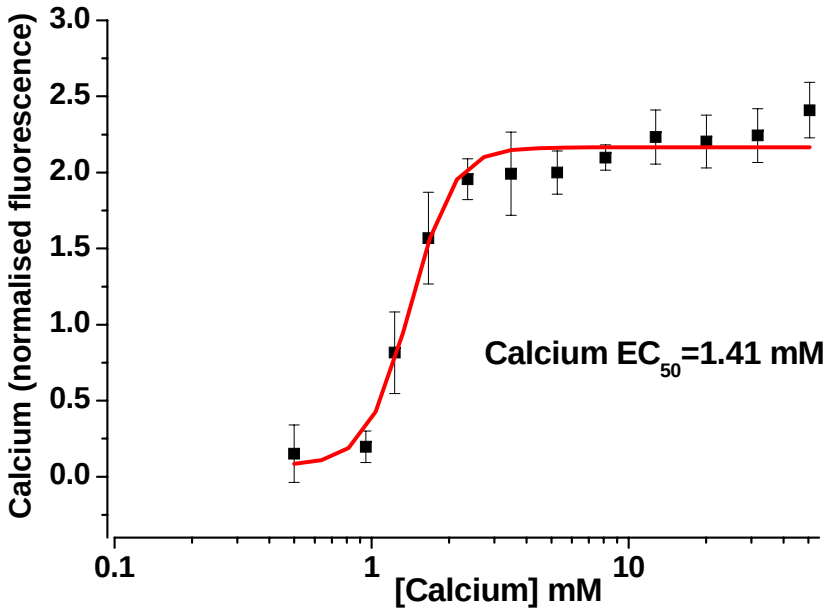
- Uses circulating calcium as an “agonist”
- Can be blocked by allosteric negative modulators
- Controls PTH release
- Role in bone formation

Effect of calcium on IP1 & Calcium mobilisation

HTRF / IP1

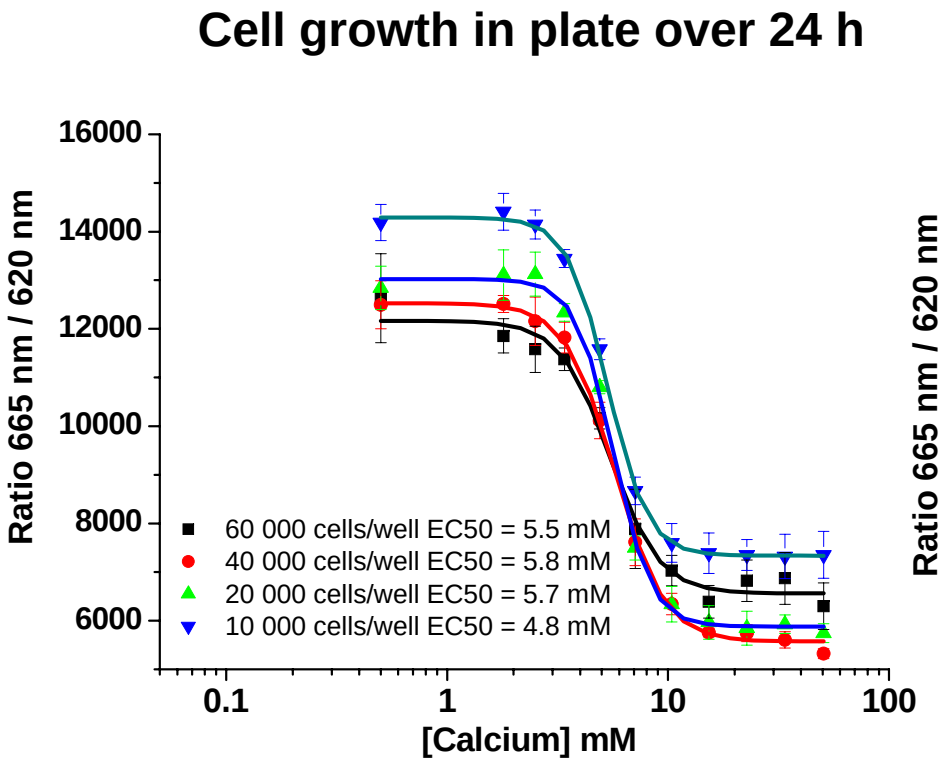


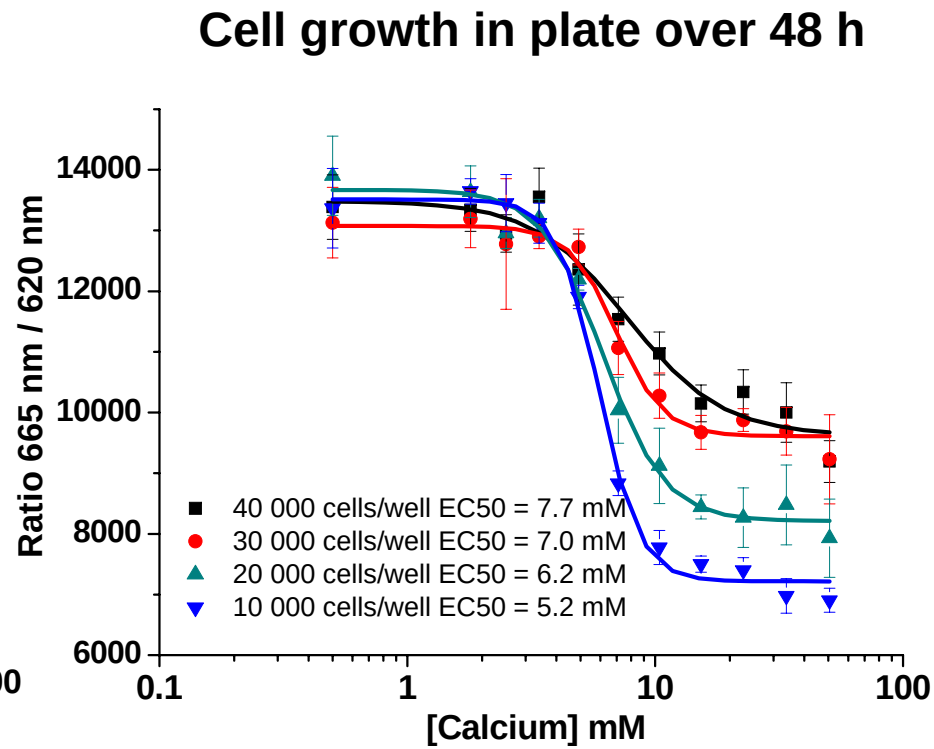
FLIPR Fluo4 calcium



10000 cells/well, 08.07.05

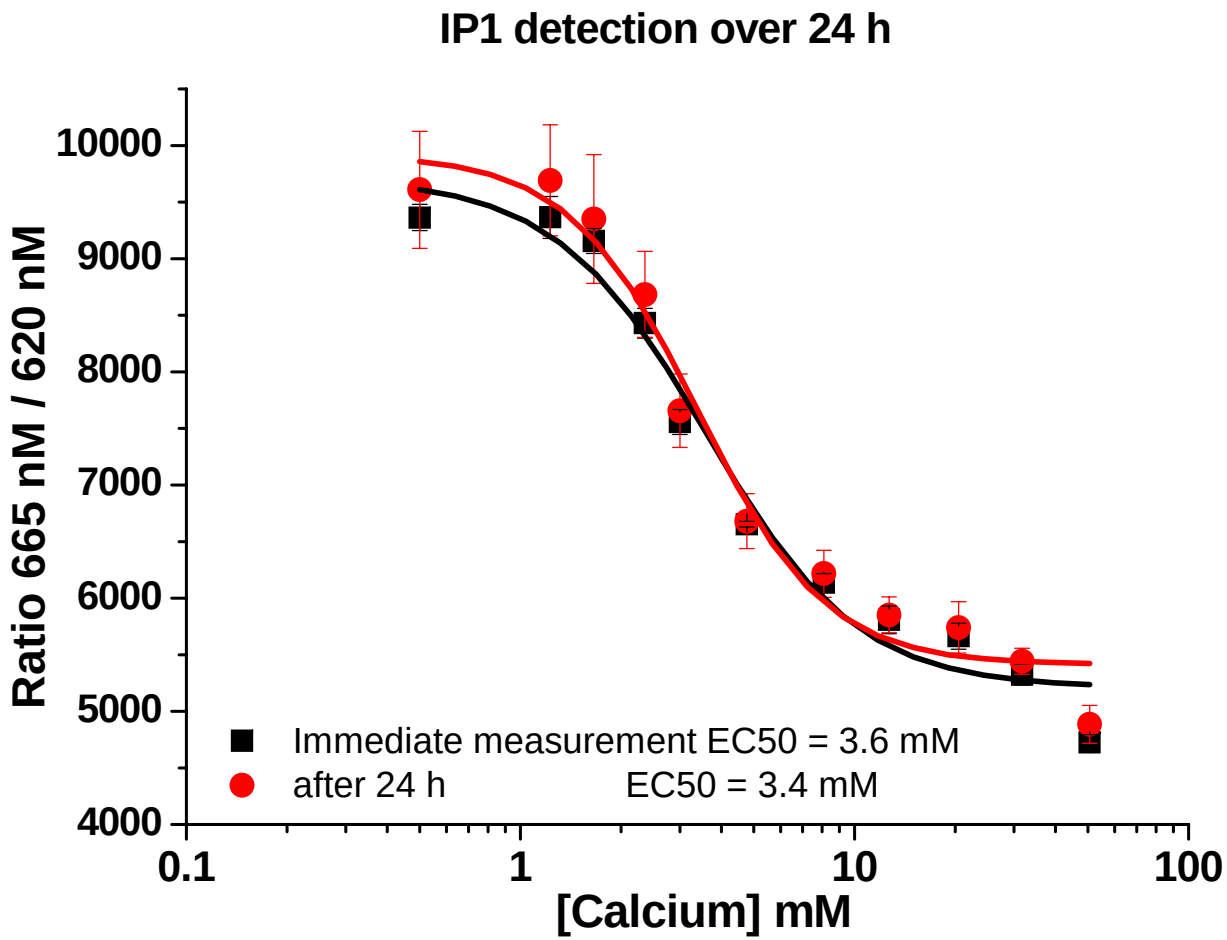
HupCaR: Evaluation of HTRF IP1 assay parameters: cell density



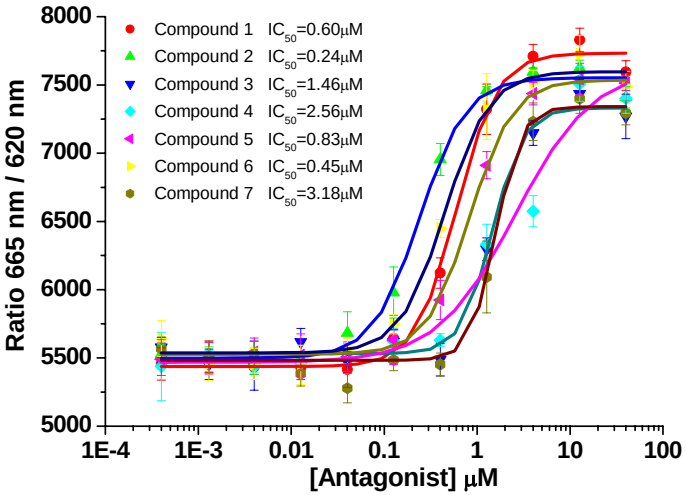


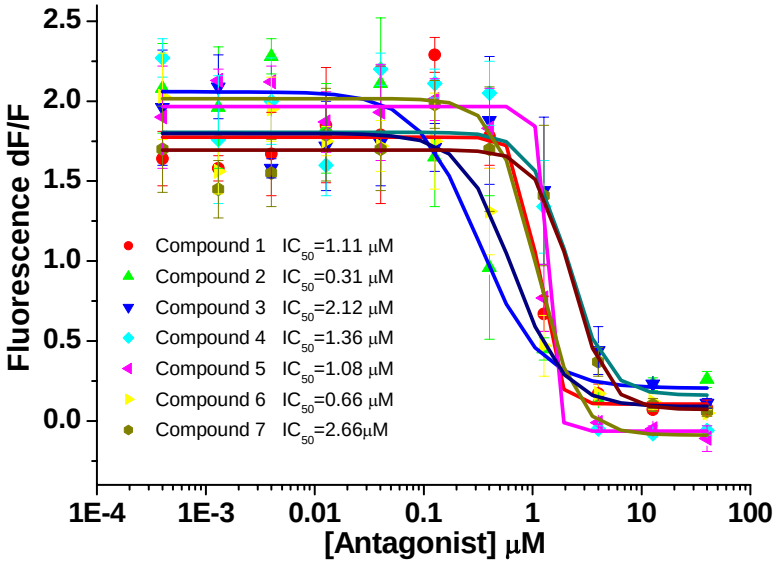
- Potencies unchanged
- Cell density can be reduced to 5000-10000 cells /well over 24 or 48 h

HTRF signal stability over time



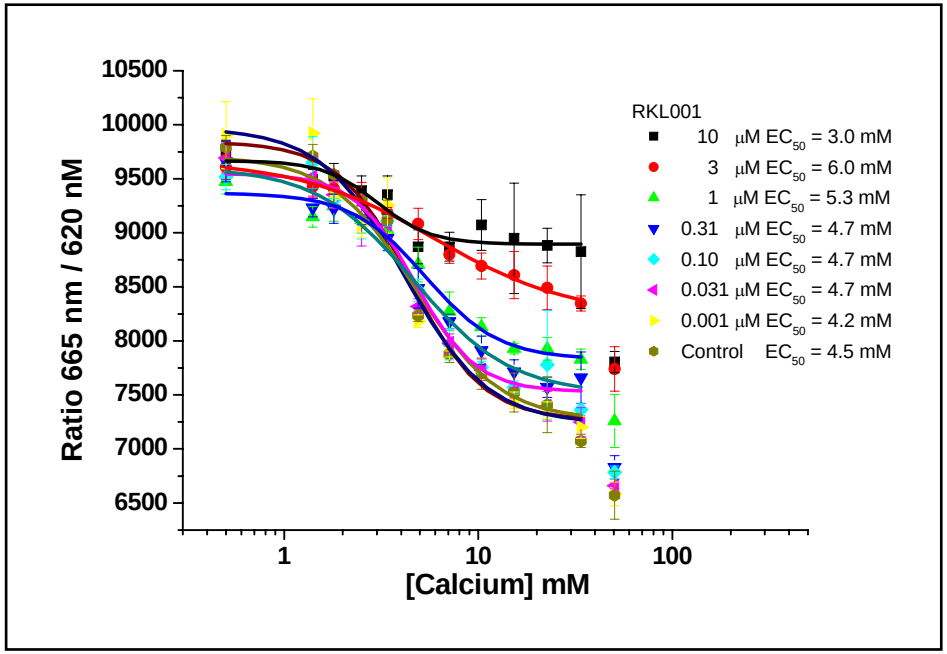
Antagonist effects in the HTRF & Calcium assays

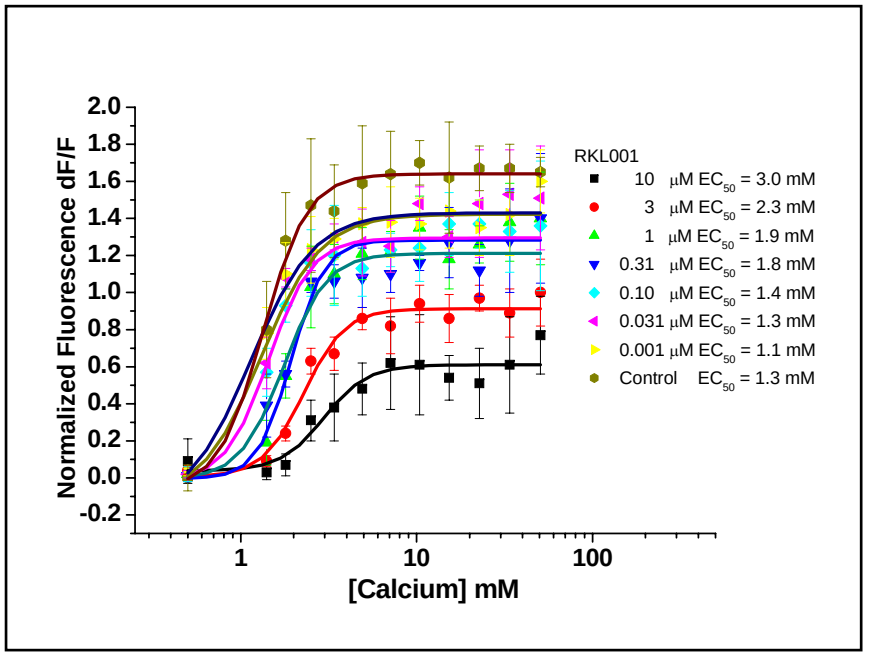




Compound ID	IP1 (HTRF)	Calcium (FLIPR)
	IC50 (µM) n =3	
1	0.58 ± 0.02	0.74 ± 0.32
2	1.00 ± 0.67	0.66 ± 0.51
3	1.23 ± 0.38	1.75 ± 0.84
4	2.91 ± 0.81	1.98 ± 0.54
5	0.61 ± 0.21	1.32 ± 1.04
6	2.51 ± 1.87	1.32 ± 1.43
7	1.88 ± 1.13	3.06 ± 1.14

Antagonist effects: RKL001





– Varying blocker concentration depresses maximal activity without maj changes in calcium EC50

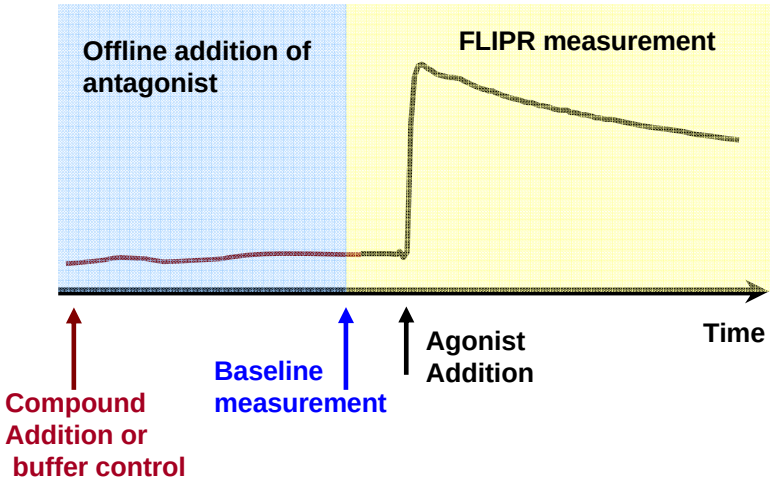
IP1 in HTS

Study aim

- Use the IP one assay in a productive screening campaign
- Comparison to calcium mobilization screen
- 7744 natural product tested

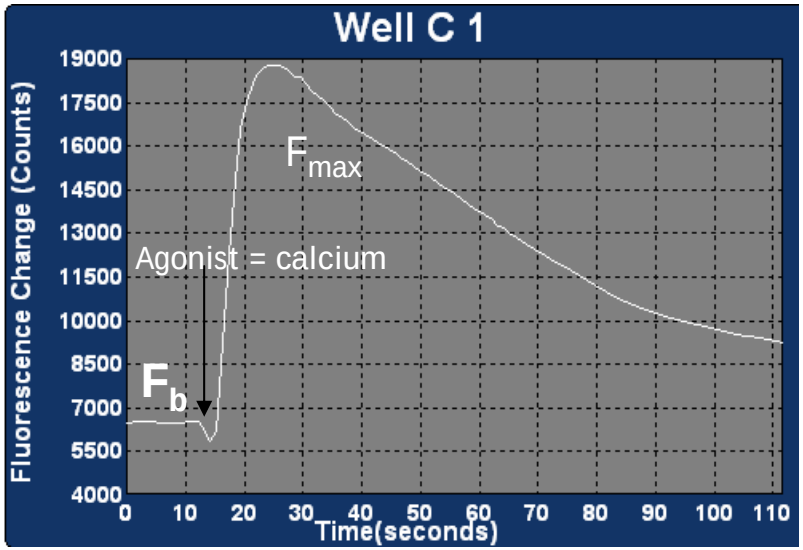
FLIPR assay types: Data Analysis

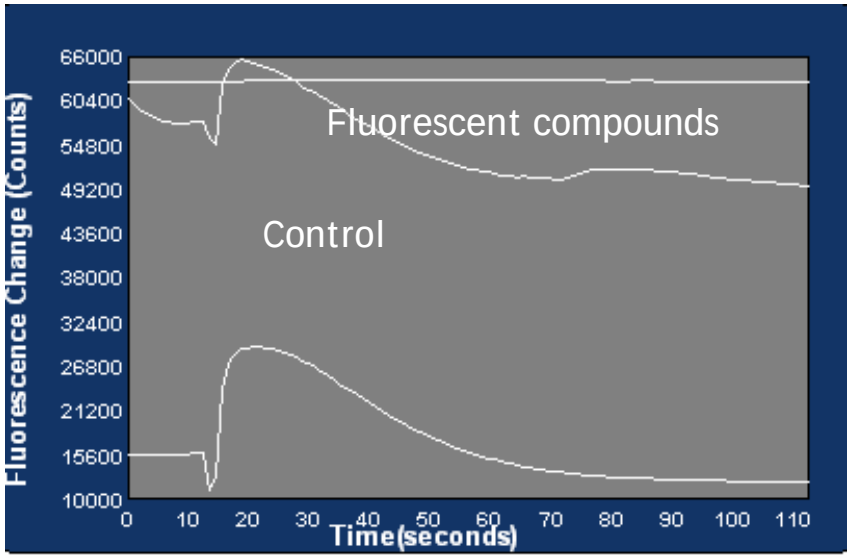
Antagonist FLIPR assay set-up



- F_{basal} or F_b = Fluorescence before agonist injection
- F_{max} = Fluorescence maximum or peak
- dF/F = Normalised Fluorescence = $(F_{max} - F_b) / F_b$
- $F_{b\ sample}$/average $F_{b\ controls}$ = high ratio indicates autofluorescence/ toxicity of compound

FLIPR data handling for antagonist assays





Two values of fluorescence calcium responses are exported

F_b corresponding to the value prior to agonist injection, Fmax, the fluorescence at the signal peak.

From these the values two parameters were then calculated and exported

Calculate $dF/F = F_m - F_b / F_b$ for High controls H, Low controls (L, Buffer) and Samples (S).

Activity (A) expressed as a percent of the maximal stimulation induced by agonist .

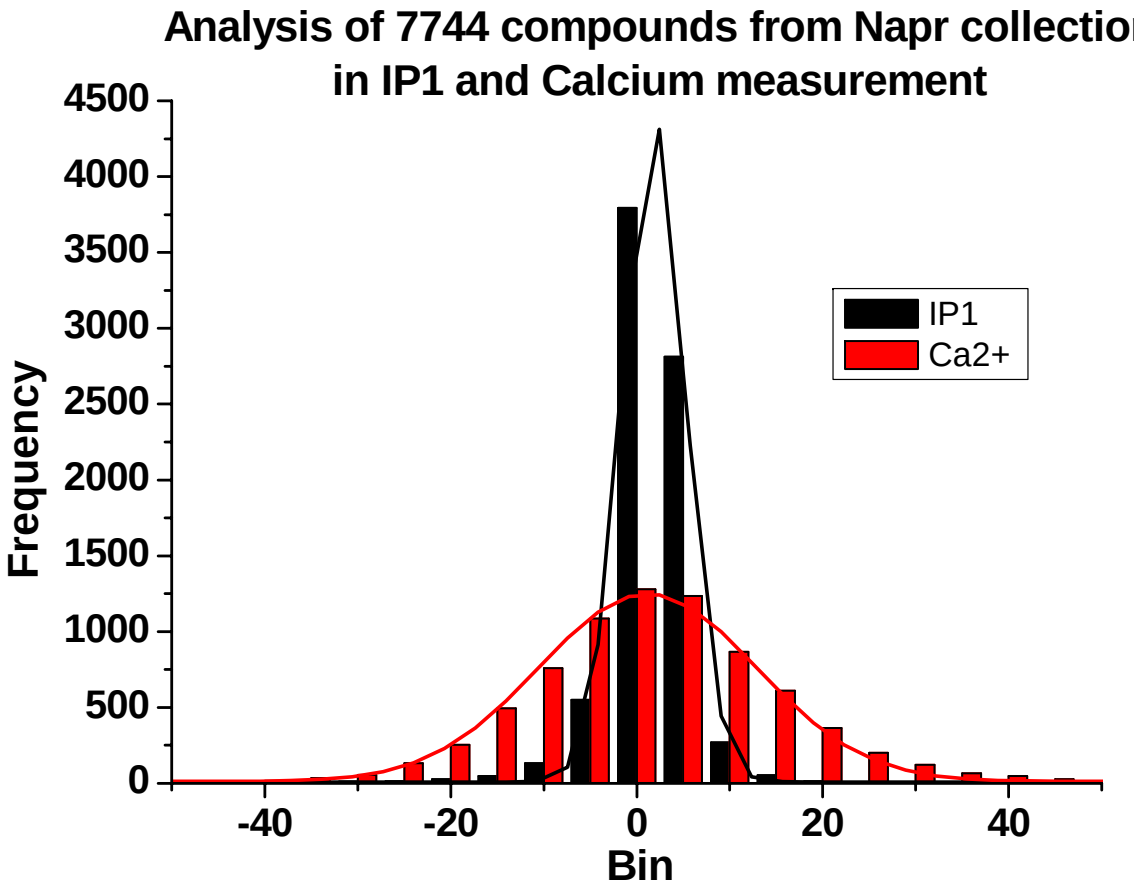
— $A (\%) = (S-H) /(H-L)* 100$

— A quality parameter (R_b) which indicates whether the compounds acted through a physiologically relevant mechanism)

$R_b = F_{b,s} / F_{b,H}$

— This parameter is used to reject compounds showing a sustained activity (toxicity / autofluoresence)

Calcium receptor HupCaR miniscreen - HTRF & Calcium assay



Threshold		-30	-40	-50	-60	-70
Hit number	IP1	17	4	2	2	1
	Ca^{++}	165	75	50	37	28

Miniscreen: Data quality

	IP1	Ca^{2+}
$EC_{50,Ca}$ [mM]	4.94 ± 3.39 n=9	1.97 ± 0.53 n=7
$IC_{50,\ Anta}$ [µM]	0.48	1.3

	IP1	Ca^{2+}
mean % change	-0.45	-0.57
sd	4.74	15.02
3*sd	14.2	45.0
Z'	0.85 ± 0.05	0.56 ± 0.10
Z	0.86 ± 0.05	0.66 ± 0.06

IP1 and Calcium: Compound interference

Compound	IP1	Calcium	
	% change	% change	Ratio Rb
1	6	-59	1.34
2	-4	-64	2.03
3	-3	-85	2.13
4	-3	-102	3.65
5	0	-102	4.07
6	-3	-100	4.09
7	-3	-102	3.96
8	0	-102	4.57
9	-2	-102	4.04
10	1	-93	3.8
11	5	-102	4.38
12	3	-100	3.02
13	-1	-93	2.33
14	-4	-72	1.45
15	-1	-72	1.58
16	-3	-97	1.77
17	-12	-102	2.59
18	-3	-91	2.42

— **Frequent hitters are compounds scoring positive in all FLIPR screening campaigns due to their toxicity, their fluorescence at 488 nM or their interaction with common pathways.**

— **These compounds are not detected in the HTRF IP one assay**

IP1 and Calcium: Common hits

Compound	IP1	Calcium	
	% change	% change	Ratio Rb
1	-37	-78	0.75
2	-31	-93	0.82
3	-22	-50	0.70
4	-35	-78	0.73
5	-34	-68	0.73
6	-30	-74	0.74
7	-38	-65	0.77

- Hits interfering with the receptor of common mechanism

IP1 and Calcium: PS hits in calcium

Compound	IP1	Calcium	
	% change	% change	Ratio Rb
1	-9	-54	0.91
2	1	-88	1.00
3	13	-67	0.86
4	3	-55	0.71
5	7	-63	0.93
6	2	-58	1.24
7	1	-55	1.02
8	-1	-64	0.71
9	-2	-72	0.70
10	3	-62	0.63
11	4	-86	0.66
12	11	-67	0.70
13	3	-54	0.78
14	-2	-55	0.85
15	-1	-88	0.81
16	-3	-56	0.84
17	-2	-70	0.62
18	-1	-61	1.05
19	2	-71	0.98
20	-5	-72	0.54
21	13	-53	1.05
22	12	-52	1.21
23	6	-73	0.71

- **A number of compounds are found only in the calcium assay without modification of the calcium baseline**

Common FLIPR hits, i.e found in several FLIPR assay (

False positives in calcium assay; Known for FLIPR assays

Other mechanisms ?

Conclusion / outlook

- IP1-one is a useful HTS assay using IP1 as a reporter for IP3
  - Homogenous assay. Some parameters were optimised (Cell density & culture condition
  - Similar data obtained compared to calcium FLIPR with a slightly higher sensitivity for calcium (amplification mechanisms)
  - Hit rates lower
    - Can be used for secondary screening to exclude false positives and FLIPR specific hits
  - More robust. Better assay quality in productive screening set-up
- Further studies
  - More studies with controlled conditions with endogenous GPCRs.
  - More data to be produced with other recombinant system (SMW agonist GPCR assay with competitive antagonists. Potential calcium interference with IP1 assay ?

Acknowledgements

Novartis

K. Seuwen

D. Gabriel

Cisbio France

E. Trinquet

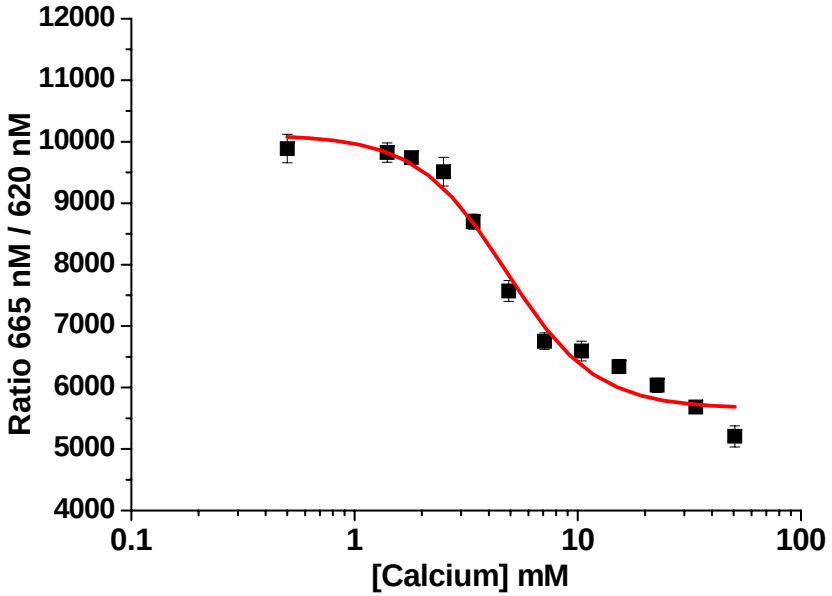
M. Fink

P. Seguin

J-L Tardieu

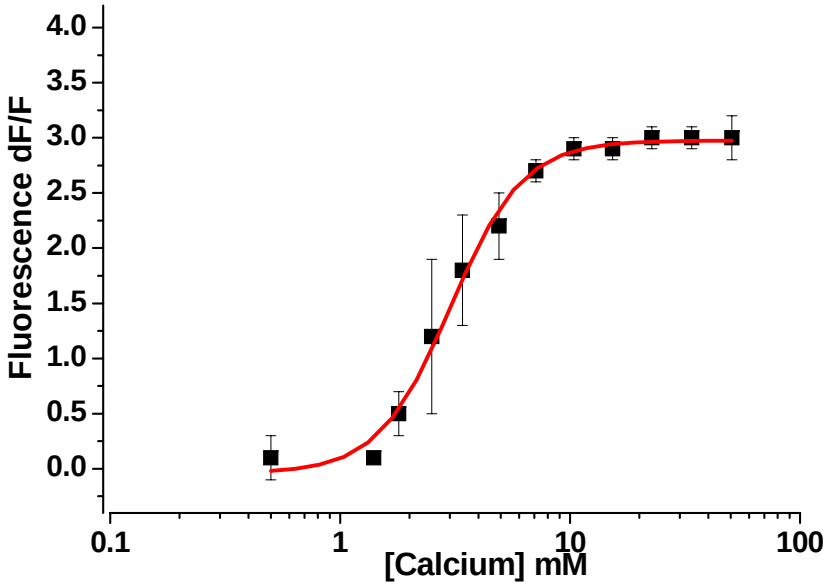
The calcium sensing receptor HupCaR

HTRF / IP1



EC_{50} = 4.79 mM

FLIPR Fluo4 calcium



EC_{50} = 3.0 mM