APPLICATION NOTE

Upstream/ downstream GPCR readout monitoring with GTP Gi binding and cAMP Gi HTRF assays.

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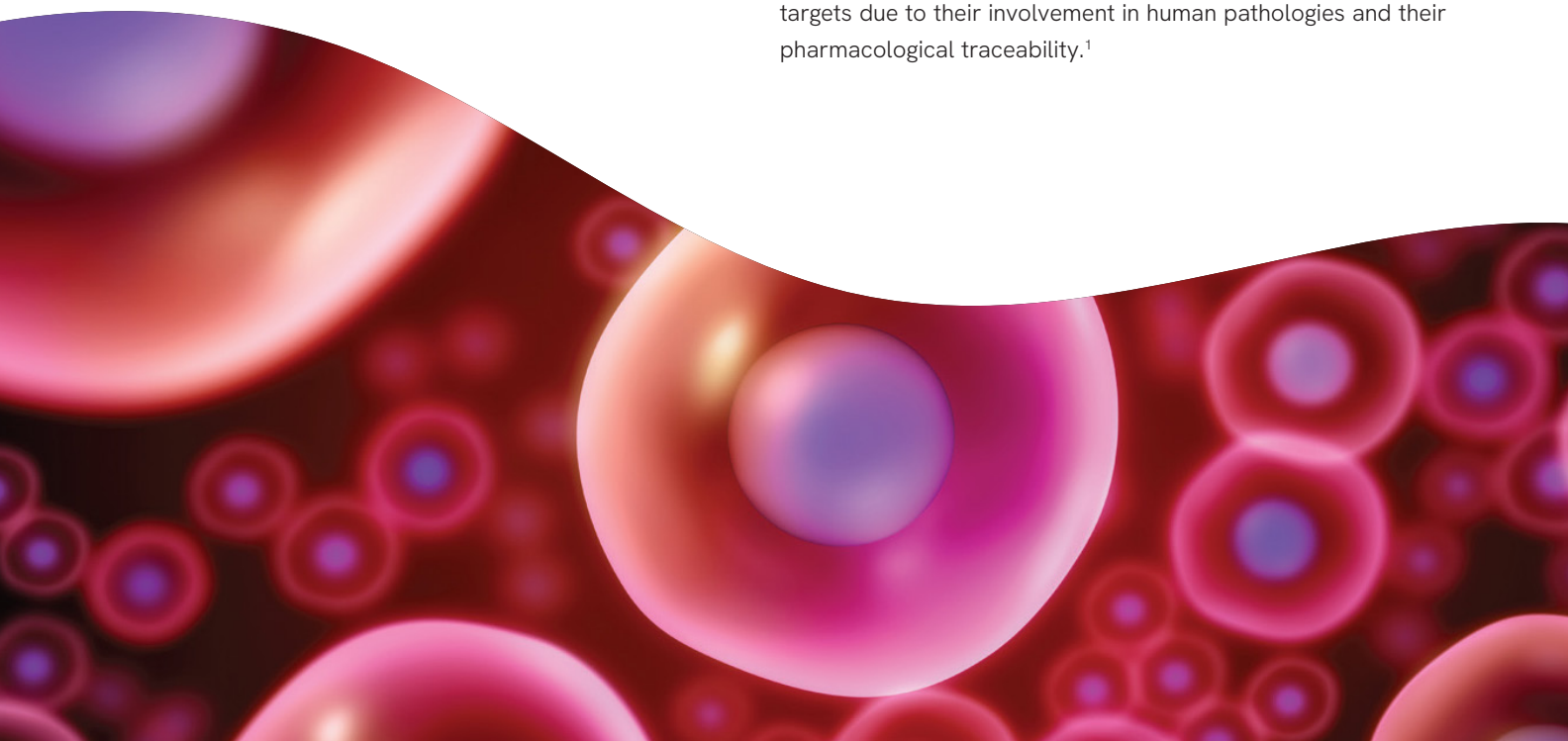
Abstract

This application note presents and compares the pharmacological characterization of a panel of reference compounds (full agonists, partial agonists, antagonists) in δ-opioid and D2 receptor models with two different Gi-coupled receptor activity monitoring assays: the HTRF® GTP Gi binding assay and the HTRF cAMP Gi assay. The GTP Gi binding assay is an upstream readout that has the advantage of revealing the functional response of GPCRs at the level of one of the earliest receptor-mediated events. The cAMP Gi assay, however, is a second-messenger downstream readout monitoring GPCR-activation related events further from the receptor. The results show the relevance and suitability of both assays for the pharmacological characterization of compounds. They also indicate that their use in parallel enables the identification of subtle downstream alterations and/or amplifications of GPCR signaling that are described in the literature, and which may contribute to the incorrect profiling of partial agonists when investigated with a downstream approach alone.

Introduction

G-protein-coupled receptors (GPCRs), also known as seven transmembrane receptors, are the largest group of membrane receptors in eukaryotes and mediate a wide variety of physiological functions. Representing ~34% of all FDA-approved therapeutic drugs, they are the most intensively studied drug targets due to their involvement in human pathologies and their pharmacological traceability.[1]



GPCR activation upon agonist binding induces conformational changes in their intracellular portion, which in turn recruits and activates heterotrimeric G proteins (composed of α, β, and γ subunits). These act as mediators and transduce various external stimuli into cellular responses.[2,3] The G protein activation process is catalyzed by the exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) on the α-subunit, which in turn engages conformational changes that lead to the dissociation of Gα from the dimeric Gβγ subunits.[2,3] There are four major classes of G proteins, defined by their α subunits ($G\alpha_i$, $G\alpha_s$, $G\alpha_q$, $G\alpha_{12}$). Each Gα protein signals through a specific biochemical pathway. In this study, we focus on the Gi-coupled receptor model. $G\alpha_{i/o}$ signals through adenylate cyclase inhibition, i.e. the enzyme responsible for converting adenosine ATP to cAMP, a second messenger that activates downstream effectors. Thus, this pathway leads to a decreased cAMP level (Figure 1).[2,3,4]

There are several functional assays available for GPCR compound screening and characterization. They are either upstream readouts, focusing on events close to the receptor (such as GTP binding assays), or downstream readouts monitoring events and analytes further from the receptor (such as second messenger assays). The respective merits of these different functional assay categories have been compared and discussed in several reviews, without conclusive evidence that one is more "suitable" than the another.[3,5] Moreover, they are considered as complementary for pharmacological compound studies. There is evidence that some GPCRs' biological activity becomes increasingly altered and/or amplified as part of the signal transduction process downstream. This means that upstream and downstream readouts can result in different compound potencies and efficacies, which could be more accurately studied by a combination approach using both assay categories.[5,9]

In this Application Note, two Gi-coupled GPCR models (δ opioid receptor DOR or DOP, and dopamine D2 receptor) were taken as case studies for the pharmacological characterization of several compounds, using two different readouts of the Gi protein signaling pathway: the upstream Gi activation assay (HTRF® GTP Gi binding kit), and the downstream cAMP measurement assay (HTRF®cAMP Gi Kit).

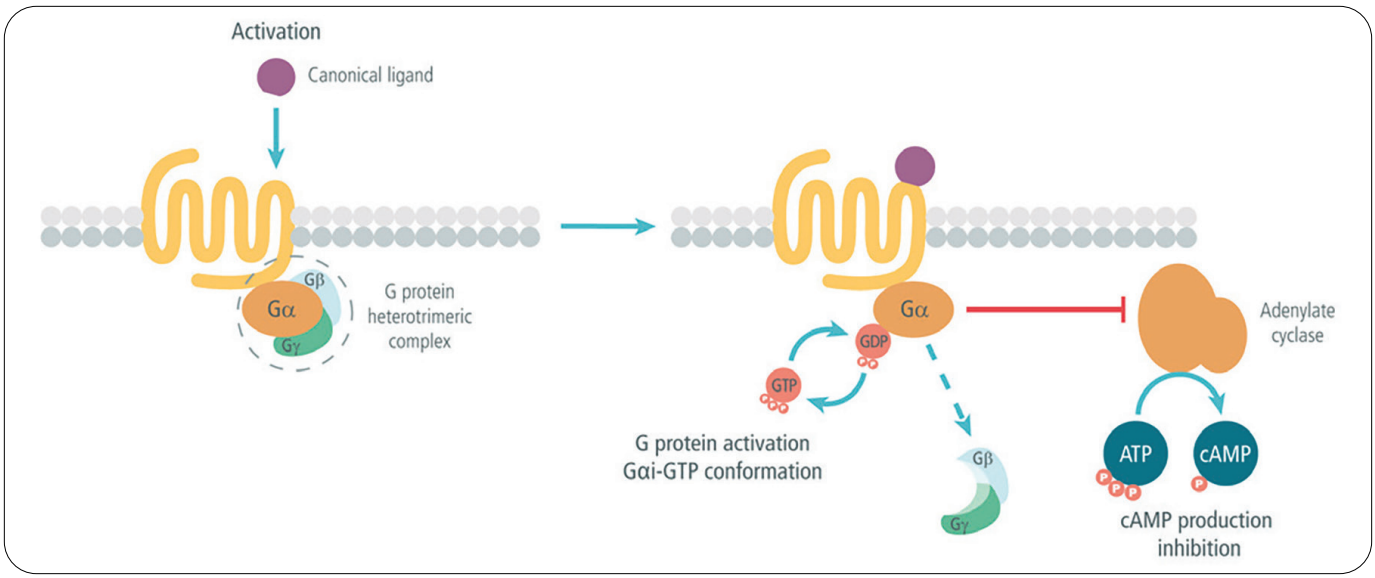


Figure 1. Gi-coupled receptor signaling process. The HTRF GTP Gi binding assay monitors GDP/GTP nucleotide exchanges at the receptor-coupled Gαi subunit. The HTRF cAMP Gi assay measures the drop in second-messenger cAMP levels following adenylate cyclase inhibition by active Gαi.

Protocols

1- GTP binding assay

The HTRF® GTP Gi Binding assay (#62GTPPET/G) measures the level of Gi protein activation, and has the advantage of studying the functional response of GPCRs at the level of one of the earliest receptor-mediated events. It detects Gi protein activation via the binding of a Eu-cryptate-labeled non hydrolysable GTP analog (donor) and a d2-labeled anti-Gi monoclonal antibody (acceptor) (Figure 2).

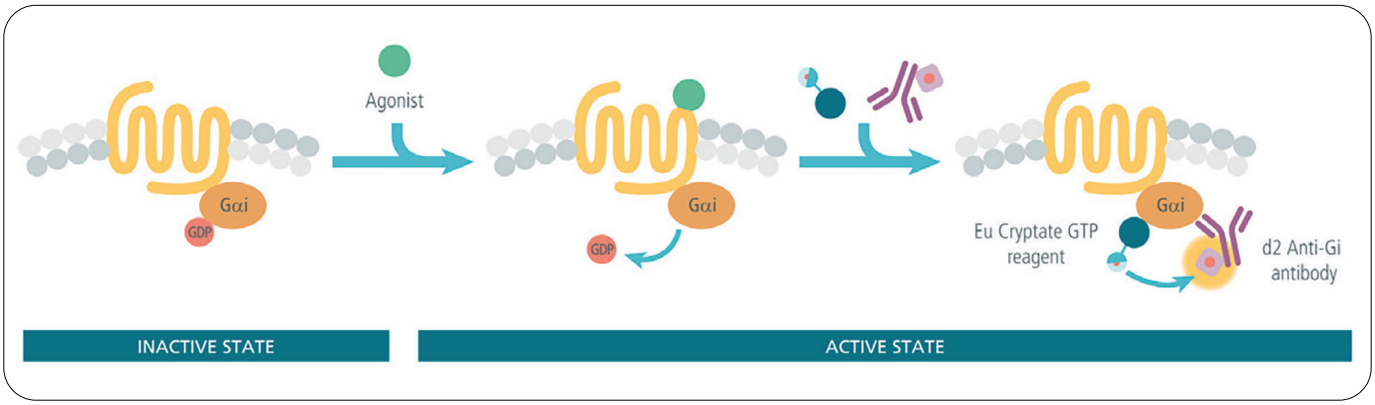


Figure 2. HTRF GTP Gi binding assay principle.

The biological models used for the study were membranes from CHO cells overexpressing delta opioid receptors (CHO-DOR membranes# RBHODM400UA), or D2 (CHO-D2 membranes# RBHD2CM400UA) receptors. The assay was performed in 20 µl final volume. The study presented here shows experiments performed as described in the HTRF GTP Gi binding kit protocol.

Typically, GTP assays require an optimization step to select the optimal conditions for each biological membrane model. Methods and recommendations to optimize the assay for agonist and antagonist studies are presented in detail in the associated kit guides.

The following table summarizes the final optimized conditions for each receptor membrane model in this study.

Table 1. Optimized conditions of the GTP Gi binding assay using CHO-DOR and CHO-D2 membrane models.

Membrane model	CHO-DOR	CHO-D2
Membrane quantity/Well	5 µg	5 µg
[GDP]	0.5 µM	0.25 µM
[MgCl2]	50 mM	50 mM
Incubation time	Overnight	Overnight

1- cAMP assay

The cAMP kit is specifically designed for the direct quantitative determination of cyclic AMP. The HTRF cAMP Gi assay (#62AM9PEB/C) is based on a competition between native cAMP produced by cells and cAMP labeled with the cryptate for binding to a d2-labeled antibody. The specific signal (i.e. energy transfer signal) is inversely proportional to the concentration of cAMP in the sample (Figure 3).

Tag-Lite® CHO stable cell lines expressing delta opioid receptor (#C2SU1DOP*) and dopamine D2 receptor (#C2SU1D2*) were used to perform the cAMP assay. The study presented here shows experiments performed as described by the HTRF cAMP Gi kit protocol.

**: custom request references*

Preliminary experiments to optimize the cell density were performed to select the optimal conditions to work within the dynamic range of the kit. The assay was performed with cells in suspension, in 20 µl final volume. Guidelines and data analysis for agonist and antagonist studies are presented in the associated guides.

The optimal conditions selected are summarized in the following table.

Table 2. Optimized conditions for the cAMP Gi assay using DOR and D2 Tag-Lite stable cell lines.

Stable cell line model	Tag-Lite® DOR	Tag-Lite® D2
Optimal Cell Density	6000 Cells / Well	6000 Cells / Well
[Forskolin]	1 µM	1 µM
Agonist Stimulation Time	45 min	45 min
Antagonist Stimulation Time	45 min	45 min

Biological case studies

After GPCR stimulation by an agonist, GDP-GTP exchange occurs at the Gαi subunit, resulting in a Gαi-GTP complex which directly inhibits adenylyl cyclase and thus indirectly leads to a decrease in intracellular cAMP levels. Intracellular signal amplification from the upstream readout to the

downstream readout may already be detectable at this point.[2,3,5,9] Two Gi coupled receptor case studies are presented here for the functional characterization of different classes of reference compounds (full agonists, partial agonists, and antagonists) using both the GTP Gi Binding assay and the cAMP Gi assay.

The δ-opioid receptor is a member of the opioid receptor family, a group of inhibitory G protein-coupled receptors with opioids as ligands. This receptor is broadly expressed in the brain, binds endogenous opioid peptides, and shows a functional profile clearly distinct from that of μ- and κ-opioid receptors. The important role of this receptor in reducing chronic pain, as well as in psychiatric and other neurological disorders, has been extensively investigated and the beneficial effects of DOR agonists are now well established in the contexts of chronic pain, emotional responses, and mood disorders. It has also shown reduced adverse effects compared to other opioid receptors, such as μ-opioid.[6]

The dopamine D2 receptor belongs to the D2-like dopamine receptor subfamily. It is highly expressed in the brain, and is related to pathological disease processes such as schizophrenia and Parkinson's Disease in addition to other disorders, such as depression, substance abuse, or cancer. It has been widely studied, and many pharmacological compounds that target it, such as agonists or antagonists, are considered promising targets.

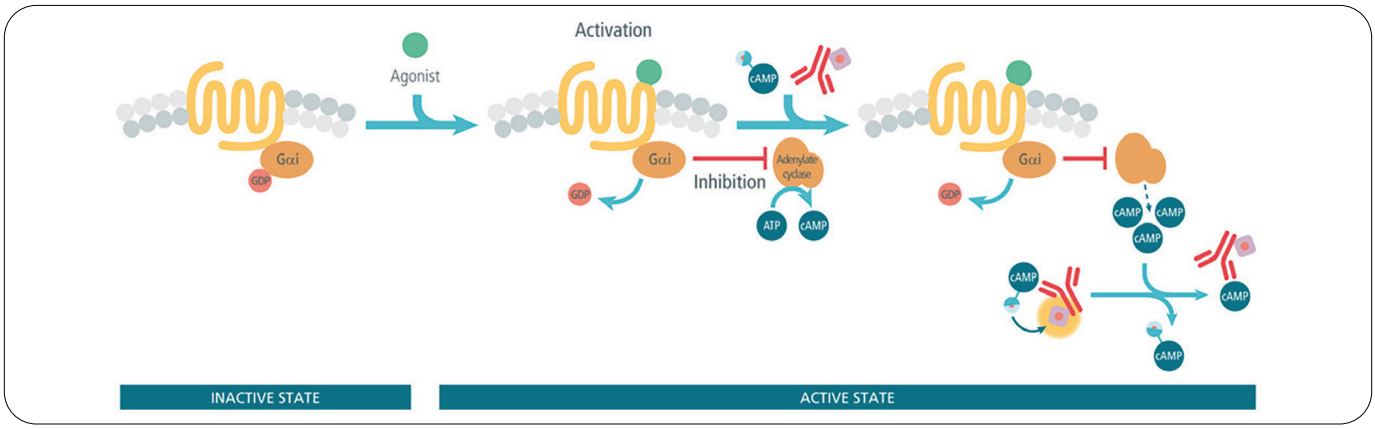


Figure 3. HTRF cAMP Gi assay principle.

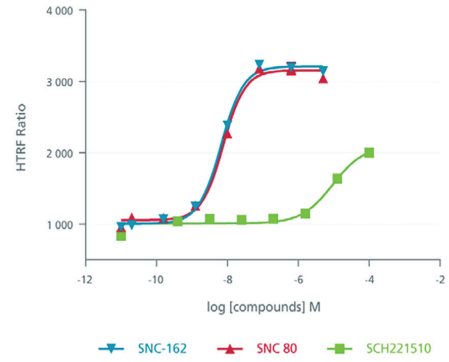
Full agonist characterizations

A panel of full agonists were characterized and ranked with both the GTP Gi binding assay and cAMP Gi assay on the δ-opioid and dopamine d2 receptor models.

In the first case study, the δ-opioid receptor agonists characterized were SNC-162, SNC-80 (both selective for DOR), and SH221510 (more selective for the Nociceptin receptor than for δ-opioid receptor). The agonists' dose-response curves are presented in Figure 4, and the pharmacological data are reported in the associated table. All agonists showed similar potencies and ranking with the two assays.

In the second case study, the D2 receptor agonists PPHT, 7-OH-DPAT, and dopamine were characterized. The same rankings of these agonists were achieved in both the GTP Gi binding and cAMP Gi assays. However, the cAMP assay showed them to be more potent than the GTP binding assay, with EC50 values inferior to the latter's by over one log in every instance (Figure 5). This difference is accounted for in the literature, where a majority of agonists are described as appearing more potent in a cAMP assay than in a $^{[35S]}$GTPγS binding assay.[5,9] Mainly, the signal transduction amplification mechanism that takes place downstream artificially increases the apparent potency of agonists. But the literature also reports that different receptor expression levels and subtle differences in agonists' abilities to activate different G-proteins may occur, and also contribute to these differences (different species of Gi/o proteins which may act differently on different isoforms of adenylate cyclase).[9] Interestingly, Soto *et al* published the following affinity values for the dopamine agonist on the D2 receptor: EC50 of 6.7 nM in an adenylyl cyclase inhibition-BRET experiment (related to the cAMP assay), and around 100 nM in protein activation-BRET experiments (related to the GTP binding assay).[10]

A



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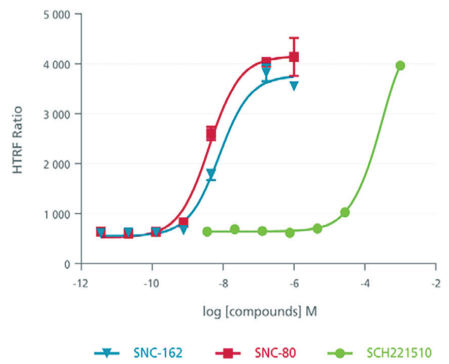

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Agonist potencies (EC50) nM	GTP Gi binding kit	cAMP Gi kit
SNC-162	6.6	7.8
SNC-80	7.8	4.1
SCH221510	> 10,000	> 10,000

Figure 4. Characterization of full agonists (SNC-162, SNC-80, and SCH221510) in the δ-opioid receptor models with the GTP Gi binding assay and the cAMP Gi assay.

A: GTP Gi Binding kit - Agonist dose-response on the CHO DOR membranes
B: cAMP Gi kit - Agonist dose-response on the Tag-lite DOR stable cell line
C: Potencies of DOR agonists SNC-162, SNC-80, and SH221510, with the GTP Gi Binding and cAMP Gi kits

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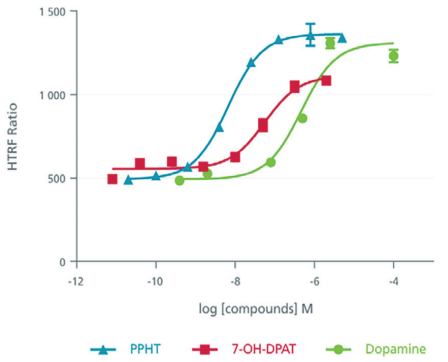

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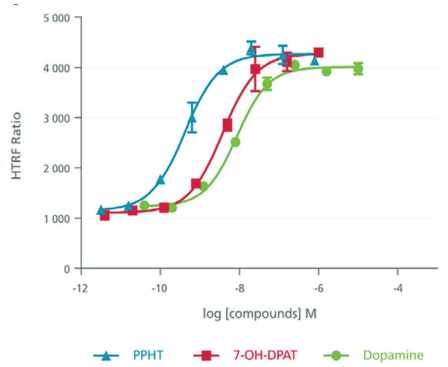

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Agonist potencies (EC50) nM	GTP Gi binding kit	cAMP Gi kit
PPHT	6.6	0.4
7-OH-DPAT	56	3.8
Dopamine	468	8.6

Figure 5. Characterization of full agonists (PPHT, 7-OH-DPAT, and Dopamine) in the dopamine D2 receptor models with the GTP Gi binding assay and the cAMP Gi assay.

A: GTP Gi Binding kit - Agonist dose-response on the CHO D2S membranes
B: cAMP Gi kit - Agonist dose-response on the Tag-lite D2 stable cell line
C: Potencies of D2 receptor agonists PPHT, 7-OH-DPAT, and dopamine with the GTP Gi Binding and the cAMP Gi kits

1- Partial agonist pharmacological characterization

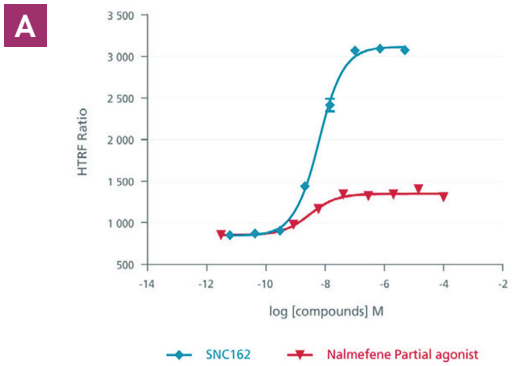
It is widely stated in the literature that functional assays monitoring downstream effectors of GPCR activity (such as second-messenger cAMP) are less successful in discriminating partial agonists from full agonists than their upstream effector-based counterparts. In practice, the efficacy of partial agonists may be exaggerated in these downstream assays, and can result in their wrongful characterization as full agonists. The higher efficacy measured in cAMP assays compared to GTP binding assays may be explained by an intracellular signal amplification mechanism, which artificially enhances a partial agonist's efficacy to the point where it might be mistaken for a full agonist.[5,9] This point was addressed in the following study with a highly partial agonist pharmacological parameter comparison with the GTP Gi binding assay and the cAMP Gi assay in the δ-opioid receptor models.

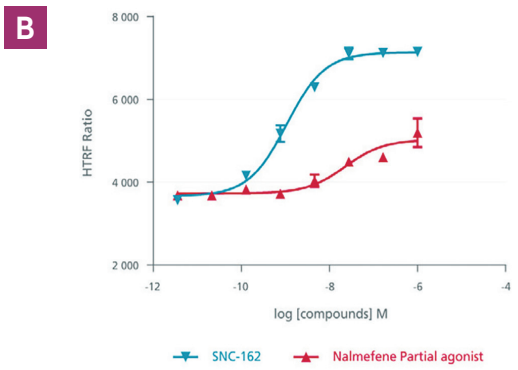
The partial agonist used in the following experiments was Nalmefene, a drug approved for alcohol management and other dependence treatments. It has been primarily described as a modestly selective opioid antagonist with a higher potency in μ- and δ-opioid receptors than δ-opioid receptors, but its partial agonism has been reported in the literature for μ- and κ-opioid receptors using [35S]GTPγS radioactive assays.[7] The results obtained with the GTP Gi binding assay and the cAMP Gi assay are presented in Figure 6. The potency of Nalmefene is shown to be in the same nM range for both assays. Both assays correctly assess the partial agonism of Nalmefene (with the full agonist SNC-162 as reference); however, the efficacies obtained are different. The downstream cAMP assay shows an efficacy of 44%, while the GTP binding assay has an efficacy of only 20%. This difference provides an insight into the contribution of amplification mechanisms to Nalmefene biological activity.

2- Antagonist pharmacological characterization

A panel of reference antagonists were characterized and ranked with the GTP Gi binding assay and the cAMP Gi assay on the δ-opioid and dopamine d2 receptor models. All compounds displayed correct antagonist profiles in all experiments.

The δ-opioid antagonists showed overall similar potencies and rankings in the two assays, with a slight difference for Naltrindole in the nM range with the cAMP assay. The graphs and results are presented in Figure 7.





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Compounds	GTP Gi binding kit		cAMP Gi kit	
	PPHT	PPHT	PPHT	PPHT
PPHT	6.6	6.6	6.6	0.4
7-OH-DPAT	56	56	56	3.8

Figure 6. Characterization of a partial agonist (Nalmefene) in the δ-opioid receptor models with the GTP Gi binding assay and the cAMP Gi assay.

A: GTP Gi Binding kit - Partial agonist dose-response on the CHO DOR membranes
B: cAMP Gi kit - Partial agonist dose-response on the Tag-lite DOR stable cell line
C: Potencies of DOR agonists SNC-162 and Nalmefene with the GTP Gi Binding and cAMP Gi kits

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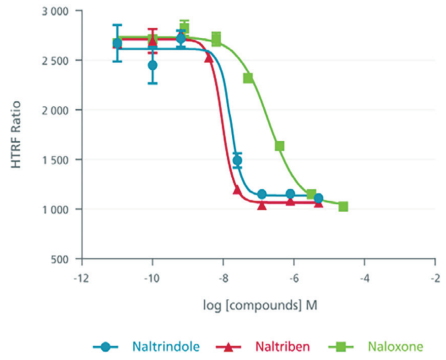

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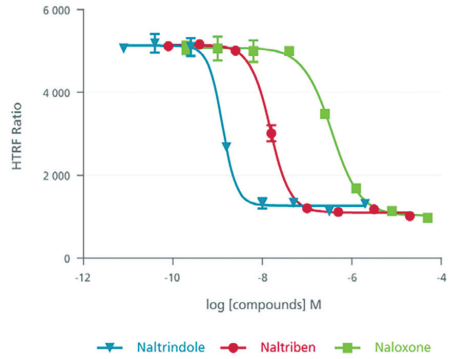

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Agonist Potencies (EC50) nM	GTP Gi binding kit	cAMP Gi kit
Naltrindole	13	1.5
Naltriben	9.3	14
Naloxone	193	356

Figure 7. Characterization of antagonists in the δ-opioid receptor models with the GTP Gi binding assay and the cAMP Gi assay.

A: GTP Gi Binding kit - Antagonist dose-response on the CHO DOR membrane
B: cAMP Gi kit - Antagonist dose-response on the Tag-lite DOR stable cell line
C: Potencies of DOR antagonists Naltrindole, Naltriben, and Naxolone with the GTP Gi Binding and cAMP Gi kits

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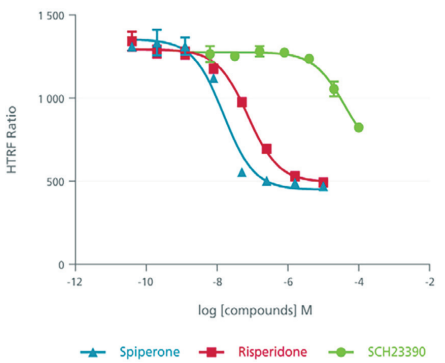

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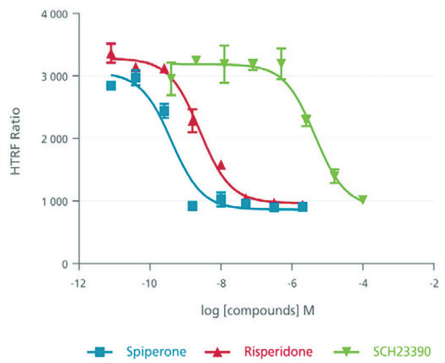

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Agonist Potencies (EC50) nM	GTP Gi binding kit	cAMP Gi kit
Spiperone	15	0.4
Risperidone	74	2.5
SCH23390	> 10,000	4,400

Figure 8. Characterization of full agonists in the Dopamine D2 receptor model with the GTP Gi binding and cAMP Gi assays.

A: GTP Gi Binding kit - Antagonist dose-response on the CHO D2S membranes
B: cAMP Gi kit - Antagonist dose-response on the Tag-lite D2 stable cell line
C: Potencies of D2 receptor antagonists Naltrindole, Naltriben, and Naxolone with the GTP Gi Binding and cAMP Gi kits

The D2 receptor antagonists are presented in Figure 8. All antagonists were ranked the same in the two assays. However, the potencies obtained with the cAMP assay were dramatically higher than for the GTP binding assay. This difference is in line with the increased potencies of agonists measured with the cAMP assay in the same D2 receptor model (Figure 5). The results indicate that D2 receptor activity and signaling seem to be consistently altered and/or amplified by downstream mechanisms.

Conclusion

In the context of GPCR studies and pharmacological characterizations of compounds, Revvity presents the application of two Gi-coupled receptor readout assays, the GTP Gi binding assay and the cAMP Gi assay. Through real case studies of the δ-opioid and dopamine D2 receptors, a panel of reference compounds (full agonists, partial agonists, and antagonists) were characterized and ranked using these two assays. The results show that all compounds were correctly profiled and were overall ranked similarly for both receptors and by both assays.

Interestingly, while the two assays determined the same potencies for all compounds in the δ-opioid receptor models, they achieved different results in the D2 receptor models. In this case, the cAMP assay consistently resulted in much higher potencies than the GTP binding assay for all compounds except the partial agonists.

Moreover, partial agonist characterization showed a higher efficacy detected with the cAMP Gi assay than the GTP binding assay (with similar potency in both). These findings are in accordance with possible intracellular amplification signaling effects described in the literature, and are a convincing argument for the complementarity of using both upstream and downstream assays for fine-tuned characterization and studies of GPCR signaling. Identifying the relative actions of compounds via upstream and downstream readouts could therefore offer an interesting approach in the accurate pharmacological study of therapeutics.

References

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