Development of a Multiplex Assay for Studying Functional selectivity of Human Serotonin 5-HT_{2A} Receptors and Identification of Active Compounds by High-Throughput Screening

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Introduction

Serotonin 5-HT_{2A} receptor is a GPCR that is involved in diseases such as schizophrenia. It was one of the first GPCRs where the existence of functional selectivity was described between two different signaling pathways: the phospholipase C (PLC) and phospholipase A_2 (PLA_2) activation, that mediate IP accumulation and AA release, respectively (Berg *et al*., 1998; Martí-Solano *et al.*, 2015).

We have previously demonstrated the existence of 5-HT_{2A} homooligomers (Brea *et al*., 2009) which evidenced a negative cooperative phenomenon that was observed for certain ligands, such as clozapine, through the PLA_2 signaling pathway.

Hypothesis

The development of a multiplex and miniaturized methodology that can simultaneously measure activation of the PLC and PLA_2 signaling pathways by 5-HT_{2A} receptors would allow the evaluation of functional selectivity and cooperativity phenomena in the first stages of drug discovery

Objective

Our aim in this work was to develop a miniaturized functional assay for the simultaneous measurement of the PLC and PLA_2 signaling pathways coupled to the 5-HT_{2A} receptor using the Prestwick® Chemical Library for technology validation.

Results

1. AA release was optimized in 96 well plates using 2% BSA in assay buffer.

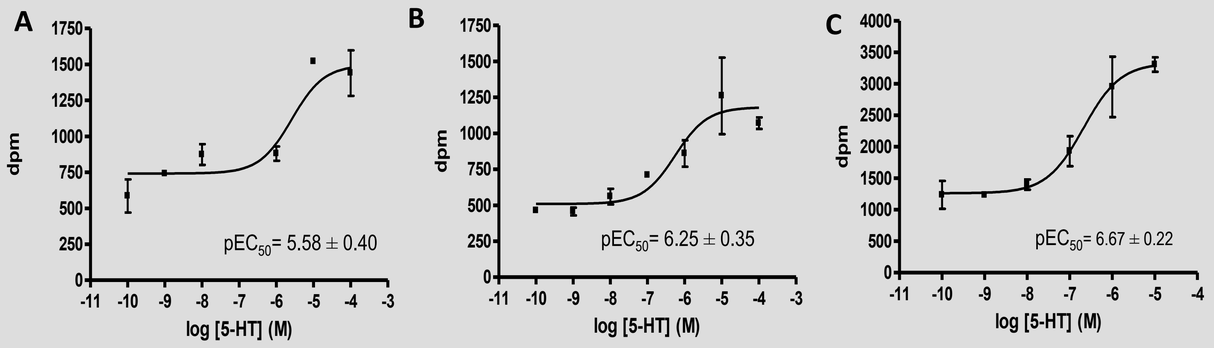


Fig 1. Concentration-response curves for 5-HT induced release of AA (1 μCi/mL [^{3}H]AA). Results from increasing the percentage of BSA in assay buffer from 0,5% (**A**), 1,5% (**B**) to 2% (**C**). 2% BSA was chosen because it provides the highest signal/background ratio. Points represent the mean ± SEM(vertical bars) of triplicate measurements, n=2.

Materials and methods

CHO-FA4 cells expressing human 5-HT_{2A} receptor were seeded into 96 well plates. 24 hours later, the media was replaced with 10 μCi/mL [3H]*myo*-inositol (PerkinElmer). 20 hours later, the media was again replaced with 1 μM [^{3}H]arachidonic acid (PerkinElmer) or 0.2 μCi/mL [^{14}C]arachidonic acid (PerkinElmer) for 4 hours at 37 °C. After the labelling period, cells were washed with different experimental buffer containing different concentrations of BSA, and incubated with tested compounds for 20 minutes at 37 °C. Then, an aliquot of the media was collected to measure AA release, and cells were lysated with 100 mM formic acid to measure IP accumulation in two different ways: with AG 1-X8 resin (Bio-Rad) and with RNA YSi SPA Beads (PerkinElmer). The Prestwick® Chemical Library was used for technology validation. Data analysis was performed using Prism 4.0 software (GraphPad Software, La Jolla, CA). Statistical analysis was performed using extra sum-of-squares *F* test and Student *t* test. Statistical significance was set up at $p<0,05$.

2. Simultaneous measurement of [^{14}C]AA release and [^{3}H]IP accumulation revealed that RNA binding YSi SPA Beads are more suitable for the automated assay

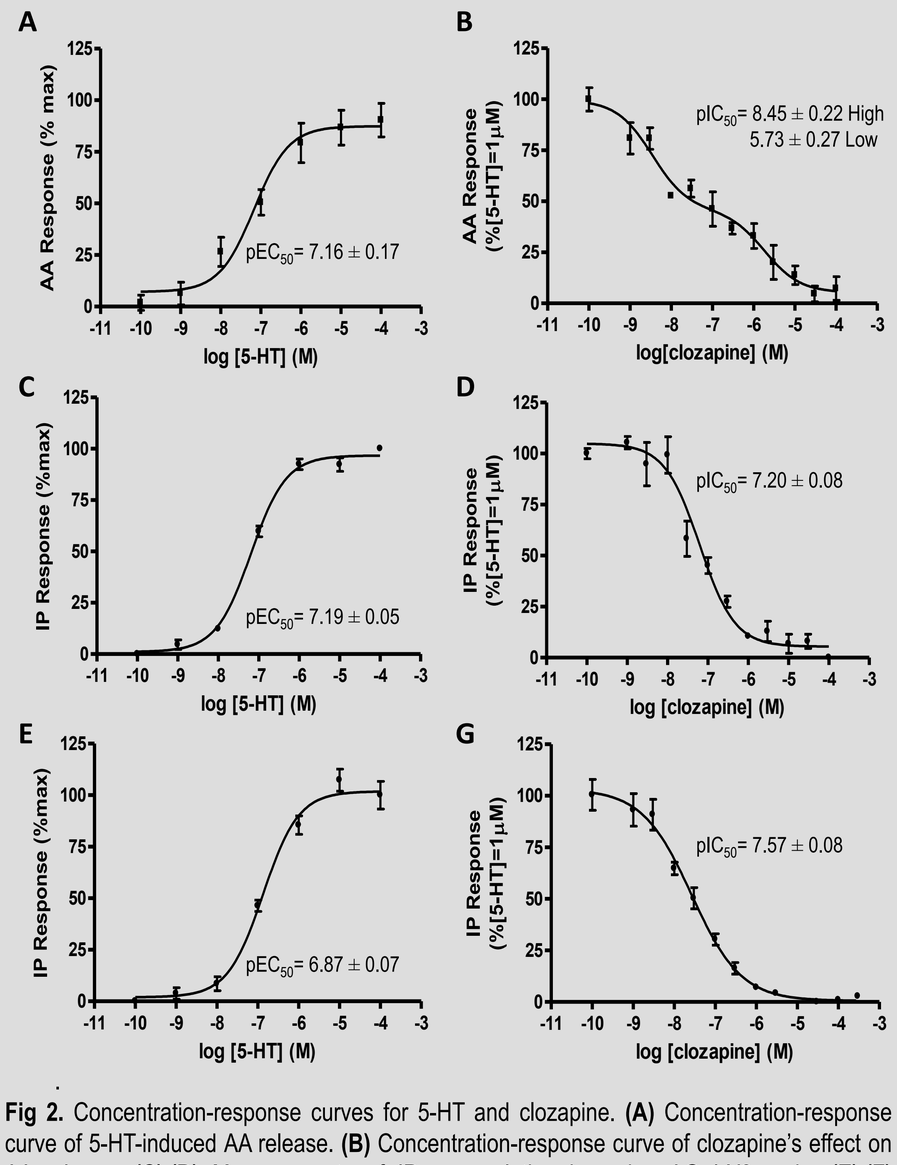
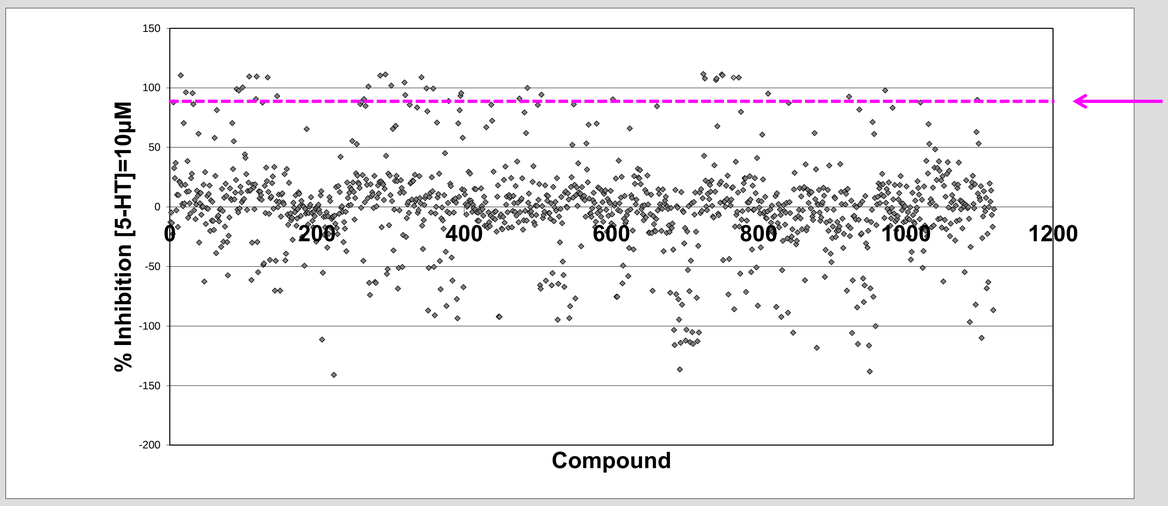


Fig 2. Concentration-response curves for 5-HT and clozapine. **(A)** Concentration-response curve of 5-HT-induced AA release. **(B)** Concentration-response curve of clozapine's effect on AA release. **(C),(D)** Measurements of IP accumulation by using AG I-X8 resin. **(E),(F)** Measurements of IP accumulation by using RNA binding Ysi SPA beads. Functional selectivity is demonstrated by the two different profiles got to the PLC and PLA_2 pathways when clozapine is used to inhibit 5-HT-induced AA release **(B)**. Besides, the SPA technology was chosen to perform the future assays because of its miniaturization potential. Points represent the mean ± SEM (vertical bars) of triplicate measurements, n=2 for **A-D** and n=3 for **E-F**, respectively.

3. The 96-well multiplex allowed hit identification and also detects functional selectivity as demonstrated by using the Prestwick® Chemical Library as proof-of-concept.



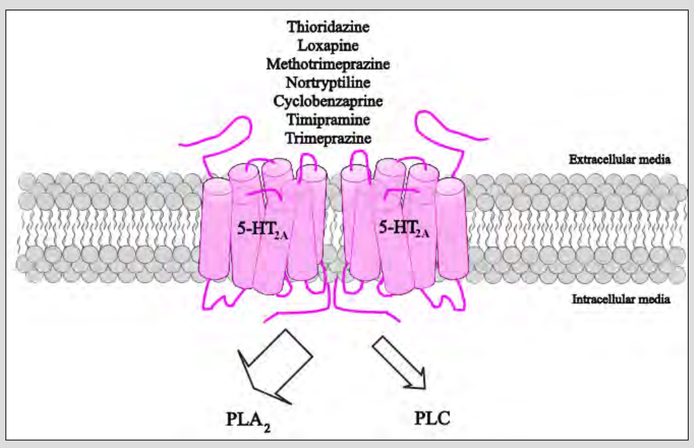


Table 1. pIC_{50} values for compounds exhibiting functional selectivity to PLA_2 pathway.

Compound	pIC_{50} AA (PLA_2)	pIC_{50} IPs (PLC)
Thioridazine	7,23 ± 0,21*	6,03 ± 0,27
Loxapine	7,52 ± 0,27*	6,35 ± 0,29
Methotrimeprazine	7,71 ± 0,39*	6,56 ± 0,12
Nortryptiline	8,52 ± 0,20*	7,05 ± 0,25
Cyclobenzaprine	8,41 ± 0,22**	7,07 ± 0,09
Trimipramine	7,69 ± 0,24**	6,28 ± 0,17
Trimeprazine	7,89 ± 0,54*	6,07 ± 0,11

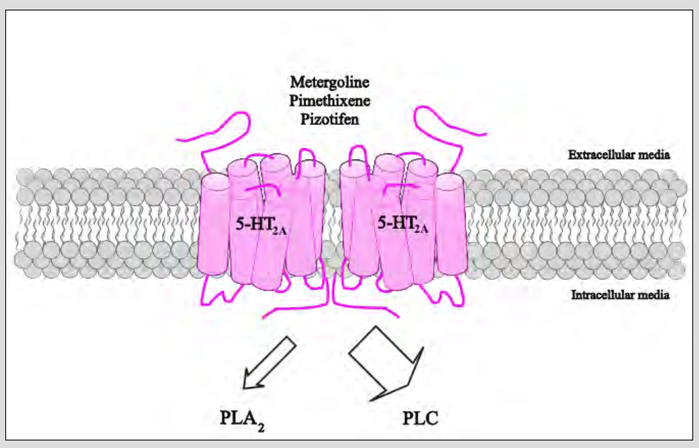


Table 2. pIC_{50} values for compounds exhibiting functional selectivity to PLC pathway.

Compound	pIC_{50} AA (PLA_2)	pIC_{50} IPs (PLC)
Metergoline	6,80 ± 0,25**	8,39 ± 0,12
Pimetixene	7,24 ± 0,37*	8,43 ± 0,06
Pizotifen	7,10 ± 0,30*	8,50 ± 0,08

Values represent the mean ± SEM of triplicate measurements. *$p<0,05$, **$p<0,01$ (Student *t* test) of pIC_{50} at PLA_2 pathway vs PLC pathway.

Conclusions

1. We have developed a miniaturized and robust multiplex 96-well plate assay that allowed us to simultaneously analyze the PLA_2 and PLC effector pathways.
2. As proof-of-concept, we used the Prestwick® Chemical Library. The identified hits are known to interact with the target receptor studied, and the assay have detected previously undiscovered functional selectivity for some of them.
3. This novel multiplex methodology would allow the detection of 5-HT_{2A} ligand functional selectivity in hit finding campaigns.

Grants

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