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Lighting up G protein-coupled purinergic receptors with engineered fluorescent ligands

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Abstract

The use of G protein-coupled receptors fluorescent ligands is undergoing continuous expansion. In line with this, fluorescent agonists and antagonists of high affinity for G protein-coupled adenosine and P2Y receptors have been shown to be useful pharmacological probe compounds. Fluorescent ligands for A_1R, $A_{2A}R$, and A_3R (adenosine receptors) and $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{14}R$ (nucleotide receptors) have been reported. Such ligands have been successfully applied to drug discovery and to GPCR characterization by flow cytometry, fluorescence correlation spectroscopy, fluorescence microscopy, fluorescence polarization, fluorescence resonance energy transfer and scanning confocal microscopy. Here we summarize recently reported and readily available representative fluorescent ligands of purinergic receptors. In addition, we pay special attention on the use of this family of fluorescent ligands revealing two main aspects of purinergic receptor biology, namely ligand binding and receptor oligomerization.

Keywords

Fluorescent ligands; Purinergic receptors; FRET; GPCR Oligomerization

1. Introduction

The purinergic neurotransmission system involves two main extracellular effectors, namely adenosine and adenosine 5′-triphosphate (ATP) (Burnstock, 1972). In the central nervous system (CNS), while adenosine acts as a neuromodulator, ATP also operates as a neurotransmitter. Despite the initial resistance to acceptance of ATP as a genuine extracellular signaling molecule, the existence of consistent physiological effects and specific extracellular enzymes regulating this molecule eventually supported its role as

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neurotransmitter, and consequently the existence of a purinergic neurotransmission system (Burnstock, 1972). Afterwards, ATP was identified as a co-transmitter in peripheral nerves and subsequently as a co-transmitter with glutamate, noradrenaline, GABA, acetylcholine and dopamine in the CNS (Burnstock, 2004). Interestingly, extracellular ATP is quickly hydrolyzed into adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine plus inorganic phosphate and pyrophosphate, through the action of extracellular nucleotidases (ecto-NTs) (e.g. ecto-NTPDases and ecto-5′-nucleotidase) (Zimmermann, 1996). Adenosine, which consists of a purine base (adenine) attached to the 1′ carbon atom of ribose, has been historically considered a retaliatory metabolite modulating a large array of physiological processes (Newby, 1984). Accordingly, it has been postulated that this purine nucleoside is a mediator of metabolic distress, thus having considerable impact on homeostatic cellular functioning. In addition, there is supporting information confirming the involvement of adenosine and ATP in the pathophysiology of the CNS, including neurodegenerative diseases, neuropsychiatric disorders and cancer (i.e. neurogliomas), which have lead to an explosion of interest related to the purinergic neurotransmission system.

Extracellular ATP and adenosine exert their effects via specific plasma membrane receptors. In 1976 purinergic receptors were first defined (Burnstock, 1976), and two years later 2 types of purinoceptors, named P1 and P2 for adenosine and ATP/ADP, respectively, were proposed (Burnstock, 1978). The dual effect of adenosine on cAMP accumulation served as initial subclassification of adenosine receptors into R_i and R_a (Londos et al., 1980), or alternatively, A_1 and A_2 adenosine receptors (van Calker et al., 1979). Nowadays, it is well established that adenosine mediates its actions by activating specific P1 purinergic G protein-coupled receptors (GPCRs), for which four subtypes (i.e. A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) have been identified (Table 1). These receptors have a distinguishable pharmacological profile, tissue distribution and effector coupling (Jacobson and Gao, 2006) (Table 1), and its functioning have been largely studied in the CNS (Sebastião and Ribeiro, 2009). In 1985, P2 receptors were subdivided into P2X and P2Y subtypes on the basis of pharmacology (Burnstock and Kennedy, 1985). While P2X are ligand-gated ion channel receptors, the P2Y subtypes are GPCRs (Burnstock and Williams, 2000). Currently, eight subtypes of the P2Y family (i.e. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been cloned and functionally characterized (Abbracchio et al., 2006). Receptors of the P2Y family are variously activated by an expanded list of nucleotides, including ATP, ADP, UTP, UDP and UDP-sugars.

The wide-spread expression of purinergic receptors, in addition to the diverse and abundant roles of these receptors in extracellular signaling, has lead, as commented above, to a great deal of interest for pharmacologists in general and for neuropharmacologists in particular. Indeed, the physiological and pathophysiological consequences of targeting these receptors with specific ligands may offer new opportunities for a purinergic-based pharmacotherapy in various neurological and neuropsychiatric diseases. Accordingly, this scientific interest has prompted a dramatic growth in the purinergic pharmacology field, in which a large number of ligands have been designed and synthesized. Noteworthy, an important contribution to the purinergic field has been the development of selective ligands labeled with fluorescent moieties that remain active at their receptors. Interestingly, these compounds have become

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powerful tools to address fundamental unmet questions regarding the purinergic receptor biology. For instance, these novel fluorescent ligands of purinergic receptors may permit the visualization of ligand-receptor binding at the cell surface of living cells, or help revealing the existence of purinergic receptor-containing oligomers in native tissue. Accordingly, in the present review, we not only provide an extensive overview of the currently reported fluorescent ligands of purinergic receptors, but also focus on aspects of the biology of adenosine and P2Y receptors to characterize ligand-receptor binding and receptor oligomerization by means of fluorescent ligands.

2. GPCR fluorescent ligands

Fluorescent ligands were introduced in the GPCR field about forty years ago as pharmacological tools to visualize receptors in native conditions and thus boosting the spatial resolution provided by other methodologies (Melamed et al., 1976). The synthesis and purification of these compounds was not easy in those days and thus only accessible to a few researchers. Later on, in the 1990s, the chemistry of fluorescent ligands substantially advanced, and new high-affinity compounds with improved kinetics (i.e. slow dissociation rates) were synthesized (McGrath et al., 1996). Therefore, by using these ligands it was possible to detect receptors not only at the cell surface level but also within intracellular compartments, thus allowing the study of receptor endocytosis (Lutz et al., 1990). Currently, the use of GPCR fluorescent ligands is undergoing continuous expansion (Ciruela et al., 2014a; Hill et al., 2014; Vernall et al., 2014). In this manner, GPCR-based fluorescent ligands have been successfully introduced in a vast number of techniques, such as flow cytometry (FCM), fluorescence correlation spectroscopy (FCS), fluorescence microscopy (FM), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), or scanning confocal microscopy (SCM) (Kozma et al., 2013), for the study of receptor physiology and pathophysiology at both the cellular and the subcellular level (Kuder and Kieć-Kononowicz, 2008). In addition, they have been implemented as screening tools in drug discovery (Middleton and Kellam, 2005).

The use of GPCR-selective fluorescent ligands in receptor binding studies has proved to be complementary, and eventually superior, to the classical radioligand-based techniques. Certainly, these compounds show several advantages over conventional radioisotopically-labelled ligands, for instance avoiding the cost of scintillation detection; enhanced safety and reduced costs as no radioisotope use and disposal is needed; ‘real-time’ readout of the ligand-receptor interaction, which allow kinetic experiments; direct visualization of the receptor localization allowing both local binding and internalization experiments; and finally the possibility of miniaturization, which facilitates a more economical technology (Ciruela et al., 2014b). On the other hand, there are some important considerations when engineering a fluorescent ligand, especially considering that biological activity must be preserved. Thus, among other important factors to be contemplated are: selection of the type of intrinsic activity of the pharmacophore (i.e. agonist *vs.* antagonist; orthosteric *vs.* allosteric ligand); the fluorophore type based on specific photophysical properties (e.g. wavelength, quantum yield, fluorescence lifetime, etc.) and the linker between them, which should allow flexibility but maintain ligand activity. Importantly, these chemical challenges have not prevented the synthesis of novel highly potent and selective fluorescent ligands, and the

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number of marketed GPCR fluorescent ligands is growing rapidly. Hence, GPCR fluorescent ligands might become a major component of investigation of the physiological and pathophysiological functions of GPCRs and consequently aid the development of new drugs for GPCR-based pharmacotherapies.

2.1. Adenosine receptor fluorescent ligands

The first examples of fluorescent purinergic receptor ligands were synthesized by Jacobson et al. in 1987 (Jacobson et al., 1987). These early compounds were synthesized from both xanthine and adenosine amine congeners (XAC and ADAC, respectively) (Table 2) as bifunctional ligands containing a fluorescent dye (i.e. fluorescein isothiocyanate, FITC and 7-nitrobenz-2-oxa-1,3-diazol-4-yl, NBD) (Fig. 1). Indeed, the initially functionalized congeners were versatile adenosine receptor antagonist (C^8-modified alkylxanthines, XAC) and agonist (N^6-modified adenosines, ADAC) probes to which chemically-reactive fluorescent dye derivatives (Fig. 1) were coupled (Table 2) (Jacobson et al., 1987). Accordingly, several fluorescent antagonists (**1**) and agonists (**3,4**) showing moderate to high affinity for A_1R in rat brain were initially synthesized (Table 1 and 2) (Jacobson et al., 1987). Importantly, these initial compounds demonstrated the feasibility of using a tethering approach for adenosine receptor fluorescent ligands. In addition, conjugation to biotin provided an additional strategy for receptor-labeling (Jacobson, 1990), with option of using fluorescently-labeled avidin or streptavidin. Nearly ten years later, a series of fluorescent derivatives of the widely used non-selective and potent adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) were synthesized by the insertion of dansylaminoalkyl moieties (Fig. 1) to the N^6-position of NECA with a linear alkyl spacer of increasing length (from 3 to 12 carbons) (Table 2) (Macchia et al., 1998). Interestingly, a compound of this series with a 6-carbon spacer chain (**5**) allowed visualizing A_1R at the level of the rat molecular layer of the rat cerebellar cortex by means of fluorescence microscopy (Macchia et al., 1998). Subsequently, with the aim of overcoming difficulties related to the short excitation wavelength of certain fluorophores (i.e. dansyl), new fluorescent derivatives of NECA containing a 4-nitro-2,1,3-benzoxadiazol-7-yl (NBD) moiety (Fig. 1) were synthesized (Macchia et al., 2001). A compound of these series (**6**) (Table 2) showed moderate to high affinity for the A_3R and thus allowed the detection of the receptor at the cell surface level of Chinese hamster ovary (CHO) cells (Macchia et al., 2001). Collectively, these results showed the impact of the fluorescent dye and linker length on receptor selectivity and affinity, respectively, a phenomenon that has been object of analysis (Baker et al., 2010). More recently, several red-shifted fluorescent NECA derivatives without selectivity for adenosine receptors were synthesized (Middleton et al., 2007). In this case, the fluorescent moiety used was BODIPY630/650-X (Fig. 1), which showed superior photophysical properties when compared to previously used fluorophores. Indeed, compound **7** allowed visualizing ligand-receptor interactions at the cell surface of CHO cells expressing the A_1R (Middleton et al., 2007) and A_3R (Cordeaux et al., 2008). Interestingly, by using FCS, the membrane diffusional characteristics of these two adenosine receptors were investigated, and two populations of agonist-occupied receptor complexes with different motilities within the membrane of single living cells were identified (Cordeaux et al., 2008; Middleton et al., 2007). Interestingly, similar results were obtained with a similar red-shifted compound (**8**) (Table 2) that bound to the A_1R (Briddon et al., 2004b), thus

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sustaining the notion of the existence of different adenosine receptor populations at the cell surface of living cells. Overall, these results suggested that A_1R and A_3R are not homogeneously distributed through the plasma membrane; instead, they are located within different membrane microdomains (i.e. caveolae and lipid-rich regions) that may determine their differential motility and more importantly their signaling capabilities.

In addition to the abovementioned agonists, other adenosine receptor fluorescent ligands with similar intrinsic activity (i.e. agonism) were described. Indeed, by using as scaffold a more selective A_3R agonist containing a (N)-methanocarba (bicyclo[3.1.0]hexane) ring (i.e. MRS3558) (Table 2) (Tchilibon et al., 2005), several nanomolar and highly selective fluorescent agonists for the A_3R were synthesized (**9, 10, 11**) (Table 2) (Tosh et al., 2012, 2010, 2009). Interestingly, the most potent fluorescent A_3R agonist in the (N)-methanocarba series is a cyanine 5 (Cy5) derivative (**11**) (Table 2), which showed high selectivity for the A_3R (Tosh et al., 2009). In addition, two functional agonists (**12, 13**) (Table 2) at the adenosine $A_{2A}R$ in which the fluorescent dyes Alexa488 and 532, respectively, were covalently attached to the $A_{2A}R$ agonist APEC (Table 2) were synthesized. These ligands were mostly used in ligand-receptor binding experiments (Brand et al., 2008; Fernandez-Duenas et al., 2012), a topic that will be further illustrated in the following sections. Finally, apart from the aforesaid agonists, some adenosine receptor fluorescent antagonists were also engineered. For instance, a non-selective fluorescent antagonist (**2**) (Table 2) was synthesized using XAC (Table 2) and used for the visualization of A_1R and A_3R using FCS in living cells (Briddon et al., 2004a; Corriden et al., 2014). In addition, an $A_{2A}R$-selective red fluorescent antagonist (**14**) (Table 2) was recently used to study receptor oligomerization (Fernández-Dueñas et al., 2015). Overall, a plethora of adenosine receptor fluorescent ligands exists, and it is noteworthy that some of them are commercially available (**2, 7, 14**) (Table 2).

2.2. P2Y receptor fluorescent ligands

Extracellular nucleotides activate G protein-coupled purinergic receptors of the P2Y family. Significantly, while a large array of fluorescent ligands have been described for adenosine receptors, only three compounds are currently reported for P2Y receptors. Specifically, fluorescent ligands of the $P2Y_2R$ (ATP- and UTP-activated) and $P2Y_4R$ (UTP-activated), $P2Y_6R$ (UDP-activated), and $P2Y_{14}R$ (UDP- and UDP-glucose-activated) have been reported. High affinity radioligands for these GPCRs are not available; thus, the P2YR fluorescence ligands currently fulfill a need unmet by other ligand tools. The first compound described (**17**) (Table 3) was a potent and selective $P2Y_6R$ agonist containing the fluorophore Alexa Fluor 488 (Fig. 1) through a spacer chain constructed by click chemistry (Jayasekara et al., 2013). Interestingly, compound **17** was suitable for studying ligand-receptor binding and receptor internalization kinetics on $P2Y_6R$-expressing 1321N1 astrocytoma cells using FCM and FM. Thus, while additional pharmacological characterization was still needed (i.e. ligand-receptor binding in membranes of $P2Y_6R$ expressing cells), the identification of the N^4-benzyloxy group as a structurally permissive region for the synthesis of fluorescent functionalized congeners constituted a significant step forward enabling further $P2Y_6R$ drug discovery (Jayasekara et al., 2013). Subsequently, another $P2Y_6R$ fluorescent agonist (**18**) (Table 3) showing similar potency at $P2Y_2R$ and

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$P2Y_4R$ was synthesized (Jayasekara et al., 2014). Accordingly, this potent $P2Y_{2/4/6}R$ pan-agonist fluorescent probe was also used for studying ligand-receptor binding in $P2Y_6R$-expressing 1321N1 astrocytoma cells using FCM and FM (Jayasekara et al., 2014). Therefore, it was proposed that compound **18** could be useful routinely for the detection of this subset of G_q- protein-coupled P2Y receptors (Jayasekara et al., 2014). Finally, a high-affinity $P2Y_{14}R$ fluorescent compound (**19**) (Table 3) based on the potent and highly selective nonnucleotide $P2Y_{14}R$ antagonist PPTN (Table 2) was synthesized (Kiselev et al., 2014). Compound **19** showed an exceptionally high affinity for the $P2Y_{14}R$ and allowed its detection and characterization by using FCM (Kiselev et al., 2014). These results illustrated that potency of this series of 2-naphthoic acid derivatives such as PPTN can be preserved by chain functionalization, leading to highly potent fluorescent antagonists for $P2Y_{14}R$ (Kiselev et al., 2014). Overall, the development of selective and potent fluorescent ligands for purinergic P2Y receptors is a highly important goal given the role of this receptor family in health and disease. Consequently, further investigation will be needed to achieve a full pharmacological palette of fluorescent compounds for the eight subtypes of P2YRs.

3. Uncovering the biology of G protein-coupled purinergic receptors using fluorescent ligands

The utility of GPCR fluorescent ligands as pharmacological tools to unravel receptor biology has been recently reviewed (Ciruela et al., 2014a). Indeed, the biology of G protein-coupled purinergic receptors in general and adenosine receptors in particular has been the primary object of study using fluorescent ligands. Thus, the analysis of ligand-receptor binding, receptor internalization and receptor-receptor interactions (i.e. GPCR oligomerization) has largely benefited from using such compounds, which in addition have permitted unraveling certain mechanistic aspects unsolvable by classical experimental approaches. Accordingly, we will analyze here in greater detail the impact of purinergic receptor fluorescent ligands on two of these aspects that deserve, in our opinion, special attention, namely ligand-receptor binding and receptor oligomerization.

3.1. Ligand-receptor binding at the surface of living cells: FRET and beyond

The first step of any receptor-mediated signal transduction is ligand-receptor binding. This phenomenon has been classically studied by means of radioligand binding experiments in which the receptor is challenged with a radioisotope-labelled ligand, and the bound fraction is collected and detected using various techniques, such as filtration combined with radioactivity counting, scintillation proximity analysis and autoradiography (Bylund and Toews, 1993). While these assays are extremely sensitive they present several drawbacks, which have been briefly outlined before. Accordingly, there is a growing need to switch from radioactivity to fluorescence ligand binding assays, not only for safety reasons but also for experimental purposes. Indeed, the use of GPCR fluorescent ligand binding assays enables the real-time visualization and quantification of ligand-receptor interactions, thus allowing fine kinetic monitoring of this essential process for receptor biology. Interestingly, most of the purinergic receptor fluorescent ligands used for binding assays in intact cells are actually antagonists, as they do not promote GPCR internalization; conversely, the use of fluorescent agonists are thus useful in the study of receptor internalization. For example, a

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fluorescent agonist of the $A_{2A}R$ was used in FM to follow receptor internalization (Brand et al., 2008). The internalization of a fluorescently labelled agonist with high affinity for the $P2Y_6R$ was determined in astrocytoma cells expressing the $P2Y_6R$ (Jayasekara et al., 2013).

Two basic methods were initially developed to perform ligand-receptor binding experiments using fluorescent compounds. First, the binding of the corresponding fluorescent ligand to a receptor of interest was assessed and monitored either by measuring fluorescence intensity or FP in membrane preparations of living cells upon binding conditions. Indeed, adenosine receptor fluorescent ligands in binding experiments were early described for the $A_{2A}R$ using a derivative of APEC (an amine functionalized $A_{2A}R$-selective agonist) in striatal bovine membranes (McCabe et al., 1992). In addition, an $A_{2A}R$ fluorescent antagonist suitable for FP (**15**) (Table 2) allowed the easy determination of the association and dissociation parameters, thus providing an accurate estimation of the ligand affinity (Kecskés et al., 2010). Interestingly, the acquired data highly correlated with that obtained from radioligand binding experiments, thus supporting the notion that adenosine receptor fluorescent ligands are comparable to the classical radioligands in binding assays. The second method developed consisted of assessing and monitoring the binding of fluorescent ligands to intact living cells expressing the receptor of interest either by FCM or FCS. In such way, a new fluorophore-conjugated A_3R antagonist (**16**) (Table 2) was synthesized and used for cell-based applications. This compound clearly labelled the A_3R at the plasma membrane of CHO cells expressing the receptor and by means of FCM a very high specific-to-nonspecific binding ratio was obtained (Kozma et al., 2012). Interestingly, by using this last approach an equilibrium binding constant comparable to that calculated from kinetic radioligand experiments in membranes was observed (Kozma et al., 2012). Another adenosine receptor fluorescent ligand used in FCM was compound **12** (Table 2), which consisted of an Alexa Fluor 488-conjugated $A_{2A}R$ agonist. This same ligand was also used in the study of ligand-$A_{2A}R$ binding modulation by antiparkinsonian D_2R agonists (Fernandez-Duenas et al., 2013). Finally, the remarkable implementation of a high-content screening assay in living cells using compound **2** (Table 2) and a confocal imaging plate reader was reported (Stoddart et al., 2012). This methodology allowed the determination of the binding affinities of A_1R and A_3R ligands and also demonstrated that adenosine fluorescent ligands can be used in high-throughput screening (HTS) using living cells with enough sensitivity (Stoddart et al., 2012).

A significant advance in the use of GPCR fluorescent ligands in ligand-receptor binding studies consisted of the incorporation of these compounds into RET-based technologies, either as donor or as acceptor chromophores engaging fluorescence-RET (FRET) processes with RET-compatible tagged receptors (Fig. 2). In brief, FRET assays are based on the close proximity (<10 nm) established between donor and acceptor chromophores labelling both the ligand bound and the receptor, which upon ligand binding allows the energy transfer (Fig. 2A). Interestingly, we recently reported the use of a N-terminally cyan fluorescence protein (CFP)-tagged $A_{2A}R$ (i.e. $A_{2A}R^{CFP}$) in combination with compound **13**, which allowed the dynamic evaluation of ligand-receptor binding in single living cells and in a real-time mode (Fernandez-Duenas et al., 2012). Hence, we used the elicited FRET signal between $A_{2A}R^{CFP}$ (i.e. donor) and compound **13** (i.e. acceptor) as readout for the ligand-

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receptor binding in living cells (Fig. 2B). Accordingly, by using this FRET-based ligand-receptor binding approach we were able to demonstrate the existence of an allosteric modulation of the $A_{2A}R$ by the dopamine D_2 receptor (D_2R), which was $A_{2A}R/D_2R$ oligomer-dependent (Fernandez-Duenas et al., 2012). Indeed, a negative allosteric effect of the D_2R agonist quinpirole on $A_{2A}R$ ligand binding and activation was observed (Fernandez-Duenas et al., 2012). Therefore, D_2R agonists could be considered oligomer-dependent negative allosteric modulators (NAMs) of the $A_{2A}R$. In addition, by using this FRET-based ligand-receptor binding approach, we evaluated the $A_{2A}R$ NAM activity of different D_2R agonists (i.e. pramipexole, rotigotine and apomorphine) currently used in clinics as antiparkinsonian agents (Fernandez-Duenas et al., 2013). Interestingly, a differential D_2R-mediated negative allosteric modulation with apomorphine being the best antiparkinsonian drug attenuating $A_{2A}R$ agonist binding was confirmed (Fernandez-Duenas et al., 2013). Overall, the number of approaches using purinergic receptor fluorescent ligands is continuously growing, and these go from the easy primary detection of receptors by FCM, FCS, FM and FP to the study of the ligand-receptor interaction dynamics by means of real-time FRET-based approaches.

3.2. Fluorescent ligands for the study of purinergic receptor oligomerization

One exciting application of fluorescent ligands when studying GPCR biology consists of visualizing receptor oligomers. Such oligomers are thought to display distinct pharmacological properties from the monomers. Thus, any tools for distinguishing heteromers, homomers and monomers of GPCRs will be valuable. In such way, several experimental approaches using fluorescent ligands have been designed to study purinergic receptor oligomerization. A basic approach consists of measuring the impact of receptor oligomerization on the thermodynamics of fluorescent ligand-receptor binding in a real-time mode. In such way, A_1R/A_3R oligomerization was assessed by monitoring the dissociation kinetics of a fluorescent agonist (**7**) from the A_1R and/or A_3R expressed in CHO cells. Interestingly, the data obtained shed some light about the cooperative interactions taking place among protomers within the oligomer (May et al., 2011, 2010). Thus, these studies provided evidence for an allosteric modulation occurring between orthosteric binding sites of the adenosine receptors across their homodimeric interface, which supported the spatial and temporal specificity of drug action. A more sophisticated experimental approach consists of using FRET compatible fluorescent ligand pairs tagging the different receptors within the oligomer (Fig. 2C). Therefore, if two putative protomers are in close proximity (<10 nm), the binding of the fluorescent ligands to their binding sites may engage a FRET process (Fig. 2C) (Gandia et al., 2008). Interestingly, several studies have taken advantage of the abovementioned approach, and have demonstrated the existence of GPCR oligomers in heterologous expression systems and, more importantly, in native tissue. Importantly, fluorescent ligands are especially valuable to reveal GPCRs in native conditions, since physiologically expressed unmodified receptors can be readily recognized by properly-designed selective fluorescent ligands. In such way, by means of this latter strategy we recently studied the well-known receptor-receptor interaction between $A_{2A}R$ and D_2R occurring at the level of the striatum, a region of major interest in certain pathologies like Parkinson's disease (Fernández-Dueñas et al., 2015). Of note, although $A_{2A}R$-D_2R oligomerization was postulated quite a long time ago based on receptor binding in model

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systems (Ferre et al., 1997; Fuxe et al., 1998; Torvinen et al., 2002), evidence for the existence of $A_{2A}R/D_2R$ oligomers in native tissue was indirect. For instance, these receptors were able to co-immunoprecipitate in rat striatum homogenates, and also co-distribute within striatal neurons (Cabello et al., 2009). In addition, a potential direct receptor-receptor interaction between $A_{2A}R$ and D_2R was suggested in view of the proximity ligation assay results shown in mouse and monkey brains (Bonaventura et al., 2014; Trifilieff et al., 2011). However, the final proof of the close $A_{2A}R$-D_2R proximity (<10 nm) or oligomerization was provided by means of time resolved-FRET (TR-FRET) experiments using specific $A_{2A}R$ and D_2R fluorescent ligands (Fernández-Dueñas et al., 2015). In brief, after validating previous results by means of immunoelectron microscopy and proximity ligation assays, we applied a TR-FRET-based approach using a selective $A_{2A}R$ fluorescent antagonist (**14**) in combination with a D_2R antagonist (*N*-(p-aminophenthyl)spiperone) labelled with Lumi4-Tb. Importantly, the use of antagonists targeting the protomers within the oligomer may provide a higher range of detection signal to that obtained using agonist pairs, as previously reported (Albizu et al., 2010). Indeed, when a GPCR oligomer is challenged with two TR-FRET-compatible fluorescent agonists some negative cooperative binding may appear, which may result in a lower TR-FRET signal (Albizu et al., 2010). However, when a functional output of GPCR oligomerization is needed to assess the existence of receptor-receptor cooperativity phenomena, two TR-FRET-compatible fluorescent agonists are recommended (Durroux, 2005). The latter approach using TR-FRET compatible ligands was remarkable in the respect that one of the ligands was labelled with a lanthanide (i.e. terbium) as a donor in the TR-FRET process, which presents important advantages with respect to classical FRET paradigms. First, the long-lived emission fluorescence of lanthanides allows the temporal separation of excitation and detection events of the energy transfer (Bazin et al., 2001; Mathis, 1995). And second, the signal-to-noise ratio is extremely high, since the bleed-through of lanthanide emission into the acceptor wavelength (i.e. dy647) is negligible (Fig. 1) (Bazin et al., 2002; Terrillon et al., 2003). Overall, the engagement of a TR-FRET process between fluorescent ligands may represent a definitive tool to demonstrate receptor oligomerization in native conditions. However, as commented above, other approaches are possible using fluorescent ligands. In such way, a fluorescent ligand directed to a specific receptor can elicit a FRET process with a fluorescent-labelled antibody directed to the other protomer within the oligomer (Fig. 2C). This strategy was successfully applied to demonstrate the existence of D_2R-ghrelin GHS-R1a receptor oligomerization (Kern et al., 2012). However, the use of antibodies might involve some drawbacks: i) the size of antibodies, which may hinder proper labelling of both receptors due to steric incompatibilities, ii) the fact that most GPCR antibodies are directed to intracellular domains, thus requiring permeabilization processes that disrupt the plasma membrane and alter native conditions. In addition, when using antibodies, the FRET process is indeed less probable depending on the accessibility of the epitope or the orientation of the fluorophores. On the other hand, it may be considered, as commented above, that selecting an agonist instead of an antagonist can diminish the FRET signal, since although no cooperativity may exist with the antibody, receptor agonist-mediated activation may lead to possible internalization, thus hindering binding equilibrium between the ligand and the antibody. In conclusion, GPCR fluorescent ligands in general and purinergic receptor fluorescent ligands in particular have recently emerged as powerful tools to ascertain receptor oligomerization

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in native conditions. Needless to say, especially when thinking of first characterizing new receptor-receptor interactions or screening specific oligomer-based drugs in a straightforward manner, i.e. in heterologous systems, the combination of fluorescent ligands with fluorescent-tagged receptors may represent an advantage that may also permit developing HTS systems. In such way, it was recently described a heterodimeric binding assay, as a proof of concept to screen for oligomer selective ligands (Hounsou et al., 2014). Interestingly, by using this assay it was possible to discriminate between the fluorescent ligand-receptor binding to heterodimers *vs.* homodimers, thus conferring this kind of approach the ability for identifying ligands that exhibit selectivity for heterodimers. This fact, as commented previously, may facilitate the search for new drugs for treating such pathologies (i.e. Parkinson's disease) in which an inbalance occurs between distinct GPCRs forming an oligomer (i.e. $A_{2A}R$-D_2R). Accordingly, this approach might become a useful tool for the characterization of another already described purinergic receptor-containing oligomer, namely the adenosine A_1 and the $P2Y_1$ receptor-receptor interaction described both in heterologously transfected cells and in rat brain tissues (for review see (Nakata et al., 2005)), thus new FRET compatible fluorescent ligands for this pair of purinergic receptors will be needed.

5. Concluding remarks

Since the discovery of the purinergic neurotransmission system by Burnstock in the early seventies, characterizing purinergic receptors has constituted a major goal for neuropharmacologists. Hence, adenosine and P2Y receptors are abundantly expressed through the organism, and they therefore represent relevant targets in a number of pathologies in which the functioning of these receptors has been shown to be altered. The development of fluorescent ligands directed to purinergic receptors has signified an important step, not only to achieve the former objectives, but also in modern GPCR pharmacology. Thus, the number of available GPCR fluorescent ligands is growing rapidly, and purinergic receptors fluorescent ligands have somehow led the progress of this field. Importantly, the synthesis of fluorescent ligands has permitted overcoming technical obstacles found in classical approaches (i.e. radioactivity), thus more detailed knowledge of receptors biology (i.e. ligand binding, receptor oligomerization) may be fulfilled in the near future. These advances are expected to be readily translated into clinical diagnostics and in facilitating the development of novel drugs for the management of GPCR-related pathologies.

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- Fluorescent ligands propel G protein-coupled purinergic receptor pharmacology.
- Dynamic FRET-based ligand-adenosine receptor binding at the surface of living cells.
- Purinergic fluorescent ligands allow visualization of adenosine receptor-containing oligomers in native tissue.

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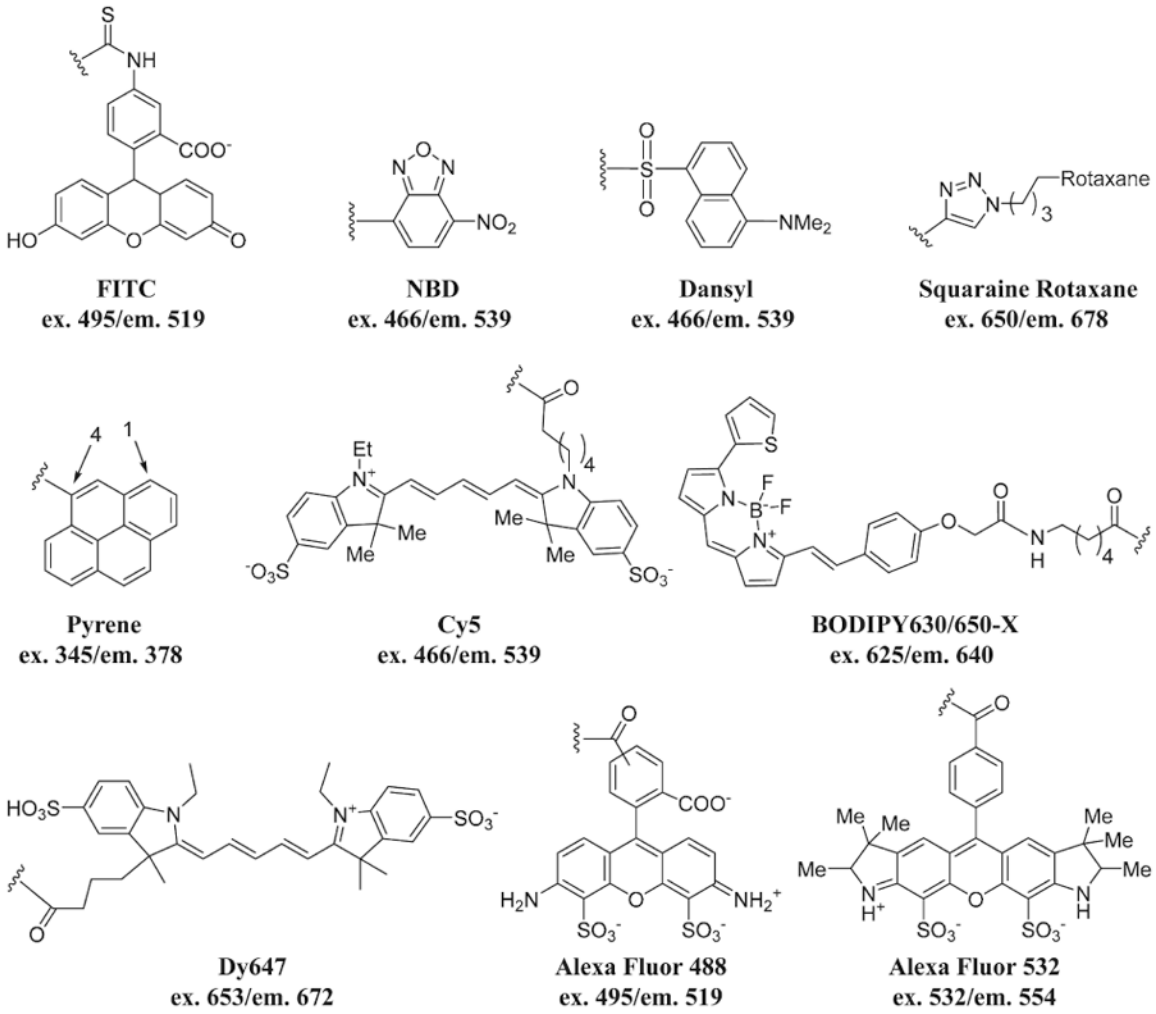

Fig. 1. Structures of fluorophores used to tag purinergic receptor ligands

Abbreviations: FITC: fluorescein isothiocyanate; NBD: 7-nitrobenzofurazan-4-yl;

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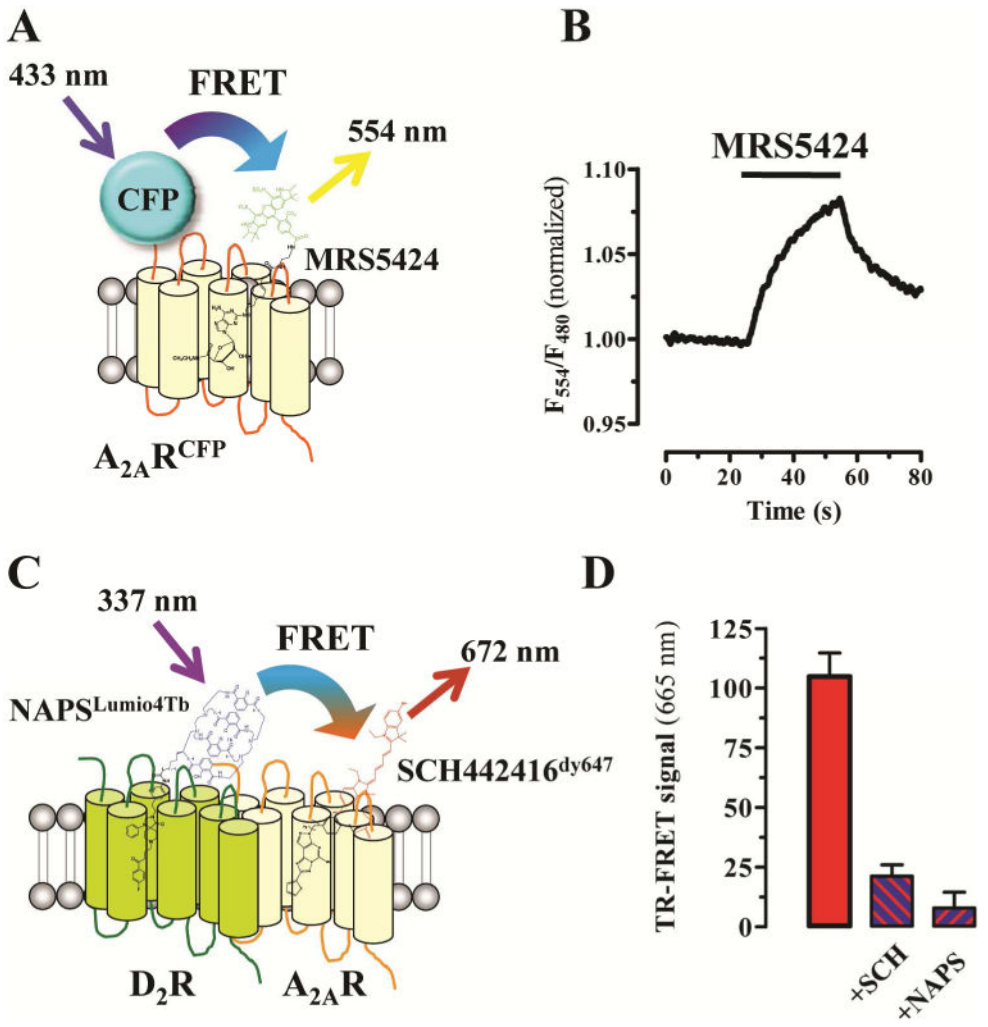

Fig. 2. FRET-based purinergic receptor ligand binding assay in living cells

(A) Schematic representation of FRET between a fluorescent ligand (i.e. MRS5424) and the corresponding fluorescently tagged adenosine receptor (i.e. $A_{2A}R^{CFP}$). (B) Single-cell time-resolved changes in receptor-ligand FRET signal. The recording shows the changes in the F554/F480 ratio upon rapid superfusion of the fluorescent ligand. Adapted from (Fernandez-Duenas et al., 2012). (C) Endogenous receptor oligomers (i.e. $D_2R/A_{2A}R$) in native tissue can be readily detected by TR-FRET compatible specific fluorescent ligands (i.e. $NAPS^{Lumio4Tb}$ and $SCH442416^{dy647}$), which upon binding to their respective receptors (i.e. D_2R and $A_{2A}R$, respectively) within the oligomer can engage in a FRET process. Adapted from (Fernández-Dueñas et al., 2015). (D) Representative example of $D_2R/A_{2A}R$ oligomer detection in membrane preparations from rat striatum incubated with $NAPS^{Lumi4Tb}$ plus $SCH442416^{dy647}$. The corresponding TR-FRET signal (1st column, red) is significantly higher than that observed in the presence of an excess of unlabelled NAPS or SCH442416. Adapted from (Fernández-Dueñas et al., 2015).

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Table 1

Adenosine receptors and representative fluorescent ligands used to label these receptors

Receptor	G protein	Transduction mechanisms[¥]	Physiological actions	Ligand No.
A_1	$G_{i/o}$* $G_{q/11}$ G_s	• Inhibits: AC* • Activates: PLC, AC	Vasoconstriction (Murray and Churchill, 1984); hypothermia and sedation (Anderson et al., 1994); analgesia (Yamamoto et al., 2003); neurotransmitter release (De Lorenzo et al., 2004; Scholz and Miller, 1992); chemotaxis (Schnurr et al., 2004); neuroprotection (MacGregor et al., 1993).	**1** **2** **4** **5** **7**
A_{2A}	G_s* G_{olf} $G_{15,16}$ §	• Activates: AC*, PLC • Inhibits: Ca^{2+} channels	Platelet aggregation inhibition (Varani et al., 2000); vasodilation (Carroll et al., 2006); neurotransmitter release (Popoli et al., 1995); regulation of sensorimotor integration in basal ganglia (Nagel et al., 2003); sleep promotion (Scammell et al., 2001);	**2** **7** **12** **13** **14** **15**
A_{2B}	G_s* $G_{q/11}$	•Activates: AC*, PLC	Vasodilation (Kemp and Cocks, 1999); vasoconstriction (Donoso et al., 2005); cytokine production (Zhong et al., 2004); inhibition of cell proliferation (Dubey et al., 2005);	**2** **7**
A_3	$G_{i/o}$*	• Inhibits: AC* • Activates: PLC	Mast cell activation (Zhong et al., 2003); preconditioning (Das et al., 2005);coronary vasodilation (Hinschen et al., 2003); regulation of intraocular pressure (Avila et al., 2002); hypotension (Stella et al., 1998).	**2** **6** **7** **9** **10** **11** **16**

*Main mechanism of coupling.

¥AC, adenylyl cyclase; PLC, phospholipase C; PLA2, phospholipase A2; PLD, phospholipase D; GIRKs, G protein-dependent inwardly rectifying K^+ channels.

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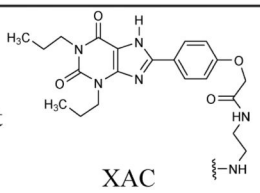
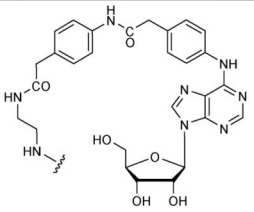
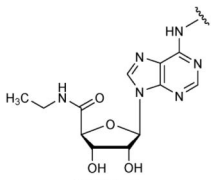
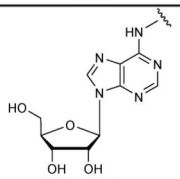
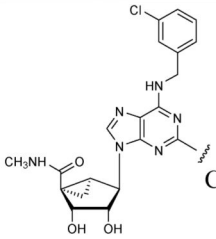
Table 2

Structures of representative fluorescent ligands used to label adenosine receptors

The conjugates shown are structurally divided into three moieties following pharmacological, covalent linking and fluorescent properties. See Fig. 1 for structures of fluorophores and Table 1 for references.

No.	Compound: Name (Ref.)	I.A.	Pharmacophore	Linker	Fluorophore
1	**XAC^{FTIC}** (Jacobson et al., 1987)	Antagonist	 XAC	–	FITC
2	**XAC^{BY630}** (Briddon et al., 2004a)	Antagonist		$(CH_2)_6NH$	BODIPY 630/650-X
3	**ADAC^{FTIC}**	Agonist	 ADAC	–	FITC
4	**ADAC^{NBD}** (Jacobson et al., 1987)	Agonist		–	NBD
5	**NECA^{dansyl}** (Macchia et al., 1998)	Agonist	 NECA	$(CH_2)_6NH$	Dansyl
6	**NECA^{NBD}** (Macchia et al., 2001)	Agonist		$(CH_2)_8NH$	NBD
7	**ABEA^{BY630}** (Middleton et al., 2007)	Agonist		$(CH_2)_4NH$	BODIPY 630/650-X
8	**ABA^{BY630}** (Briddon et al., 2004)	Agonist	 Adenosine	$(CH_2)_4NH$	BODIPY 630/650-X
9	**MRS5243** (Tosh et al., 2010)	Agonist	 MRS3558	$C\equiv C(CH_2)_4$	Squaraine-Rotaxane
10	**MRS5704** (Tosh et al., 2012)	Agonist		$C\equiv C$	1-Pyrene
11	**MRS5218** (Tosh et al., 2009)	Agonist		$C\equiv C(CH_2)_4CONH(CH_2)_2NH$	Cy5

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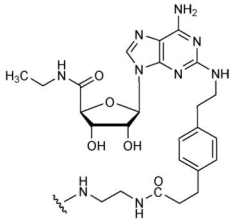
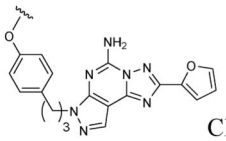
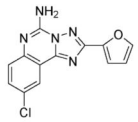
12	**MRS5206** (Brand et al., 2008)	Agonist	 APEC	–	AF488
13	**MRS5424** (Fernández-Dueñas et al., 2012)	Agonist		–	AF532
14	**SCH442416^{dy647}** (Fernández-Dueñas et al., 2015)	Antagonist	 SCH442416	–	dy647
15	**MRS5346** (Kecskés et al., 2010)	Antagonist		$CH_2CONH(CH_2)_2NH$	AF488 5-isomer
16	**MRS5449** (Kozma et al., 2012)	Antagonist	 CGS15943	$CO(CH_2)_3$	AF488 azide 5-isomer

Abbreviations: ADAC: adenosine amine congener; AF Alexa Fluor; APEC: 2-[(2-aminoethylamino)carbonylethyl-phenylethylamino]-5′-ethylcarboxamidoadenosine; CGS15943: 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine; I.A.: intrinsic activity; MRS3558:(1′S, 2′R,3′S,4′R,5′S)-4-(2-chloro-6-(3-chlorobenzylamino)-9*H*-purin-9-yl)-2,3- dihydroxy-N-methylbicyclo [3.1.0] hexane-1-carboxamide; NECA: 5′-N-ethylcarboxamidoadenosine; SCH442416: 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; XAC: xanthine amine congener;

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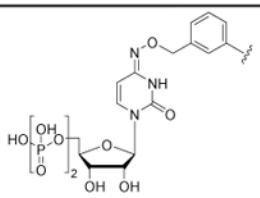
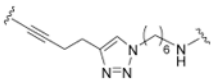
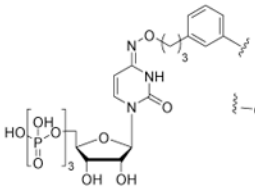
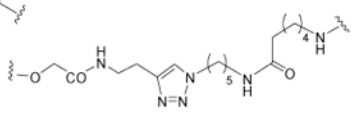
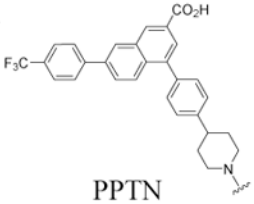
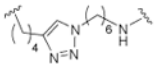
Table 3

Structures of fluorescent ligands used to label P2Y receptors

The conjugates shown are structurally divided into three moieties following pharmacological, covalent linking and fluorescent properties. See Fig. 1 for structures of fluorophores and Table 1 for references.

No.	Compound: Name (Ref.)	I.A.	Pharmacophore	Linker	Fluorophore
17	**MRS4129** (Jayasekara et al., 2013)	Agonist	 MRS2964		AF488
18	**MRS4162** (Jayasekara et al., 2014)	Agonist	 MRS4062		BODIPY 630/650-X
19	**MRS4174** (Kiselev et al., 2014)	Antagonist	 PPTN		AF488

Abbreviations: CDP: Cytidine 5′-diphosphate; MRS2964: N^4-benzyloxy-CDP; MRS4062: N^4-phenylpropoxycytidine-5′-O-triphosphate; PPTN: 4-(4-(piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)- phenyl)-2-naphthoic acid.