The challenge of interpreting drug potency under non-equilibrium conditions using Ca^{2+} assays

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1. Introduction

The development of HTS in the pharmaceutical industry has resulted in calcium assay becoming the most widely utilized for the characterization of receptors ligands interactions within whole cells. Due to the rapid and complex nature of calcium signal, the system is unlikely to reflect equilibrium binding conditions. Thus, there are potential issues associated with the interpretation of drug potency when performing SAR for GPCRs. Shild regression analyses are arguably the most appropriate method to determine antagonist potency from functional experiments. pA2 is used as a universal determinant of antagonist potency to overcome the potential bias associated with non equilibrium conditions. It is then possible to estimate the affinity of insurmountable antagonists by calculating pA2 from dose ratios at low agonist responses. Additional experimental parameters should be taken into account when performing calcium assays. The diffusion properties for agonists appeared as important parameters to be considered during evaluation of antagonist compounds.

Precise and defined agonist diffusion parameters have to be closely determined for any given GPCR when implementing calcium assays. Here we focus on studying depth and rate of agonist injection.

2. Experimental procedures

Plated CHO recombinant cells (5 to10 x10^4 cells per well in 96 well plate) are washed once by HBSS-buffer, 20 mM HEPES buffer pH=7.4. Cells are loaded by 80 µl/well of HBSS/HEPES buffer containing Fluo-4-AM (2.8 µM), 0.04 % Pluronic F-127, probenecid (2,5 mM). The plates are incubated for 45 min at room temperature.

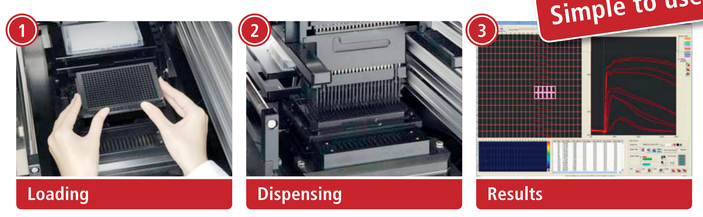
Cells are then washed twice by HBSS/HEPES buffer to remove excess dye and resuspended in 140 µl of HBSS/HEPES buffer. 20 µl of 10X antagonist solution are added and additional incubation is performed in dark for 30 min at room temperature.

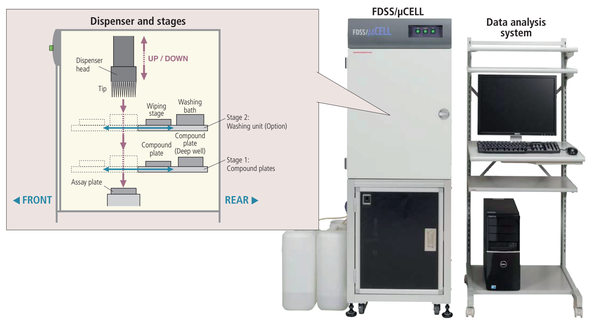
The plate is placed in the FDSS/µCell (Hamamatsu). 40 µl of 5X agonist solution (aspiration rate at 40µl/sec) are injected per well into the 160 µl assay buffer at appropriate depth and rate.

Reading is performed for 90 secs (1 lecture/sec), exposure time 200ms (gain 1), Excitation: 480 nm; Emission: 540 nm.

Responses are calculated by substraction of the minimum fluorescence counts from the maximum fluorescence counts (Fmax – Fmin). Data are plotted on a log scale to determine concentration response curves and to calculate EC50, IC50 as well as intrinsic activity.

Simple to use!





3. Principe of Schild plot

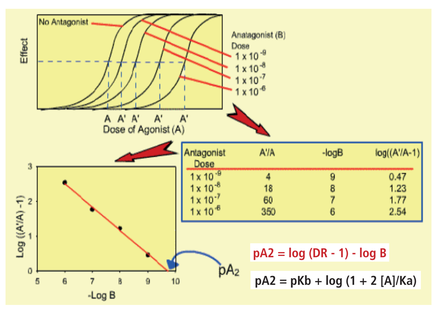


Fig. 1: principle of shild regression analyses*

Use of the pA2 as a universal determinant of antagonist potency: The dose-effect curve for an agonist is determined in the presence of various concentrations of a competitive antagonist.

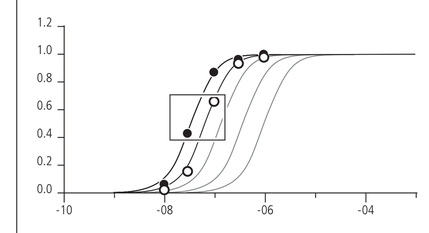
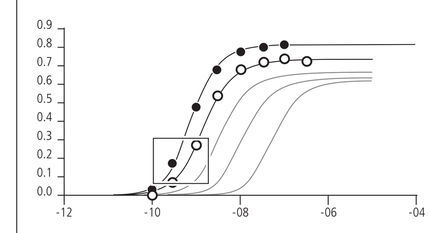
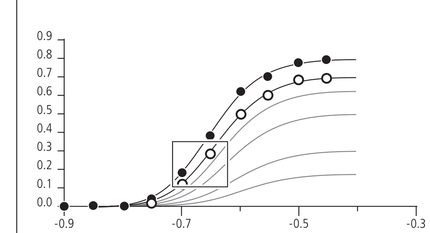
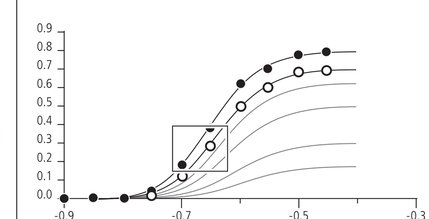
At low [agonist] occupancy → [A] < < < Ka → pA2 tend towards the pKb

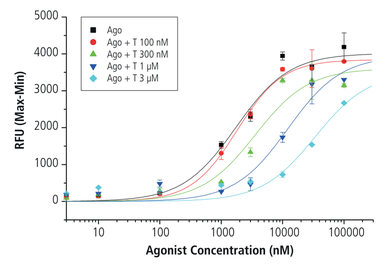
4. Results

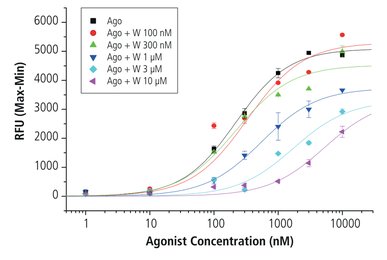
Calcium assays were performed on CHO cells expressing a recombinant aminergic GPCR using the Hamamatsu FDSS/µCell to determine accurate affinity and efficacy of insurmontable antagonist. To investigate the influence of its injection height, the reference agonist reponse was evaluated following injection at different depht conditions: settings at 4 mm (100 µL height), 7.2 mm (180 µL height), and 9.6 mm (240 µL height). Other parameters were constant: Rate (10 µl/sec), gain 1, sensitivity 500 ms during 90 sec reading. To estimate accurate affinity and efficacy of insurmontable antagonist, pA2 was calculated at low agonist responses to overcome the potential bias associated with non equilibrium conditions of calcium assay. pA2 = 7 was previously determined on calcium assay using Flexstation and on GTPg35 binding assay. The expected calculated pA2 value was obtained at the FDSS/µCell when injecting the agonist at low speed (10 µl/sec) and far from the bottom (240 µl height), corresponding on the panel C of the figure 3.

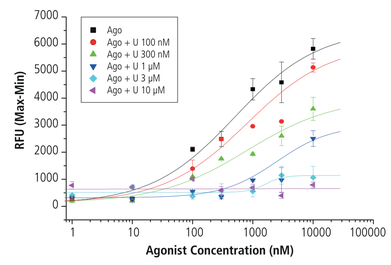
5. Summarized results

In reference to Terry Kenakin*, evaluation of different antagonists using CHO expressing recombinant hu-GPCR cell line by calcium flux measurements, several examples representing competitive or non competive antagonists under non equilibrium conditions are illustrated by experiments using FDSS/µCell.

Type of Agonism	pA2 =	Correction Factor	Curve Pattern(s)
Competitive Surmountable	pK_B	None but Schild regression must be linear with unit slope	
Hemi-Equilibria	$pK_B + \Psi Log (1 + 2 [A]/K_A)$ $\Psi << 1$	Very slight overestimation to no correction	
Orthosteric Insurmountable	$pK_B + Log (1 + 2 [A]/K_A)$	Slight overestimation (maximal error ≈ 2)	
Allosteric Insurmountable	$pK_B + Log (1 + 2\alpha [A]/K_A)$	Very slight overestimation (for modulators with $\alpha < 1$)	







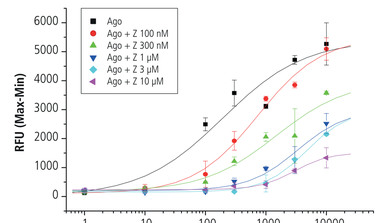


Fig. 3: Relationship between the pA2 for various mechanisms of antagonism and the pKb.

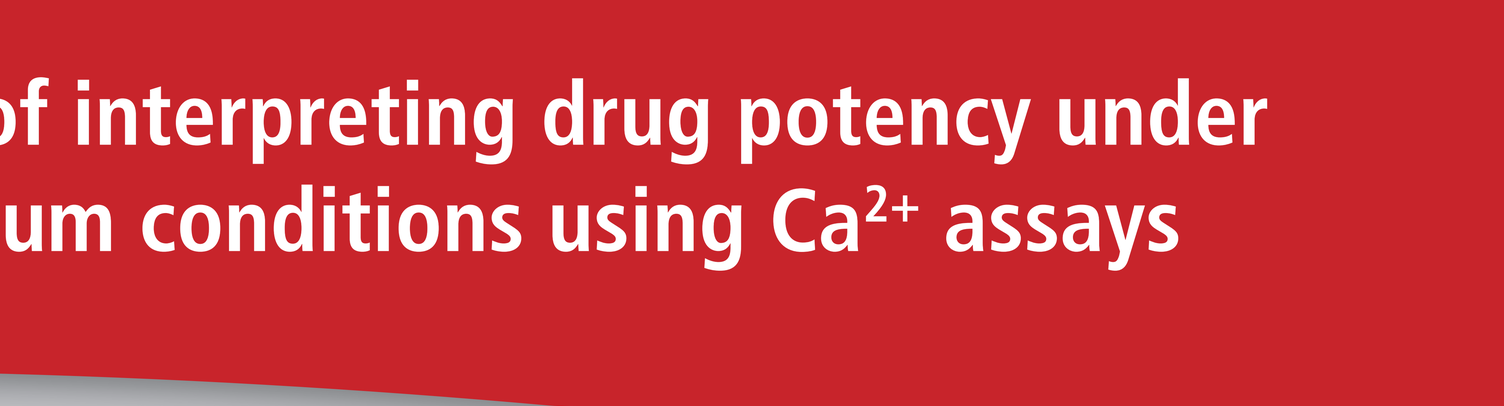


Fig. 2: Calcium assays performed on CHO cells expressing a recombinant aminergic GPCR at the Hamamatsu FDSS/µCell.

The agonist injection was performed at different depht conditions (4 mm (A), 7.2 mm (B) and 9.6 mm (C) for the readings. Rate was constant (10 µl/sec), gain 1, sensitivity 500 ms during 90 sec. pA2 = 7 was expected as previously determined on calcium assay using Flexstation and on GTPγS35 binding assay.

Expected pA2 = 7 (FlexStation)

Agonist injection height (related volume)	FDSS/µCell Determined pA2
4 mm (100 ul)	Inactive
7.2 mm (180 ul)	6.23
9.6 mm (240 ul)	**7.05**

6. Conclusions

- FDSS/µCell is a good functional drug screening system suitable for calcium assays with almost any GPCR
- Applicable for Screen as well as for Structure Activity Relationship
- Use of the pA2 as a universal determinant of antagonist potency to overcome the potential bias associated with non equilibrium conditions
- At low [agonist] occupancy, pA2 tend towards the pKb
- FDSS/µCell Settings for rhodopsin-like class A GPCR with aminergic ligands:
  - High speed and deep delivery close to cells for Agonist testing
  - Low speed and delivery above the buffer surface for Antagonist testing

Bibliography

- (*)Terry Kenakin, 2009, A pharmacology Primer: Theory, Application and Methods, Chapter 11, Academic Press
- Steven J Charlton and Georges Vauquelin, 2010, British J Pharmacol 161:1250–1265
- Terry Kenakin et al, 2006, JPET 319:710–723
- Arthur Christopoulos et al, 1999, Euro J Pharmacol, 382:217–227

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