

Development of [EU]-Gtp Binding Assay for Selection of Histamine H₃ Receptor Agonist & Antagonists

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Abstract

The range of assay technologies for the binding and signaling has been developed in HTS laboratories for the identification of hit or lead compounds acting on GPCRs. The [³⁵S] GTP_S binding assay still remains to be a useful and simple technique to demonstrate receptor activation and is one of the few functional, cell-free assays. However, its radioactive nature imposes clear limitations to its use in regular laboratory practice and in high-throughput experimentations. Herein, we have developed a new non-radioactive version of the assay using europium-labeled GTP analogue in which europium-GTP binding can be assayed using time-resolved fluorescence. In continuation with our efforts, this assay was adapted for Histamine 3 receptors. The assay format was specifically evaluated by testing known histamine 3 agonists (Imetit, Immepip, Methylhistamine, Proxifan and Histamine) and antagonists (GSK189254, Clobenpropit and Thioperamide) drugs. Under optimized assay conditions, the potencies (pEC₅₀ & pK_B) in the binding assay are in good agreement with those obtained previously in the isotopic functional activity assay. The Eu-GTP binding assay was observed to be highly robust (Z' factor 0.84) with high percentage over basal counts. This assay can be utilized as a component of screening cascade for the screening of Histamine 3 receptor antagonists.

Keywords: Alzheimer's disease; Attention-deficit hyperactivity disorders; Histamine H₃; Central Nervous System (CNS); GSK189254; 6-[(3-Cyclobutyl-2,3,4,5-tetrahydro-1H-3-benzazepin-7-yl)oxy]-N-methyl-3-pyridinecarboxamide hydrochloride; Europium labelled Guanosine -5'-triphosphate; High-throughput screening.

Introduction

The neurotransmitter Histamine exerts a wide range of biological functions, including neurotransmission, inflammation, smooth muscle contraction, dilation of capillaries and gastric acid secretion [1]. These effects are mediated through four G-protein-coupled receptor subtypes [2]: H₁, H₂, H₃ and H₄. The H₁ and H₂ receptors are involved with the allergic response and gastric acid secretion, respectively [1]. The H₄ receptor is predominantly expressed in mast cells, and critically involved in the regulation of inflammatory and immune responses [3].

The Histamine H₃ receptor was discovered in 1983 by Arrang and colleagues, using classic pharmacological approaches [4]. It has been identified in the both central nervous system (CNS) as well as the peripheral nervous system as a presynaptic receptor controlling the release of histamine [4]. It is also recognized as a heteroreceptor on non-histaminergic neurons that are capable of regulating the release of many other neurotransmitters, such as acetylcholine, nor-epinephrine, dopamine and serotonin [5].

The H₃ receptor is expressed predominantly in the central nervous system (CNS), with highest expression in the amygdala, substantia nigra, cerebral cortex and hypothalamus [6]. These brain regions have been associated with cognition (cortex and hippocampus), sleep and homeostatic regulation (hypothalamus) [7]. The CNS effect of the H₃ antagonists make them valuable candidates for the treatment of obesity, epilepsy and age-related memory disorders, such as Alzheimer's disease and attention-deficit hyperactivity disorders (ADHD) [8]. The histamine 3 receptor has been shown to couple with heterotrimeric G_{i/o} proteins and identified several signal transduction pathways activated by the receptor [9]: inhibition of adenylate cyclase, activation of phospholipase A₂, activation of mitogen-activated protein kinase and

Akt/glycogen syntase kinase-3β pathways, inhibition of calcium influx through voltage-gated calcium channel.

In this regard assessing GPCR activation by GTP binding is an important tool to study the early stage of signal transduction. Previously this has been done using the radiolabeled non-hydrolyzable GTP analogue [³⁵S] GTPγS [10]. On the other hand, because of inherent limitations associated with working with radioactivity, there is an obvious advantage to adapting the GTP binding to a non-radioactive format.

Time-resolved fluorescence technology exploits the unique fluorescence properties of lanthanide chelates. It has few advantageous like low background and high-signal to noise ratios [11]. In TRF, emission of the fluorescent label having a long decay time is recorded with a delay of about 0.4 ms after excitation, when the nonspecific fluorescence has died out, resulting in an excellent signal-to-noise ratio and thus extreme sensitivity, comparable to or even exceeding that of the radioactive compounds [12]. The high-throughput format of the TRF assay makes the Eu-GTP-binding assay ideal for GPCR-targeted drug discovery [13] and has been applied on membrane preparations containing various GPCRs such as α_{2A}-adrenergic [14], neuropeptide [15], dopamine [16], muscarinic [17], and chemokine receptors [18].

In this present study, we describe the Eu-GTP binding assay for H₃ receptor. We have employed Eu-GTP binding assay as part of our *in vitro* pharmacological characterization of novel H₃ ligands. This

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assay protocol is also employed during each step in the development and optimization of a high-throughput assay. Typically, the assay development was started by determining the optimal receptor protein concentration followed by optimizing specific assay components (MgCl₂, GDP, NaCl, saponin and dimethyl sulfoxide [DMSO]). Once the assay was developed, it was assessed for HTS compatibility by determining the critical factors reproducibility and signal to noise discrimination which govern the screening capability of the assay. Data generated using this assay is comparable with the [³⁵S] GTPγS, with the advantages and convenience of a non-radioactive assay.

Materials and Methods

Chemicals

Histamine, R(-)-α-methylhistamine dihydrochloride, imetit dihydrobromide, immapip dihydro bromide, clobenpropit dihydrobromide, thioperamide maleate, GDP, saponin, ascorbic acid, bovine serum albumin (BSA), magnesium chloride and GTPγS were purchased from Sigma Aldrich (St. Louis, MO). Proxyfan oxalate was procured from Tocris Bioscience (UK). GSK 189254 was synthesized and provided by Oxygen Healthcare Research Private Limited. Eu-labeled GTP a fluorescent chelate was obtained from PerkinElmer's DELFIA Eu-GTP binding kit (product number AD0260) (Boston, MA). Human recombinant Histamine 3 receptor membrane protein was obtained from PerkinElmer. HPLC grade water was procured from Milli-Q water system (Millipore, USA). All other chemicals used were of analytical grade and proven purity.

Equipments

Analysis was performed on Victor³™ 1420 Multilabel counter (Perkin Elmer). Software for controlling the system was Wallac 1420 version 3.0. Multichannel pipettes and single channel pipettes were of Eppendorf. Dilution of standard agonist and antagonists was done in Corning 96 well V bottom plate. Filtration and analysis of the compounds was performed in Acrowell™ 96 well filter membrane bottom plates (GHP membrane) were from Pall Life Sciences (USA). MultiScreenHTS Vacuum Manifold (Millipore) unit was used to filter out free Eu-GTP. Assay incubation was done in the Boekel Jitterbug microplate incubator shaker.

Histamine 3 Receptor Eu-GTP binding assay

The Eu-GTP binding assay was performed according to Frang et al. [14] and as suggested by the manufacturer for the DELFIA Eu-GTP binding kit (PerkinElmer). Human recombinant H3R membranes (1 Unit, 15 μg/mL) were pre-incubated in the 96-well V bottom stock plate (Corning) for 15 minutes at 30°C in final volume of 0.2 mL with 50 mM HEPES buffer, pH 7.4, supplemented with NaCl, MgCl₂, saponin, BSA, GDP and 50 μL of desired concentration of agonist in the wells. After pre-incubation of 15 minutes on the plate shaker the reaction was started by addition of Eu-GTP to a final concentration of 3 nM and was further incubated for 30 minutes at 30°C. After completion of the incubation the reaction mixture was transferred in Acrowell™ 96 GHP filter plates (Pall). The assay was terminated by vacuum filtration (MultiScreen Vacuum Manifold, Millipore), followed by two washes with ice-cold buffer (50 mM Tris-HCl, pH 7.4 & 10 mM MgCl₂). Plates were immediately allowed to Victor³™ 1420 Multilabel counter (PerkinElmer Life Sciences) to measure the bound Eu-GTP with the membrane. The plate was read using the factory set protocol for europium measurements (340 excitation/615 nm emission, 0.4 ms delay and 0.4 ms window). Nonspecific binding was determined by the

inclusion of reagent listed above plus a final buffer concentration of 10 μM GTPγS.

Data analysis

Data were measured as Europium GTP binding counts and percentage over basal level was calculated by using the following equation, the nonspecific binding (average) counts were subtracted from each data point:

$$\% \text{ Over basal} = ((\text{Stimulated Signal (average)} * 100) / (\text{Basal Signal (average)}) - 100)$$

Inhibitor potency (EC₅₀) was calculated using a 4-parameter, variable-slope, nonlinear iterative curve fitting provided by Prism® (GraphPad Software, Inc., San Diego, CA.) The EC₅₀ values of individual agonists were calculated using the normalized percentage of maximum response.

The K_B values of antagonists were calculated using the following equation:

$$K_B = EC_{50} / (1 + [\text{Ligand Concentration}] / K_d)$$

And the Z factor was calculated using the equation by Zhang et al. [19].

$$Z' \text{ factor} = 1 - ([3 * \text{SD of Max Signal} + 3 * \text{SD of Min Signal}] / (\text{Mean of Max Signal} - \text{Mean of Min Signal}))$$

Where SD of Max Signal and SD of Min Signal are the corresponding standard deviations of Mean of Max Signal and Mean of Min Signal.

Results and Discussion

In the mentioned research effort, the first stage of assay development was to determine the binding of Eu-GTP to membranes containing H3R to obtain significant difference between the basal binding activity and imetit stimulated samples. Typically four independent experiments in triplicate were performed; the basal level with 3 nM Eu-GTP was 6115 ± 231 (mean ± SD) counts in 30 minute, the stimulated level with 100 nM Imetit was 17776 ± 361.87 counts, and nonspecific binding was obtained in the presence of 10 μM GTPγS was 2238 ± 111 counts, resulting in 191 ± 5% over the basal level. This indicates the specific binding activity of the Eu-GTP to H3R- coupled G proteins. Eu-GTP binding was also tested with control membrane protein (non transfected CHO cells). Membrane from non-transfected cells did not show any stimulation of Eu-GTP binding with 100 nM Imetit, thus further confirming the specific binding of Eu-GTP.

Optimization of the Eu-GTP binding assay

The assay optimization steps were adapted from the PerkinElmer Delfia GTP binding protocol. The initial titration matrix for the each point was done in triplicate. A baseline control without agonist was included for each time point of the titration. A final concentration of 100 nM Imetit was used to activate the receptor.

Optimization of MgCl₂ and GDP concentration: The concentration of MgCl₂ and GDP was optimized by cross-titration on a 96-well Acro Well filter plate in concentration ranging from 0.1-10 μM GDP and 1-10 mM MgCl₂ as suggested by Perkin Elmer. We have prepared concentration matrix with final assay concentration of 1, 3, 5 and 10 mM MgCl₂ that was cross titrated with 0.1, 1, 3 and 5 μM GDP, keeping the NaCl concentration constant (100 mM) in 50 mM HEPES buffer, pH 7.4 as reaction buffer for human H3R. Figure 1 shows the comparison of the stimulation caused by 100 nM imetit in

different buffer conditions with membrane expressing H₃ receptors. The maximum percentage over basal values was found with 3 μM GDP and GDP effect was increased with increasing MgCl₂ concentration. Therefore, 3 μM GDP and 10 mM MgCl₂ were selected for further assay development.

Optimization of NaCl concentration: Similar protocol (used to optimize MgCl₂ and GDP concentration) was used to optimize NaCl concentration in the assay. We have selected 5 different concentration of NaCl in the range from 20 to 200 mM. For this, NaCl stock solution were prepared with the recommended final assay concentration in 50 mM HEPES buffer pH 7.4, by keeping the concentration of GDP (3 μM) and MgCl₂ (10 mM) constant. The effect of Na⁺ concentration on stimulation of H₃ receptor caused by 100 nM imetit is represented in Figure 2. The best percentage over basal ratio was found with 130 mM NaCl concentration.

Optimization of saponin concentration: Saponin is a mild detergent, which can improve the GTP binding to H₃ receptor, was tested within a range from 0-300 μg/mL. The stock solution of saponin with the recommended final assay concentration was made in 50 mM HEPES buffer pH 7.4 with the constant concentration of MgCl₂ (10 mM), GDP (3 μM) and NaCl (130 mM). Figure 3 depicts the effect of saponin concentration on imetit induced binding of GTP-Eu to H₃ receptor. The optimal signal was found with 100 μg/ml of saponin and this concentration was used in the subsequent steps of the assay development.

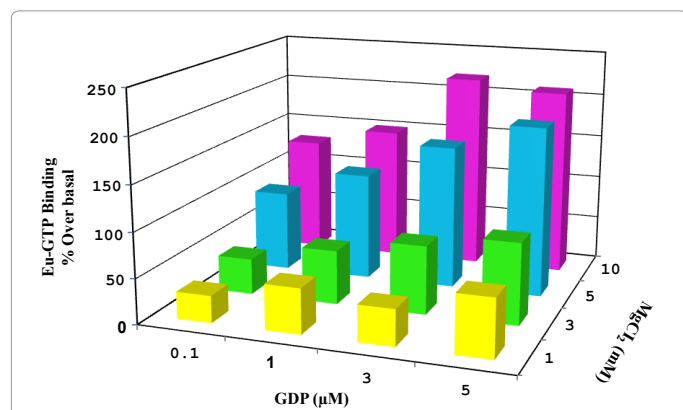


Figure 1: Optimization of the MgCl₂ and GDP concentrations for the H₃R Eu-GTP assay.

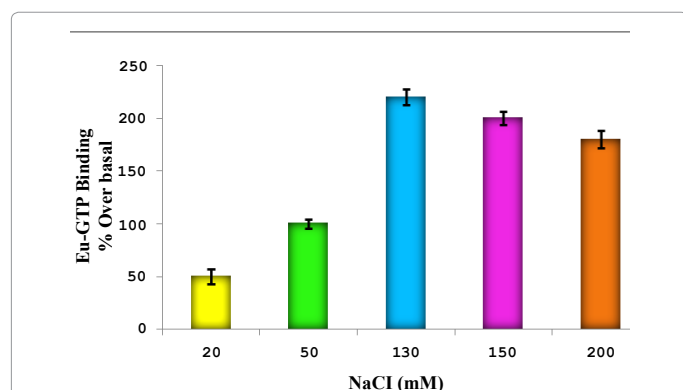


Figure 2: Optimization of the NaCl concentration for H₃R Eu-GTP binding Assay.

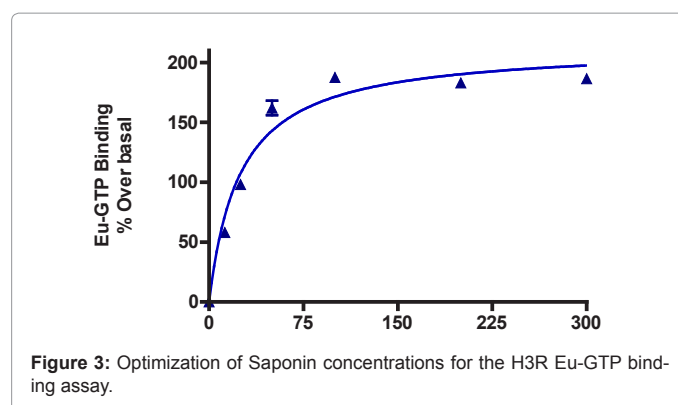


Figure 3: Optimization of Saponin concentrations for the H₃R Eu-GTP binding assay.

Optimization of H₃ membrane concentration in the binding assay: In all previous optimization steps, 15 μg (1 Unit) of H₃R membrane per well was used, which is a fairly high amount of protein per well for the antagonist screening purpose. Therefore, we tested smaller protein amounts per well, and Figure 4 shows that even 1.62 μg provided reasonable percentage over basal (96%) values compared with 15 μg per well (170). We have performed the binding assay to obtain optimal % over basal, upto 30 μg per well and the best % over basal ratio was found with 7.5 μg/well.

Validation of the Eu-GTP binding assay using known agonists: To further validate the Eu-GTP binding assay, 5 H₃R agonist previously screened in a radiometric ([³⁵S] GTPγS) assay were tested. These agonists were selected with a wide range of affinity, which includes pEC₅₀ value from low nM to High nM. As shown in the Table 1, Figure 5 the pEC₅₀ values for the 5 agonists were very similar to those determined in the isotopic [³⁵S] GTPγS functional assay [19].

DMSO tolerance study: To adapt the assay for antagonist selection, a DMSO tolerance study was performed. The H₃R activity was not significantly affected by up to 3% DMSO (data not shown). The antagonist screening was then conducted at 2% DMSO.

Adaptation of Eu-GTP binding assay for H₃R antagonists selection: The aim of these modifications to the assay is to use it to screen the H₃R antagonists. In order to utilize the assay for this purpose the H₃R membranes were pre-incubated with agonists and antagonists for 15 minutes before the addition of Eu-GTP in the 96-well V bottom assay plate (Corning), by keeping other components constant. The potencies of H₃R receptor antagonists (GSK 189254, Clobenpropit and Thioperamide) were determined for antagonism of imetit-mediated inhibition of Eu-GTP binding to human H₃ receptor. The data was analyzed in nonlinear regression mode to obtain pK_B values by using Graph pad Prism software which is shown in Table 1, Figure 6. These values are in close agreement with previously published data [20].

Z' Determination: To be of use in agonist and antagonist screening, the assay must fulfill other criteria in that it must be reproducible, give data comparable to the gold standard radiometric assay, and be free of interference from test compounds. To address these points, the Z' value for the assay, the standard measure of reproducibility and signal-to-noise discrimination, were determined [19]. The Z' value is obtained by conducting back ground and maximum stimulation for multiple times across assay plate on separate days. The data shown in Figure 7 gives calculated Z' factor of 0.847. These excellent scores indicate that this assay is viable for use in high throughput screening.

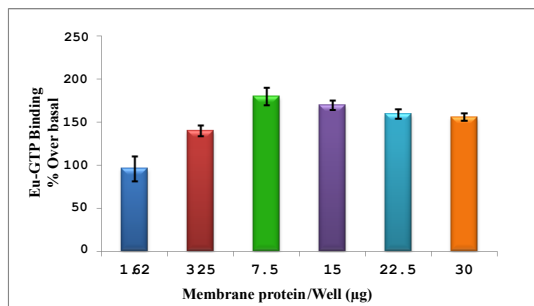


Figure 4: Effect of membrane protein concentration on % over basal values in the H3R Eu-GTP binding assay.

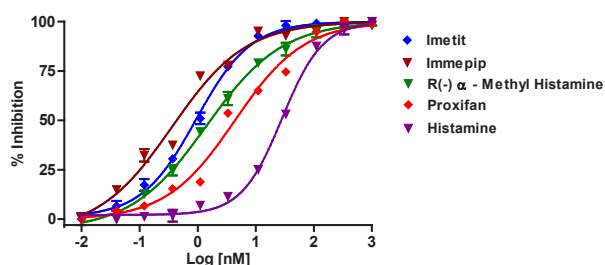


Figure 5: Dose-response curve of agonist-induced binding of Eu-GTP to H3R, investigated with 5 different H3R agonists.

Ligand	Eu-GTP	[³⁵ S]GTPγS
Agonists		
Imetit	9.03±0.11	8.82±0.07
Immepip	9.44±0.01	9.29±0.07
R(-)-α-methylhistamine	8.85±0.08	8.57±0.01
Proxifan	8.36±0.12	8.3±0.10
Histamine	7.55±0.07	7.46±0.04
Antagonists		
GSK189254	9.90±0.11	9.06±0.02
Clobenpropit	9.00±0.02	9.28±0.06
Thioperamide	8.26±0.10	8.12±0.01

Table 1: Dose-response isotherms of ligands were analyzed by non-linear regression. Agonist potency is expressed as pEC₅₀. The antagonist potency to inhibit Eu-GTP binding induced by 100 nM Imetit is expressed as PK_B. Results are presented as means ± S.E.M. of at least three independent experiments.

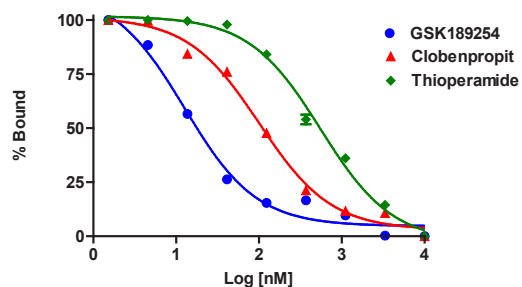


Figure 6: Antagonism of Imetit-stimulated Eu-GTP binding: [Antagonist concentration-inhibition curves for antagonism of 100 nM Imetit responses by GSK 189254, Clobenpropit and Thioperamide.] The data shown are the average of at least three experiments (± SEM), each with three determinations per condition.

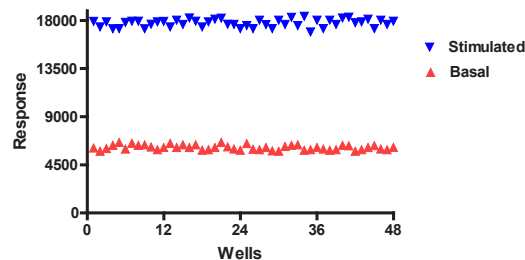


Figure 7: Z' factor estimation for Eu-GTP binding assay.

Conclusion

We have presented data showing that time resolved fluorescence (TRF) based Eu-GTP binding assay is an excellent format for the selection of H3R agonist and antagonists in that it provides a simple homogeneous format that is more affordable, less time consuming, amenable to automation and scalable to any assay volume required. However, identification of appropriate assay for the characterization of agonist and antagonist of histamine 3 receptor presents a challenge for the GPCR functional assay development and impedes progression of the target in drug discovery program. Although, radioactive assay (³⁵S GTPγS) had been developed for the selection of H3R agonist and antagonist, disposal costs and potential for the contamination of HTS facilities restricts the use of radioactive binding assay for screening purpose. The assay has an advantage over the radiometric assay, as it has long fluorescence lifetime and can detect less than one attomole of europium in a multiwell plate sample.

The assay, when compared with the gold standard radiometric formats for histamine 3 receptor functional assay, gives comparable Z' values and in actual screening trials gave nearly identical results to those of the radiometric assay.

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