BJP British Journal of Pharmacology

DOI:10.1111/bph.12265
www.brjpharmacol.org

Themed Section: Molecular Pharmacology of GPCRs

REVIEW

The evolving small-molecule fluorescent-conjugate toolbox for Class A GPCRs

Andrea J Vernall[1], Stephen J Hill[2] and Barrie Kellam[1]

[1]*School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK, and* [2]*Institute of Cell Signalling, School of Biomedical Science, Queen's Medical Centre, University of Nottingham, Nottingham, UK*

Correspondence
Dr Barrie Kellam, School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK. E-mail: barrie.kellam@nottingham.ac.uk

Keywords
G-protein coupled receptor; fluorescent ligand conjugate; binding; screening; fluorescence microscopy; imaging

Received
9 April 2013
Revised
9 May 2013
Accepted
17 May 2013

The past decade has witnessed fluorescently tagged drug molecules gaining significant attraction in their use as pharmacological tools with which to visualize and interrogate receptor targets at the single-cell level. Additionally, one can generate detailed pharmacological information, such as affinity measurements, down to almost single-molecule detection limits. The now accepted utilization of fluorescence-based readouts in high-throughput/high-content screening provides further evidence that fluorescent molecules offer a safer and more adaptable substitute to radioligands in molecular pharmacology and drug discovery. One such drug-target family that has received considerable attention are the GPCRs; this review therefore summarizes the most recent developments in the area of fluorescent ligand design for this important drug target. We assess recently reported fluorescent conjugates by adopting a receptor-family-based approach, highlighting some of the strengths and weaknesses of the individual molecules and their subsequent use. This review adds further strength to the arguments that fluorescent ligand design and synthesis requires careful planning and execution; providing examples illustrating that selection of the correct fluorescent dye, linker length/composition and geographic attachment point to the drug scaffold can all influence the ultimate selectivity and potency of the final conjugate when compared with its unlabelled precursor. When optimized appropriately, the resultant fluorescent conjugates have been successfully employed in an array of assay formats, including flow cytometry, fluorescence microscopy, FRET and scanning confocal microscopy. It is clear that fluorescently labelled GPCR ligands remain a developing and dynamic research arena.

LINKED ARTICLES
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Introduction

Recent years have witnessed a rapid expansion in the use of fluorescence-based techniques with which to interrogate biological processes and receptors of physiological and pharmacological importance. There are a number of methods by which a fluorescent probe can be generated, including genetic manipulation to label a protein with a fluorophore (Dedecker *et al.*, 2013), measurement of the inherent fluorescence of a compound (Beltran *et al.*, 2011; Burchak *et al.*, 2011) or by using synthetic chemistry to covalently link a biologically active compound to a fluorophore of choice, creating a fluorescent ligand conjugate (Daly and McGrath, 2003). Fluorescent ligands can be designed to interact with different entities, for example, as reaction-based probes, which offer a powerful technique for detecting and studying small molecules and/or metal ions of interest in living systems (Chan *et al.*, 2012). However, the most prevalent use of fluorescent ligands has been the study of protein–protein interactions (Kale *et al.*, 2012) or ligand–receptor interactions (Leopoldo *et al.*, 2009) in biological systems.

One of the most important human receptor families, from a drug discovery and development perspective, is the GPCRs (Alexander *et al.*, 2013). GPCRs are 7-transmembrane spanning receptors, which account for nearly 4% of the protein-encoding human genome (Bjarnadóttir *et al.*, 2006) and are the target of approximately 30% of all marketed drugs (Overington *et al.*, 2006). GPCRs have been classified into five different classes (http://www.gpcr.org/7tm/proteinfamily), of which Class A is the largest and generally regarded as the most understood. GPCRs are signalling powerhouses and can regulate various intracellular biological cascades via the binding of extracellular endogenous ligands, such as peptides, hormones and neurotransmitters. There is significant interest surrounding the development of fluorescent ligands with which to study GPCRs, and research reports of fluorescent GPCR ligands have been previously reviewed (Middleton and Kellam, 2005; Kuder and Kieć-Kononowicz, 2008; Böhme

and Beck-Sickinger, 2009). This article summarizes the small-molecule (non-peptide) fluorescent conjugates for Class A GPCRs that have been reported subsequent to these aforementioned reviews.

Fluorescent ligands are powerful tools to study GPCRs as they can be employed in many varied experiments to reveal insight into receptor structure and function in native, live cells (Briddon *et al.*, 2011). High-affinity fluorescent antagonists can be used to label the target GPCR, and fluorescently tagged agonists can provide a means to monitor dynamic processes such as receptor internalization and trafficking (cf. examples within this review). A fluorescent ligand can be used as the competing probe in a competition-based binding assay (Cottet *et al.*, 2011; Sexton *et al.*, 2011; Stoddart *et al.*, 2012) instead of a radiolabelled ligand, thereby avoiding the inherent safety risks, legal issues and disposal costs associated with the latter. In addition to measuring the direct displacement of a competing fluorescent ligand from a GPCR orthosteric site, fluorescent ligands have enormous potential for revealing elaborate and intricate details about receptor oligomerization through the use of FRET and BRET assays (Albizu *et al.*, 2010; Cottet *et al.*, 2011; 2012). Kinetic measurements of the fluorescent ligand–receptor interaction can reveal insight into receptor allosterism (Hill *et al.*, 2014, this issue) and receptor dimerization (May *et al.*, 2011), while ligand–receptor diffusion times measured using techniques such as fluorescence correlation spectroscopy (FCS) can be used to distinguish different receptor complexes (Briddon and Hill, 2007; Jakobs *et al.*, 2012). This brief precis highlights just some of the possible applications of fluorescent ligands in what is becoming a rapidly expanding field.

Fluorescent ligands for GPCRs

The design of a small-molecule-based fluorescent probe begins with selecting an amenable parent pharmacophore, surveying where to append the linker, determining what linker to use and, lastly, deciding what fluorophore to covalently tether (Jacobson, 2009). The linker position on the parent ligand must be tolerant to chemical change, which is often driven by existing structure activity relationship (SAR) data where available. There is an increasingly diverse range of commercially available fluorophores, and often the commercial fluorophore can be purchased as the pre-activated *N*-hydroxysuccinimidyl (NHS) ester primed for coupling to an amine on the pharmacophore-linker congener. Properties to consider when selecting the appropriate fluorophore to append to a congener include the absorption and emission profile of the fluorophore, lipophilicity (which can influence the conjugate's ability to diffuse across the cell membrane) and whether the fluorophore is quenched in certain environments. From a GPCR imaging perspective, the fluorescent ligand will ideally not enter the cells (unless bound to the internalized receptor), show very low levels of non-specific membrane binding, be quenched when not bound to the receptor and/or cell membrane and, for most applications, be displaceable using higher concentrations of a known non-fluorescent ligand that targets the same receptor. Once assembled, the fluorescent conjugate must be rigorously pharmacologically characterized, as its profile in terms of affinity and/or efficacy may be very different from that of the parent ligand. In the following sections, we review the small-molecule-based fluorescent conjugates that have been developed for Class A receptor families since publication of earlier reviews of this subject area (Middleton and Kellam, 2005; Kuder and Kieć-Kononowicz, 2008; Böhme and Beck-Sickinger, 2009).

Adenosine receptor

The use of fluorescent probes for studying the adenosine receptor has recently been comprehensively reviewed by Kozma *et al.* (2013b). The fluorescent ligand toolbox for the adenosine receptor family is relatively advanced compared with other Class A GPCRs, with many reports of both antagonist and agonist-based probes built around different pharmacophores (predominately for the A_1- and A_3-adenosine receptor subtypes) by the research groups of Jacobson and Hill/Kellam (refer to references within Kozma *et al.*, 2013b). Use of fluorescent antagonists for the adenosine A_1- and A_3-receptors is now at a stage where they can be used in place of radioligand-binding studies for screening purposes. A good example of this is a recent report from our laboratories of high-content screening of a fragment library to identify new synthetic scaffolds for the human A_1- and A_3-adenosine receptor family subtypes (Stoddart *et al.*, 2012). Since the review by Kozma and colleagues, there has been one additional account comparing the pharmacology and imaging properties of three new agonist-based fluorescent adenosine A_3-receptor probes (Kozma *et al.*, 2013a) to five alternatives that had been previously reported (Tosh *et al.*, 2009). The new compounds included an IR dye 700 DX conjugate (**1**) linked through the C2 position of the adenine nucleoside ring, and two *N*6-linked Alexa Fluor 488 probes (**2**) and (**3**) synthesized by click-coupling between an azide and alkyne. The three novel conjugates unfortunately displayed a weaker adenosine A_3-receptor potency when compared with the originally reported fluorescent ligands, and therefore, the authors proceeded with imaging studies using the previously reported Cy5-containing MRS5218 (Tosh *et al.*, 2009) as their first-choice fluorescent probe to visualize and study both the human and the mouse A_3-receptor.

Adrenoceptor

Martikkala *et al.* (2009) constructed three europium(III)-labelled probes for the β_2-adrenoceptor by coupling amino pindolol derivatives containing different linker lengths to isothiocyanate-activated europium chelates [the chemical moiety(s) of these chelates were not disclosed]. The compounds with the shortest (**4**) and longest (**5**) linker-length were employed in a competitive time-resolved fluorescence emission-binding assay using the beta-blocker propranolol as the model drug. IC_{50} values of 60 and 37 nM for the human β_2-adrenoceptor were obtained for propranolol using (**4**) and (**5**), respectively, compared to a value of 33 nM calculated from a [^{3}H]dihydroalprenolol radioligand displacement assay. It was interesting to note that an intermediate linker-length europium conjugate (**5** minus one of the heptanamide units) could not displace propranolol from the β_2-adrenoceptor. This is intriguing, as one might expect the structure–activity relationship trend of linker length to be consistent, up to a

point, in any one direction. This demonstrates that a fluorescent conjugate can possess a complex, unique and often unpredictable pharmacological profile compared with the parent pharmacophore.

In a comprehensive study from our laboratories, a series of red-fluorescent β-adrenoceptor ligands were synthesized based on three different orthosteric β-antagonist head groups; namely propranolol, alprenolol and pindolol (Baker *et al.*, 2011). Using alkyl- or polyether-based linker extensions, the resultant propranolol (**6**) and alprenolol-based (**7**) fluorescent β-blockers displayed high affinity for the human β_2-adrenoceptor. This study provided a further example of how subtle changes in the structural nature of the linker can exert a significant impact on the final conjugate's pharmacology. The 8-carbon linker analogue of **6**, where the 'PEG-like' linker was replaced with a hydrocarbon chain, showed a 10-fold lower affinity for the β_2-adrenoceptor compared to **6**. In contrast, when the linker of **6** was replaced with a shorter 4-carbon linker, the conjugate's affinity for the β_2-adrenoceptor was comparable to **6**. Conjugate **6** was used to visualize ligand–receptor binding in CHO-β_2 cells expressing the human form of the β_2-adrenoceptor using confocal microscopy, and displayed clear labelling of the membrane-bound receptors at 3 nM. This specific binding could be attenuated by incubation with various concentrations (1–100 nM) of the β_2-selective antagonist ICI 118551. In this study, it was also of interest to note that the pindolol-based fluorescent conjugates showed significant loss of affinity when compared with the native drug molecule. Even with three orthosteric ligands acting upon the equivalent receptor-binding pocket, one cannot therefore assume that installation of a fluorophore onto the analogous position of a congener will afford similar pharmacological outcomes with regard to the final conjugate.

Morishima *et al.* (2010) developed a high-affinity fluorescent probe (**8**) selective for the α_{1A}-adrenoceptor and α_{1L}-adrenoceptor (thought to be an α_{1A}-adrenoceptor phenotype) over the α_{1B}-adrenoceptor and α_{1D}-adrenoceptor subtypes. The α_{1A}-adrenoceptor subtype selective antagonist silodosin, which is used to treat bladder outlet obstruction, was labelled with an Alexa Fluor 488 fluorophore. The authors did not disclose which isomer of the fluorophore was used, and therefore the Alexa Fluor 488 mixture of 5′ and 6′ isomers has been depicted in Table 1. While fluorescent probe **8** displayed a 10-fold reduction in binding affinity across the human adrenoceptor receptor subtypes as compared with silodosin, it retained an α_{1A}-adrenoceptor selectivity profile (100- and 15-fold selective over α_{1B}-adrenoceptor and α_{1D}-adrenoceptor respectively). Fluorescent confocal microscopy demonstrated that **8** localized to the membrane of CHO cells overexpressing the α_{1A}-adrenoceptor, and this binding could be significantly reduced using the high-affinity selective antagonist prazosin. Building on this promising result, **8** was used to visualize the α_{1L}-adrenoceptor, an α_{1A}-adrenoceptor phenotype, localized to the muscle layer of the human prostate.

Angiotensin receptor

A fluorescent angiotensin II AT1 receptor (AT_1R) ligand has been reported, derived from a sartan-based pharmacophore (Giarrusso *et al.*, 2012). In place of the thiophene carboxylate moiety of the antagonist milfasartan, Giarrusso *et al.* (2012) instead installed various polyaromatic hydrocarbons or a coumarin fluorophore (e.g. **9**) by reacting an alkyl halide with the pyrimidinone pharmacophore core. The authors remark that polyaromatic conjugates, such as those containing a naphthalene moiety, were clearly unsuitable for *in vivo* use; therefore, coumarin-conjugate **9** was further evaluated as a potential visual AT_1R probe. Functional analysis using CHO cells expressing the rat $AT_{1a}R$ revealed that **9** was an antagonist of the AT_1R, with an estimated pK_b value similar to the native drug. Although the authors state that **9** was a selective ligand for the AT_1R, no pharmacological data were provided for other angiotensin receptors to confirm this statement. The ability of **9** to label the AT_1R was evaluated, but unfortunately, significant accumulation of fluorescence in the cell cytoplasm was observed even with non-AT_1R transfected CHO cells. Development of less lipophilic fluorescent ligands, to eliminate this intracellular localization, is an ongoing work in the authors' laboratory.

Cannabinoid receptor

In a recent report by Sexton *et al.* (2011), two newly designed CB_2 cannabinoid receptor (CB_2R) fluorescent probes (**10**) and (**11**) were compared with the previously reported fluorescent antagonist NIRmbc94 (**12**) (Bai *et al.*, 2008). The purpose of these new conjugates was to examine the influence of linker location around the core of the antagonist. The parent pharmacophore, CB_2R selective antagonist SR144528, lacked an intrinsic biological handle such as an amine or carboxylic acid. Therefore, a 6-(aminohexyl)aminomethyl tether was incorporated in additional positions to that previously reported for **12**, and then coupled to the near-infrared IRDye 800CW-NHS ester. The two new compounds did not demonstrate measurable binding to mouse delayed brain tumour cells that heterologously express the mouse CB_2R; consequently, the fluorescent conjugate of choice remained the previously reported NIRmbc94 (**12**). This study reinforces the importance of identifying a tolerant location on the pharmacophore for linker attachment and, as anticipated, demonstrates that different linker positions can have dramatic effects on final conjugate pharmacology. NIRmbc94 (**12**) was subsequently used as the competing probe in a competition-binding assay (Sexton *et al.*, 2011), and this methodology was further elaborated by screening a small compound library to reliably identify known CB_2R binders. The authors then went on to demonstrate that NIRmcb94 can identify endogenously expressed CB_2R in a mouse microglia cell line, BV-2.

Instead of the more common approach of conjugating a known, discrete orthosteric ligand via a linker to a fluorophore, the fluorescent moiety can instead be designed as part of the primary 'pharmacophore' scaffold with rational receptor–ligand interactions in mind. This approach was employed by Petrov *et al.* (2011), who constructed isatin acylhydrazone-based antagonist **13** that demonstrated selectivity for the human CB_2R (over human CB_1R). A methoxyisatin derivative was linked to a 7-nitro-2,1,3-benzoxadiazole (NBD) fluorophore to afford **13**, which, although displaying slightly reduced affinity to the comparable non-fluorescent compound fragment, retained the desired CB_2R selectivity profile. Using fluorescent confocal microscopy, the association of **13** with T-cells could be visualized, and this interaction could be blocked using a non-fluorescent selective CB_2R

Table 1

Recently reported small-molecule fluorescent conjugates with application to GPCRs

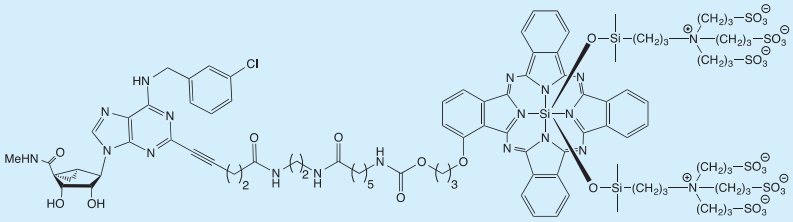
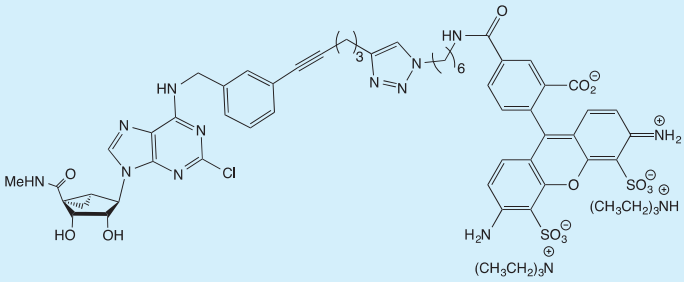
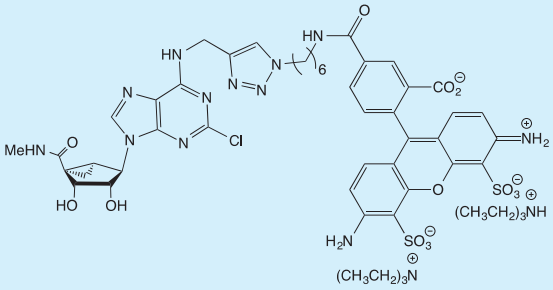
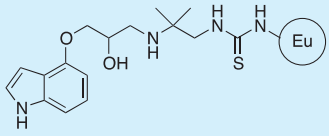
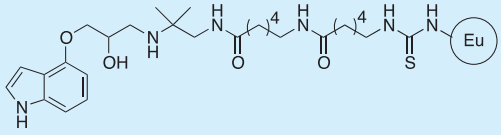
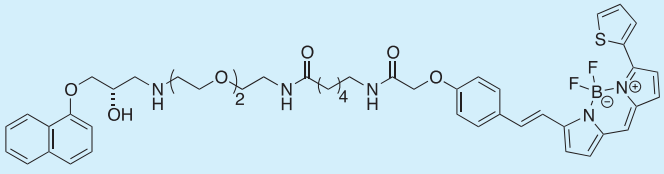
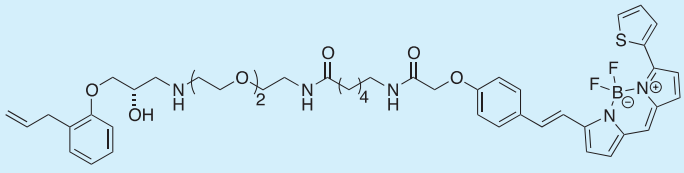
Compound No.	Target receptor	Structure	Reference
1	Adenosine receptor		Kozma *et al.* (2013a)
2			
3			
4	β_2-Adrenoceptor		Martikkala *et al.* (2009)
5			
6	β_2-Adrenoceptor		Baker *et al.* (2011)
7			

Table 1

Continued

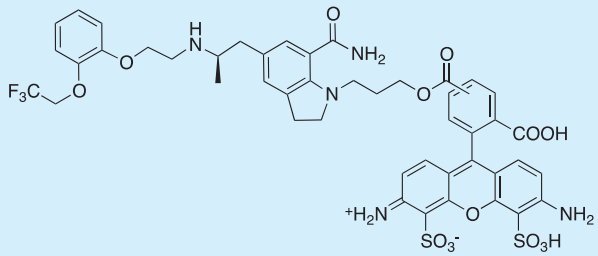
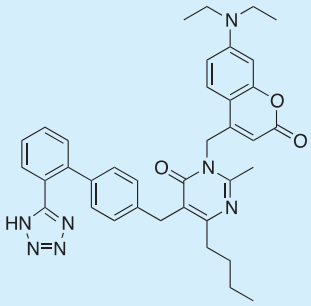
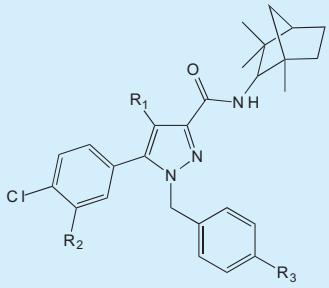
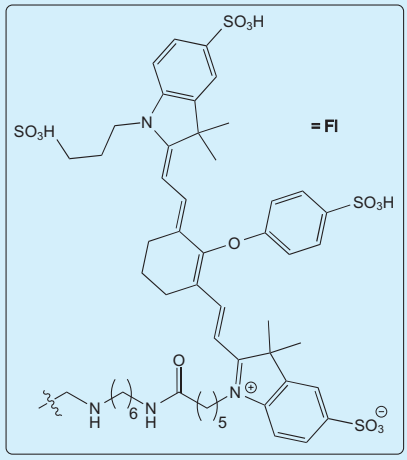
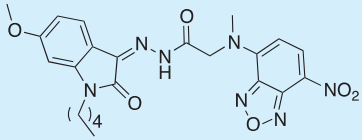
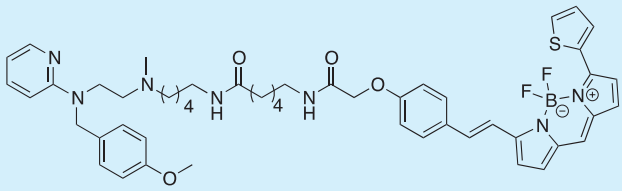
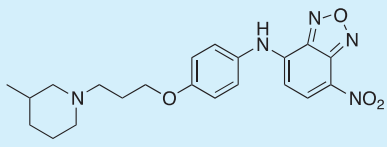
Compound No.	Target receptor	Structure	Reference
8	α_{1A}- and α_{1L}-adrenoceptor		Morishima *et al.* (2010)
9	Angiotensin AT_1 receptor		Giarrusso *et al.* (2012)
10–12	CB_2 cannabinoid receptor	 **10**: R_1 = Fl, R_2 & R_3 = H **11**: R_2 = Fl, R_1 & R_3 = H **12**: R_3 = Fl, R_1 & R_2 = H 	Sexton *et al.* (2011)
13	CB_2 cannabinoid receptor		Petrov *et al.* (2011)
14	Histamine H_1 receptor		Rose *et al.* (2012)
15	Histamine H_3 receptor		Kuder *et al.* (2009)

Table 1

Continued

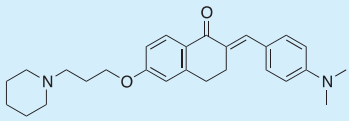
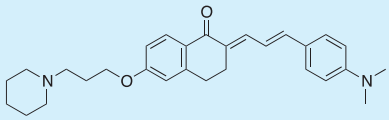
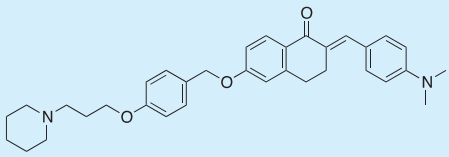
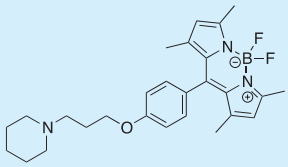
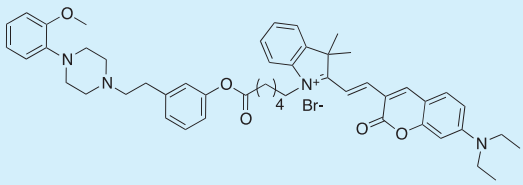
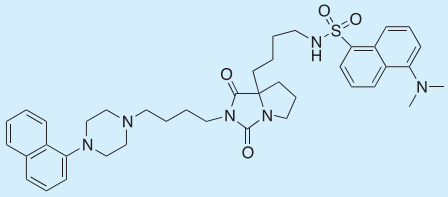
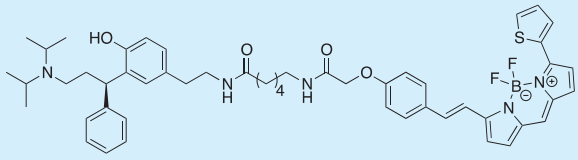
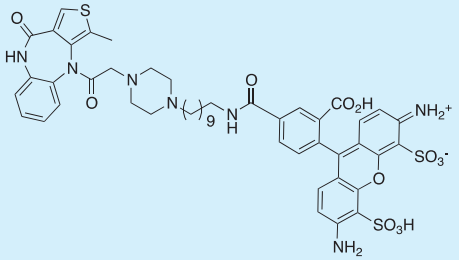
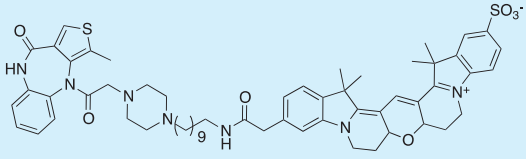
Compound No.	Target receptor	Structure	Reference
16	Histamine H_3 receptor		Tomasch *et al.* (2012c)
17			
18			
19	Histamine H_3 receptor		Tomasch *et al.* (2012b)
20	Serotonin$_{1A}$ receptor (5-HT$_{1A}$)		Lacivita *et al.* (2010)
21	Serotonin$_{1A}$ receptor (5-HT$_{1A}$)		Alonso *et al.* (2010)
22	Muscarinic M_3 receptor		Jones *et al.* (2008)
23	Muscarinic M_1 receptor		Hern *et al.* (2010)
24			

Table 1

Continued

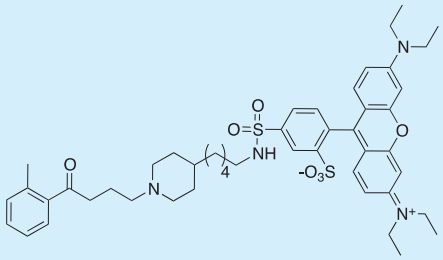
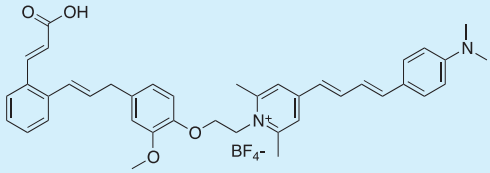
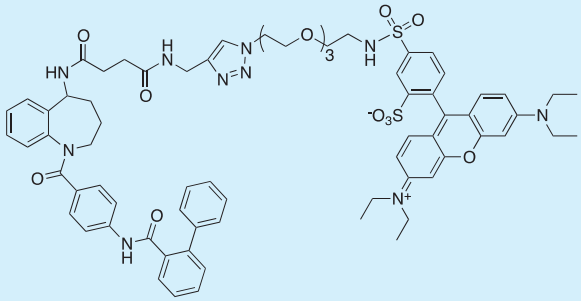
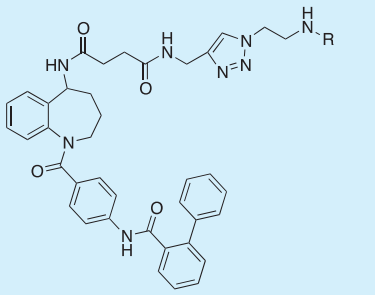
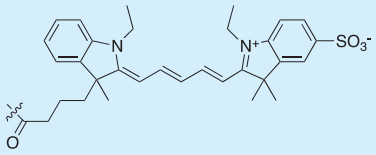
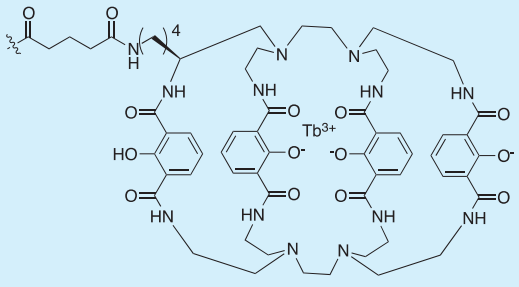
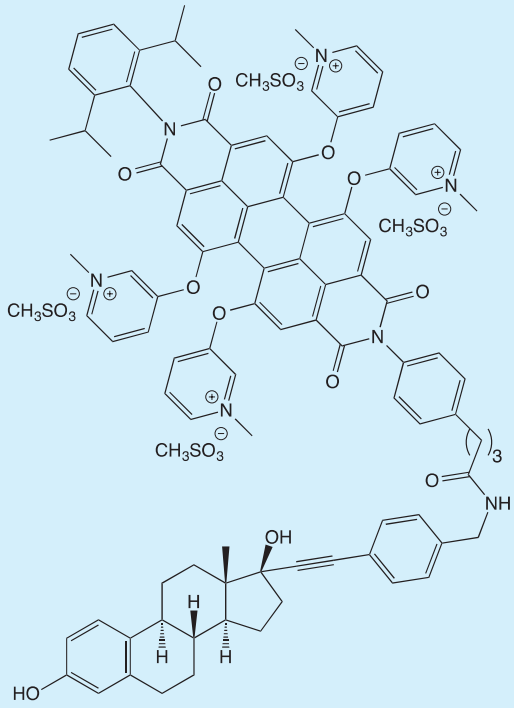
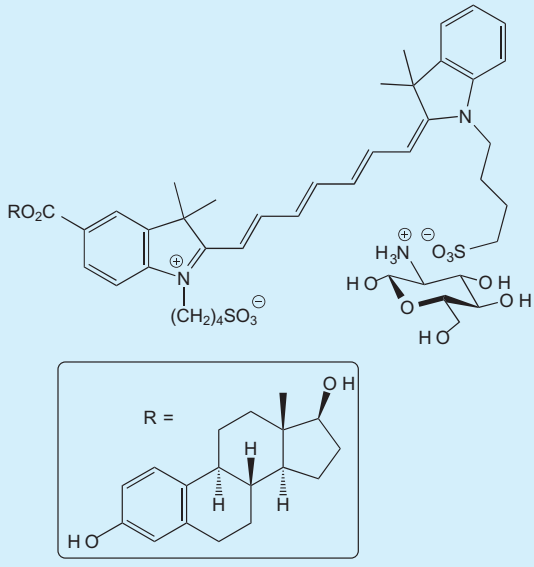
Compound No.	Target receptor	Structure	Reference
25	Muscarinic M_1 receptor		Daval *et al.* (2012)
26	Prostanoid EP_3 receptor		Tomasch *et al.* (2012a)
27	Vasopressin V_2 receptor		Loison *et al.* (2012)
28		 	
29			

Table 1

Continued

Compound No.	Target receptor	Structure	Reference
30	Nuclear oestrogen receptor		Céspedes-Guirao *et al.* (2011)
31	Nuclear oestrogen receptor		Jose *et al.* (2011)

antagonist. Using flow cytometric analysis, the authors also revealed that **13** is associated with the CB_2R in B lymphocytes.

Histamine receptor

A high-affinity fluorescent antagonist (**14**) for the human histamine H_1 receptor (H_1R) has been reported by Rose *et al.* (2012) consisting of the high-affinity and H_1R-selective antagonist mepyramine linked to the BODIPY 630/650 fluorophore. Although fluorescent ligand **14** was purchased from a commercial supplier and not synthesized by the authors in this publication, it represents the first disclosure of the chemical structure of **14**. Conjugate **14** displayed comparable affinity for the H_1R compared with the parent pharmacoph-

ore mepyramine. Confocal microscopy revealed specific and displaceable binding of **14** to the H_1R localized to the cell membrane, and despite significant non-specific intracellular uptake, this probe proved very useful for studying the receptor in single living cells. The diffusion coefficient of **14**-H_1R complexes was quantified using FCS, and these values were different for CHO-K1 cell lines transiently expressing the H_1R with and without the yellow fluorescent protein receptor tag. The authors then developed this further, by showing that FCS experiments using **14** can detect endogenously expressed H_1Rs in HeLa cells.

Kuder *et al*. (2009) have reported **15** as a selective H_3R fluorescent antagonist, which consists of a piperidine-containing pharmacophore (related to the known H_3R selective antagonist pitolisant) linked to a nitrobenzoxadiazole-based fluorophore. The only difference between **15** and a fluorescent probe previously developed by Amon *et al*. (2007) is the presence of a 3-methyl group on the piperidine ring of **15**. Conjugate **15** was twofold less potent for the human H_3R than the previously reported non-methylated derivative (Amon *et al*., 2007), and the authors did not evaluate the selectivity or imaging properties of **15**.

In another report of an H_3R fluorescent ligand, but with a goal of making red-shifted probes, Tomasch *et al*., (2012c) have tethered selective H_3R antagonists, again based on a piperidine moiety, to substituted chalcones. A series of pharmacophore–fluorophore combinations containing different linker positions and lengths were synthesized, and all exhibited nanomolar affinity for the human H_3R and selectivity over the H_1R (one log unit) and H_4R (two log units). The authors then examined the ability of three conjugates (**16**–**18**) to visualize the H_3R in hH_3-HEK-293 cells. Confocal microscopy revealed enrichment of the fluorescent signal to the cell membrane, and the authors conclude that this was specific binding to the H_3R as when HEK-293 cells that do not transiently express the H_3R were treated with the fluorescent ligands, no enriched membrane fluorescence was observed.

From the same laboratory, Tomasch *et al*. (2012b) have used the same piperidine-based pharmacophore but now with the boron-dipyrromethene scaffold as the fluorophore (**19**). Synthesis of the fluorophore moiety was completed by reaction with boron trifluoroetherate as the final reaction in a stepwise synthesis, rather than the more common convergent approach of coupling a pre-activated (and often commercially available as the NHS ester) fluorophore to a complementary pharmacophore/linker. Conjugate **19**, named Bodilisant by the authors, displayed a low nanomolar affinity for the human H_3R that was 10 times more potent that the previous generation (**16**–**18**) of conjugates (Tomasch *et al*., 2012c). Again, H_3R subtype selectivity was maintained. Fluorescence microscopy was used to visualize the human H_3R in H_3-HEK-293 cells and showed that **19** predominately localized to the cell membrane. In these experiments, the authors concluded that fluorescent probe **19** was not internalized, as it did not overlap with the nuclear stain DAPI.

5-Hydroxytryptamine (serotonin) receptor

Lacivita *et al*. (2010) designed and synthesized a fluorescent probe (**20**) for the serotonin$_{1A}$ receptor (5-$HT_{1A}R$) in an effort to improve on previous ligands from the same laboratory that showed high levels of non-specific binding. A chromenone-containing fluorophore was synthesized in-house and coupled to a 1-arylpiperizine-based antagonist, affording a conjugate with nanomolar affinity for the human 5-$HT_{1A}R$; approximately 10-fold less potent than the parent piperizine pharmacophore. The authors then evaluated the ability of **20** to visualize the 5-$HT_{1A}R$, and using a high concentration of **20**, showed fluorescent labelling of CHO-5-HT_{1A} cells that was reduced by application of serotonin. Along with the lead ligand (**20**), a conjugate containing a near-infrared fluorophore was also synthesized, and despite a similar affinity for the 5-$HT_{1A}R$ compared to **20**, the authors commented that due to the loss of fluorescent properties as measured in aqueous buffer, it was not useful as an imaging probe. However, given the location of the 5-$HT_{1A}R$, as with all Class A GPCRs, in the cell membrane, it would be interesting to study the properties of this ligand when bound to the receptor. It can be advantageous to have a fluorescent probe that is quenched in an aqueous environment (Baker *et al*., 2010) but fluoresces when associated with the receptor in a lipophilic membrane environment. For example, in competition-binding assays, this property can eliminate the need for thorough washing steps prior to analysing membrane-localized fluorescence.

Fluorescent 5-$HT_{1A}R$ probes have also been developed by Alonso *et al*. (2010) based on an arylpiperazine agonist previously reported from the same research group. A series of compounds were synthesized using the 7*a*-position of the bicyclohydantoin moiety to tether a dansyl fluorophore. Several conjugates showed an affinity for the human 5-$HT_{1A}R$ that were comparable to the starting arylpiperazine scaffold. Conjugate **21** was identified as the lead fluorescent ligand due to a high fluorescent intensity emission value. Fixed CHO-5-$HT_{1A}R$ cells could be labelled with **21** and this could be blocked using a reference non-fluorescent ligand. Despite being based on a known agonist, only radioligand competition binding assays were carried out to determine the affinity of **21** for the 5-$HT_{1A}R$ – no information was provided regarding how linkage to the dansyl fluorophore influenced ligand efficacy. The authors did not comment on localized membrane fluorescence or the potential of the fluorescent conjugate (if indeed an agonist) to internalize with the receptor. However, the timescale for the visualization experiments was only 10 min pre-incubation followed by a wash, fix and mount process, which may therefore have precluded this from occurring.

Muscarinic receptors

A fluorescent antagonist (**22**) of the muscarinic M_3 receptor (M_3R) has been reported by Jones *et al*. (2008), by linking the non-subtype selective M_3R antagonist tolterodine to the commercially available fluorophore BODIPY 630/650-NHS ester. Conjugate **22** displayed a threefold loss in affinity for the human M_3R compared with tolterodine, and also approximately the same fold-loss in affinity across the other human muscarinic receptor subtypes. The authors did not examine the use of **22** as an imaging tool, but indicated that this was an ongoing work in their laboratory.

Hern *et al*. (2010) have reported **23** as an M_1R probe, synthesized by the reaction of a telenzepine amino congener to an Alexa Fluor 488 fluorophore. Conjugate **23** had a nanomolar affinity for the human M_1R and very slow

dissociation kinetics, making it an ideal tool to visualize and monitor receptor–ligand complexes in living cells. Alongside the previously reported high-affinity M_1R ligand **24** (Harris *et al.*, 2003), total reflection fluorescence microscopy was used to track the position of fluorescent ligand–receptor complexes in live CHO cells expressing the human M_1R. Information about M_1R mobility, clustering and, in particular, dimerization could be obtained by simultaneously using probes **23** and **24** that have different fluorescence emission wavelengths.

Daval *et al.* (2012) synthesized fluorescent M_1R ligand **25** by coupling an agonist (based on AC-42) with high functional selectivity for the M_1R to the fluorophore lissamine rhodamine B sulfonyl chloride. The emphasis in this study was on investigating how the fluorescent conjugate binds to the M_1R, and teasing out possible 'non-orthosteric' binding mode(s) and receptor–ligand interactions. The parent agonist pharmacophore and conjugate **25** displayed a similar affinity towards the human M_1R receptor, but interestingly, **25** could no longer elicit a typical agonist-induced calcium response in a functional assay. Instead, **25** fully reduced the functional response to a known agonist, thereby classifying **25** as an antagonist. In a series of very comprehensive and interesting experiments involving assays with reference allosteric ligands, receptor truncation, molecular modelling and even application of **25** itself as a FRET tracer, the authors concluded that binding of **25** to the M_1R showed a bitopic (Valant *et al.*, 2012) nature. This work further demonstrates the importance of treating the ligand-linker–fluorophore conjugate as a new chemical entity, which can have subtle or quite profound pharmacological differences compared with the starting drug molecule. These differences are often not captured in a single competition-based affinity assay, and there is a fascinating road ahead in terms of rationalizing ligand–receptor interactions of fluorescent conjugates beyond the confines of the orthosteric ligand-binding pocket.

Prostanoid receptor

From the same research laboratory as fluorescent H_3R ligands **16–18** (Tomasch *et al.*, 2012b; 2012c) was the report of a fluorescent prostanoid EP_3 receptor (EP_3R) antagonist (**26**) (Tomasch *et al.*, 2012a). Based on an ortho-substituted cinnamic acid antagonist, a series of fluorescent conjugates were synthesized containing different fluorophores, with pyrylium-containing **26** showing the most promise as an imaging tool. Although with approximately threefold reduced affinity for the human EP_3R compared with the parent drug molecule, **26** maintained selectivity over the EP_1R, EP_2, and EP_4R subtypes. The authors then proceeded to demonstrate that the EP_3R receptor could be visualized using **26** in murine kidney and human brain tissue.

Vasopressin receptor

Loison *et al.* (2012) reported the first examples of selective, fluorescent, non-peptidic ligands for the vasopressin V_2 receptor (V_2R) based on a tetrahydro-1*H*-benzo[*b*]azepine antagonist. A series of conjugates containing different linker lengths and fluorophores were synthesized, and from these, three lead compounds (**27**–**29**) were identified. Interestingly, although compounds **27**–**29** displayed a slight decrease in affinity for the human V_2R compared with the parent ligand, these fluorescent conjugates were comparatively more selective for the V_2R over the $V_{1A}R$ and oxytocin receptor. Cyanine probe **28** and terbium-containing **29** (the nature of the linker housed within the fluorophore is not represented, it has been presumed this is the commercially available Lumi4-Tb-NHS ester as stated in the publication) were then used to develop an acceptor/donor V_2R TR-FRET-based assay and utilized as a tool to study V_2R-$V_{1A}R$ dimerization in association with a previously reported $V_{1A}R$ probe (Albizu *et al.*, 2010).

Other fluorescent Class A GPCR ligands

In addition to fluorescent probes specifically designed to target Class A GPCRs there have been recent reports of fluorescent ligands for alternative receptors, but which one would predict might also bind to Class A GPCRs. There are two recent reports (Céspedes-Guirao *et al.*, 2011; Jose *et al.*, 2011) outlining the synthesis and application of fluorescent oestradiol-based probes (**30**) and (**31**) to target the nuclear hormone oestrogen receptor (ER). Although the ER is not classified as a GPCR, the other type of oestrogen receptor, GPER (or GPR30), is classified as a Class A GPCR. Oestradiol is a high-affinity ligand for both the ER and the GPER; therefore, oestradiol-based conjugates developed for the ER may well be useful tools for studying GPER. In addition to the GPCR families covered in this review, there continues to be an interest in developing small-molecule fluorescent probes for the dopamine receptor, another Class A GPCR, although there have been no new reports published of novel ligands since the preceding review articles (Kuder and Kieć-Kononowicz, 2008; Böhme and Beck-Sickinger, 2009). Another Class A GPCR for which developing fluorescent ligands is an exciting prospect is the opioid receptor, but since the last GPCR fluorescent ligand reviews, there have only been reports of peptide, or peptide-based ligands conjugated to a fluorophore (e.g. Josan *et al.*, 2009), and is therefore outside the scope of this review article. In addition to the novel fluorescent conjugates reported via peer-reviewed publications that have been discussed in this review, there are a growing number of fluorescent conjugates available commercially, with and without the chemical structure disclosed. In an excellent example that showcased the power of using several commercially available fluorescent probes that emit at different wavelengths, Daly *et al.* (2010) investigated the distribution of adrenoceptors and 'cannabinoid-like' receptors in different cell types.

Conclusion

While fluorescent ligands boast over a 30-year pedigree in their application for the study of cellular receptors, it remains clear from all reported research activity within the past decade (including the more recent developments highlighted in this review) that their usage is indeed escalating. This has been accompanied by advances in microscopy and live cell imaging, which have made it possible to visualize and measure the receptor life cycle. One such receptor family class that has benefitted immensely from fluorescent ligand development is GPCRs. The fluorescent conjugates described

within this review have helped to crystallize many of the key factors for consideration when appending a fluorophore to a relatively small orthosteric drug molecule. The linker that connects the fluorescent moiety to the drug of interest must be attached to a position that is relatively insensitive to structural modification and that can potentially tolerate bulky substituents. In order to attenuate non-specific binding of the fluorescent-drug conjugate, one must also be mindful of the final physicochemical properties of the molecule. Finally, when using the generated probes for cellular-based imaging studies or certain types of assay, it has become clearer that judicious choice of the correct fluorophore is also of paramount importance. The work summarized in this and previous reviews unequivocally substantiates that if all these considerations are taken into account, the resulting fluorescent conjugates are extremely powerful pharmacological tools that can be utilized in numerous cutting-edge assay formats. An interesting road lies ahead for all researchers in this area in attempting to rationalize the ligand–receptor interactions of fluorescent conjugates beyond the confines of the orthosteric ligand-binding pocket. In so-doing though, this will undoubtedly help cement the use of these fluorescent reagents as versatile and extremely useful tools for modern-day molecular pharmacology and drug discovery.

Acknowledgements

Work in Barrie Kellam's and Steve Hill's laboratories related to this review was supported by the Medical Research Council (Grant No. G0800006), the BBSRC (Grant No. BB/0521581/1) and the University of Nottingham.

Conflicts of interest

The authors declare the following competing financial interest(s): B.K and S.J.H. are founding directors of the University of Nottingham spin-off company CellAura Technologies Ltd.

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