ELSEVIER

Developments in fluorescent probes for receptor research

Marcello Leopoldo, Enza Lacivita, Francesco Berardi and Roberto Perrone

Dipartimento Farmaco-Chimico, Università degli Studi di Bari, via Orabona, 4, 70125, Bari, Italy

Early reports on the identification of fluorescent probes for receptors date back to mid-1970s. Fluorescent probes were initially used to visualize molecular targets in an analogous way to the use of fluorescent antibodies but with the same resolution as isotopically labelled ligands. In parallel to the rapid development of techniques, such as fluorescence correlation spectroscopy, multi-photon excitation fluorescence microscopy, fluorescence polarization and *in vivo* fluorescence imaging, fluorescent probes are becoming multifaceted tools in life science. The present review will focus on how the design of fluorescent ligands for receptors has evolved to meet the needs of most recent fluorescence applications.

Introduction

Early reports on the synthesis and biological evaluation of fluorescent receptor probes were published in the1970s. In 1974, Anderson and Cohen [1] reported the labelling, with a fluorescent dye, of alpha-bungarotoxin that was used for visualization of acetyl choline receptors in vertebrate skeletal muscle fibres. In 1977 Atlas and Levitzki [2], while describing high-affinity fluorescent beta-adrenergic blockers, stated that such an approach was complementary to the well-established fluorescent–histochemical technique. The latter method involves the use of antibodies raised against a peptide sequence of the studied receptor. However, the histochemical methods detect the receptor protein but virtually never the ligand-binding site as steric considerations prevent this. For this reason, the correlation between the pharmacology and the distribution of the receptors cannot be easily addressed. In contrast with fluorescent immunoprobes, a fluorescent ligand offers the advantage of retaining the pharmacological profile of the parent unlabelled ligand, thus allowing localization and real time monitoring of processes triggered by ligand–receptor interactions (internalization, trafficking, sequestration and recycling) in living cells. The introduction of fluorescence-based technologies, such as confocal laser scanning microscopy (CLSM) and fluorescence correlation microscopy, have advanced the study of receptor pharmacology, especially G-protein coupled receptors, at the single cell level. In addition, several studies have explored the potential of fluorescent probes to the receptor's ligand-binding site to substitute for radiolabelled ligands in competition assays. This review will focus on how the design of fluorescent ligands for receptors has evolved to meet the needs of the most recent applications in receptor visualization, fluorescence correlation spectroscopy, and fluorescence polarization.

Corresponding author: Leopoldo, M. (leopoldo@farmchim.uniba.it)

Receptor visualization

The design of a fluorescent ligand starts from the selection of the appropriate ligand to be conjugated with the fluorophore. If the ligand is a large peptide, the fluorophore constitutes a relatively small proportion of the overall probe. N- or C-terminus or ε-NH_2 group of lysine residues can be targeted for conjugation, provided that this does not affect the biological activity of the peptide. For example, galanin [3] and insulin [4] retained pharmacological activity after direct N-terminal conjugation with a fluorophore. Conversely, appropriate positional attachment of fluorophores to small molecule ligands is critical to retain both receptor binding affinity and efficacy. For example, Yates *et al.* [5] conjugated the fluorescent dye, nitrobenzodiazole (NBD), directly to the cannabinoid CB_2 receptor agonist, JWH-015, but practically abolished the biological activity of the resulting molecule. The loss of pharmacological activity can be prevented by separating the pharmacophore from the fluorescent moiety through the introduction of a spacer into an area of the molecule that has minimal influence on biological activity. In this respect, the knowledge of

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2009.03.015

structure–activity relationships is crucial to the optimization of probes. For example, Balboni *et al.* [6] described the synthesis, opioid activity profile and fluorescent parameters of a H-Dmt-Tic-Glu-NH_2 analogue. Fluorescein was linked through a pentamethylene spacer at the C-terminus because N-terminus basicity is fundamental for opioid activity. In another case, Bai *et al.* [7] selected the CB_2 receptor inverse agonist, SR144528, as the pharmacophore for the development of a fluorescent probe. This compound did not, however, possess appropriate functional groups to allow facile conjugation, therefore, it was structurally modified to give the conjugable analogue mbc94. The corresponding fluorescent probe NIRmbc94 specifically labelled CB_2-expressing cells (Figure 1a).

Spacer length should ideally result from an optimization process. Only a few studies have, however, reported systematic evaluation of spacer length for the fluorescent probe under study [8,9]. From a survey of the literature, it emerges that spacers are formed by at least six atoms [10,11]. Longer linkers have been used for bulky fluorophores, such as near-infrared fluorophores (NIRF) or quantum dots (QDs) [7,12,13]. The chemical nature of the linker may influence the physicochemical properties of the fluorescent probe. Several reports have suggested that high lipophilicity may be a factor contributing to the non-specific binding of some fluorescent probes [8,14–18]. This issue has been addressed by Kshirsagar *et al.* [19] by the incorporation of a polyglycyl hydrophilic linker in the development of κ-opioid receptor fluorescent

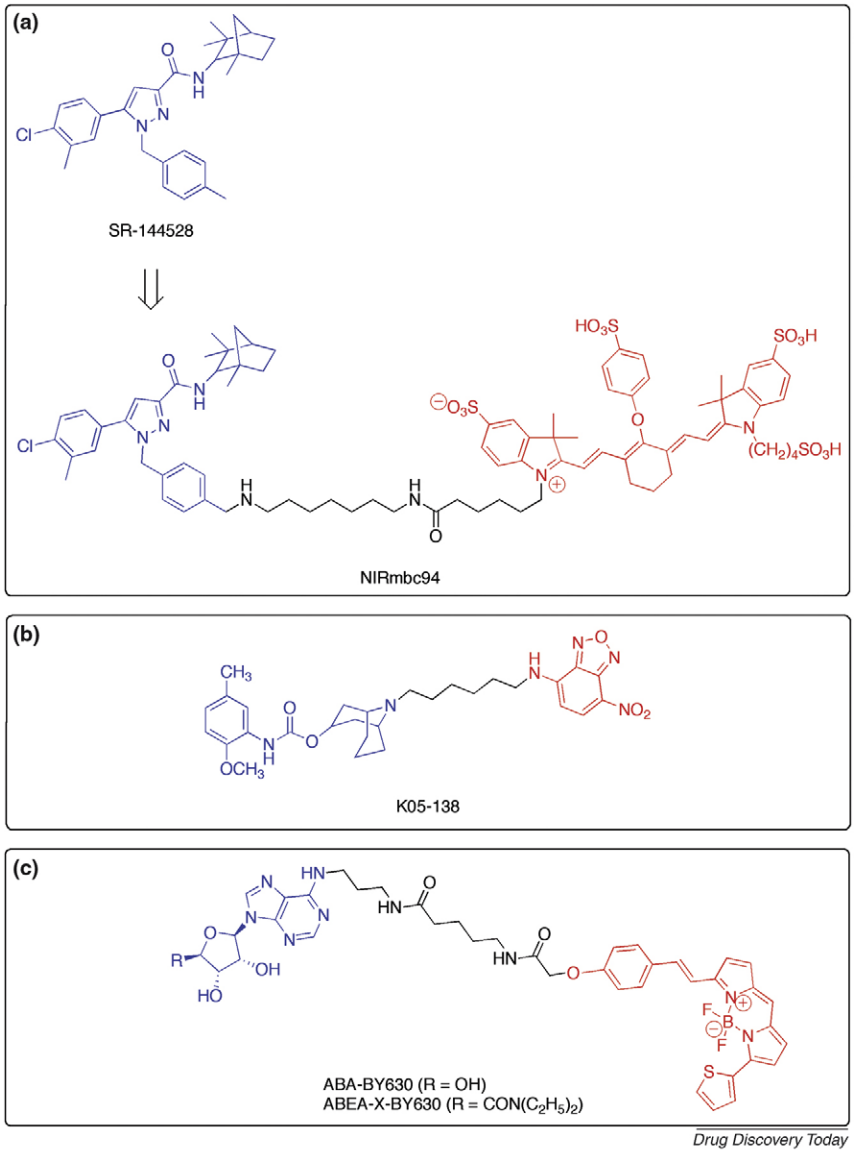

FIGURE 1

Three examples of fluorescent probes successfully used for receptor visualization are depicted. The different parts of probe structures are highlighted with different colors: pharmacophore (blue), linker (black), fluorophore (red). (**a**) The strucutral modification of the CB_2 receptor ligand SR144528 and subsequent development of the corresponding fluorescent probe NIRmbc94; (**b**) the fluorescent probe K05-138 used to visualize sigma-2 receptors by two-photon microscopy; (**c**) chemical structures of fluorescent probes ABA-BY630 and ABEA-X-BY630 that have been used to visualize adenosine receptors A_1 and A_3, respectively.

probes. Similarly, polyethylene glycol linkers have also been reported [12,13,20].

In early studies, fluorescein, coumarin, NBD, dansyl and rhodamine have been extensively used as fluorophores. They suffer, however, from several limitations, including low photostability, pH-sensitivity and excitation and emission wavelengths in the region of the spectrum where cells exhibit high levels of autofluorescence and light scattering. Nonetheless, green emitting fluorophores have permitted the study of receptor trafficking. As example, Dumont *et al.* [21] prepared several BODIPY-TMR neuropeptide Y (NPY) analogues labelled at their N-termini. It was found that each labelled peptide retained similar selectivity, high-affinity and agonist properties for the target NPY receptor subtype as the parent unlabelled peptide. These probes were able to visualize NPY receptors in living HEK293 cells overexpressing the Y_1 or Y_5 receptor. The authors showed that receptor protein expression varied from cell to cell. Furthermore, after 45 min in the presence of fluorescent probe, most of the fluorescent signal was found within the cells, suggesting the internalization of ligand–receptor complexes, in agreement with the intrinsic activity of the probe.

Environment-sensitive fluorophores have attracted attention because of their spectroscopic behaviour, which is dependent on the physicochemical properties of the surrounding environment. Particularly useful are the solvatochromic fluorophores that display sensitivity to the polarity of the local environment such as PRODRAN, 4-dimethylaminophthalimide, 4-amino-1,8-naphthalimide and dansyl. These molecules generally exhibit low quantum yield in aqueous solution but become highly fluorescent in nonpolar solvents or when bound to a hydrophobic site in proteins or membranes [9,22,23].

QDs are nanometric inorganic crystals with special characteristics: high quantum yield, colour availability, good photostability, large surface-to-volume ratio and surface functionality. Thus, QDs possess highly attractive fluorescent properties for visualization techniques [24]. Zhou *et al.* [13] reported recently a practical method for generating water soluble QDs and the necessary chemistry for covalently coupling them to receptor ligands. As a proof of principle, the authors chose to target the QDs to melanocortin and δ-opioid receptors. It was demonstrated that QD514 conjugated to the α-melanocyte-simulating hormone analogues NDP and MT-II allowed effective imaging of melanocortin receptors overexpressed on cells. Cell-surface labelling by NDP-QD514 was observed with almost no background labelling. Similarly, it was demonstrated that QDs conjugated to deltrophin-II analog could selectively image δ-opioid receptors on the cell surface. Moreover, receptor internalization was observed because deltorphin-II (Ile-Ile) is δ-opioid receptor agonist.

Fluorescent probes are becoming attractive tools in the visualization of receptor overexpression in pathology in view of a potential use in *in vivo* optical imaging. Red emitting fluorophores and NIRF possess suitable properties to be imaged deeper in living tissues. This is due to their longer excitations wavelengths, thus low energy incident light requirements, in the range of 680–800 nm. At this end of the spectrum there is much less interaction of light rays with water and other tissue components, which results in less light scattering, reduced background and, therefore, improved sensitivity.

As example Bai *et al.* [7] have described the first NIRF probe that specifically binds cannabinoid CB_2 receptors (NIRmbc94) that had potential use in *in vivo* imaging. CB_2 receptor is an attractive target for the noninvasive imaging of neuroinflammation because its expression is induced in resident microglial cells after cerebral ischemia, injury and in neuroinflammatory diseases.

The endothelin A (ET_A) receptor mediates tumorigenesis and tumor progression by activation of tumor proliferation, invasion, angiogenesis and inhibition of apoptosis. Importantly, increased ET_A receptor expression has been demonstrated in various cancers. Höltke *et al.* [12] labelled the selective ET_A antagonist PD156707 by conjugation with the near-infrared dye Cy 5.5. The fluorescent probe was tested in ET_A receptor-positive MCF-7 human breast adenocarcinoma and HT-1080 human fibrosarcoma cell lines. The signal was specific, distributed over the cell surface and was membrane-associated. These results indicated the probe as a candidate for the imaging of ET_A-overexpressing tissues *in vivo*.

Clinically validated technologies such as magnetic resonance imaging and positron emission tomography can be complemented by *in vivo* optical imaging techniques. On such bases, multimodal imaging probes have been developed during the last year. One example of multimodal imaging probe designed for targeting a receptor overexpressed in tumor tissue (i.e. somatostatin2 receptor) has been reported recently [25]. The high affinity peptide Y3-TATE was conjugated to a radiometal chelating group and to an NIRF (Figure 2). Although the probes displayed high binding

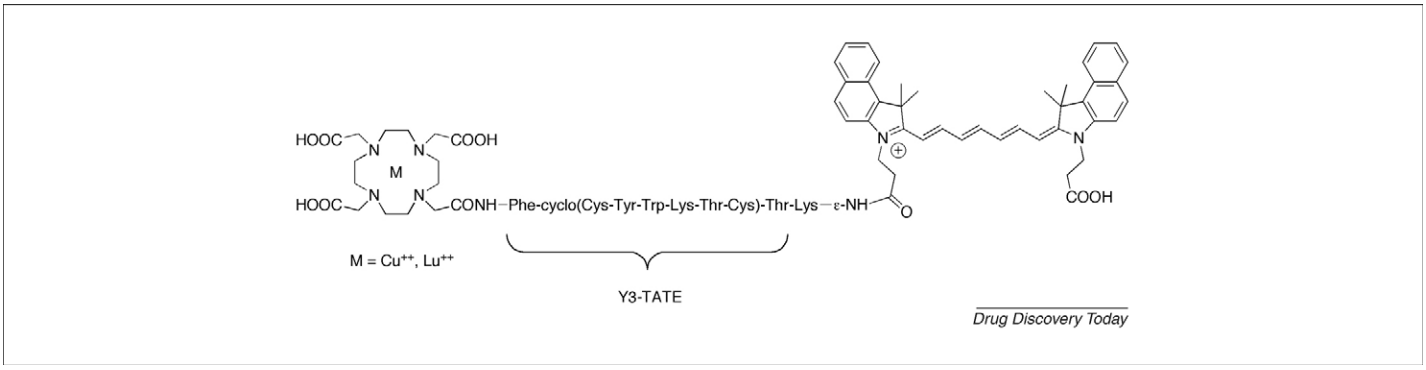

FIGURE 2

A multimodal imaging probe developed for somatostatin2 receptor visualization. The probe is constituted by a radiometal chelating group (left end) linked to the N-terminus of the Y3-TATE peptide. The C-terminus of the peptide was condensed with a lysine which bears on the ε-NH_2 the NIRF dye Cy5 (right end).

affinity for the somatostatin2 receptor *in vitro*, they did not accumulate *in vivo*, in levels consistent with their high affinity and high target expression in tumor xenografts.

One of the newest innovations in fluorescence microscopy is multi-photon fluorescence microscopy [26]. In two-photon microscopy (2PM), the sample is illuminated by a focused beam of light of approximately twice the wavelength (half the photon energy) of the normal excitation light, but of such high intensity that two photons can be absorbed simultaneously and together supply sufficient energy to excite the fluorophore. The necessary high intensity is typically achieved by using an ultrafast-pulsed laser. In 2PM the laser used to excite the fluorophores lies in the near infrared range (700–1000 nm). This translates into: (a) use of near infrared light that allows for deeper penetration into tissues, much less scattering, and absence of deleterious effects of ultraviolet illumination; (b) use of fluorophores not widely employed to conventional CLSM (excitation wavelength 400–500 nm). Near infrared light, however, causes an inherent loss in resolution compared to ultraviolet or visible illumination. Moreover, 2PM dye should have as large absorption cross-section as possible [27].

Two-photon microscopy has been used for real time differentiation of agonists from antagonists of the melanocortin receptor system (MC1-5) through the use of fluorescent probes [28]. In particular, tetramethyl rodamine (Rho) was linked to the N-terminus of the universal melanotropin agonist MTII and to the N-terminus of SHU-9119, which is an antagonist at the MC3 and MC4 receptor subtypes and an agonist at the MC1 and MC5 receptors. It was found that the fluorescent agonist, Rho-MTII, was distributed throughout those cells expressing MC4 receptors, whereas the antagonist Rho-SHU9119 was totally retained on the cell surface. Similar results were observed using the MC1, MC3 and MC5 receptor subtypes. On the other hand, Rho-SHU-9119 was internalized in those cell lines expressing MC1and MC5 receptors, in the same way as the universal agonist MTII.

Zeng *et al.* [29] have described two fluorescent sigma-2 ligands, SW107 and K05-138 (Figure 1b) that were able, selectively, to image by 2PM the subcellular localization of the receptor in the mitochondria, endoplasmic reticulum and in lipid rafts in live cells. The rapid internalization of the fluorescent probes suggested that the sigma-2 receptors internalized via endocytosis. As a result, the authors argued that sigma-2 selective ligands may potentially serve as receptor-mediated probes for delivering cytotoxic agents to solid tumors.

The advent of viable physical concepts for overcoming the limiting role of diffraction has led to readily applicable and widely accessible fluorescence microscopes with nanoscale spatial resolution. Current far-field optical nanoscopy techniques (STED, GSD, RESOLFT among many others) resolve objects that are closer together than the diffraction limit [30,31] (Figure 3).

A recent study elegantly illustrated the potential of STED microscopy in the study of the spatial distribution of nicotinic acetylcholine receptors (AchRs) at the cell surface [32]. The receptors were labelled with Alexa594-α-bungarotoxin or with the anti-AchR monoclonal antibody mAb210, followed by staining with secondary antibodies conjugated to the dye Atto 532. CLSM displayed AChR clusters as diffraction-limited dots of ~200 nm diameter, whereas STED microscopy yielded nanoclusters with a size distribution peaking at ~55 nm. At this resolution, it was shown that cholesterol depletion altered the short and long range organization of AChR nanoclusters at the cell surface, providing information on the supramolecular architecture of the AchRs. It is noteworthy that for STED applications, fluorophores need to be particularly photostable [33,34]. To date, not one fluorescent receptor probe especially designed for STED has been reported.

Fluorescence correlation spectroscopy (FCS)

The technique of FCS is based on measuring fluctuations in fluorescence intensity as fluorescently labelled particles diffuse

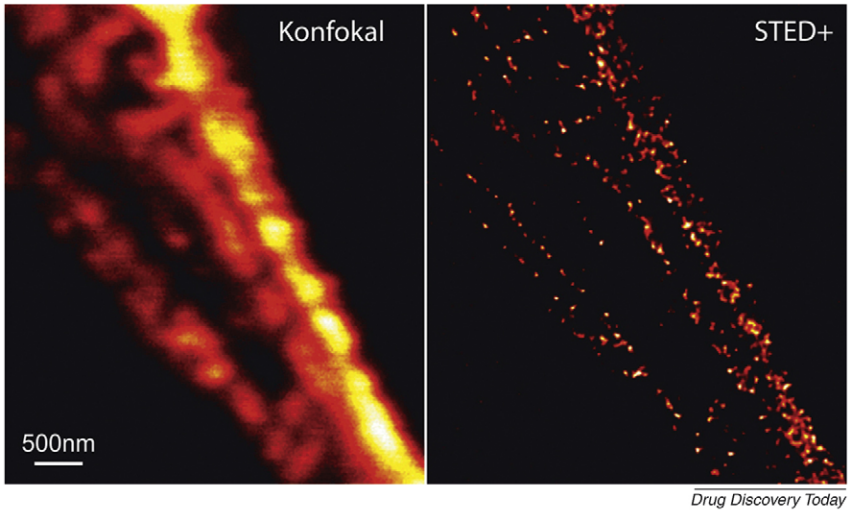

FIGURE 3

Visualization of the filaments in a human nerve cell: (left) with a common confocal microscope, (right) with a STED microscope plus mathematical deconvolution. The resolution of the STED microscope is better by more than an order of magnitude (courtesy: Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany).

through a small defined detection volume, of approximately 0.25 fl. Statistical analysis of these fluctuations using autocorrelation analysis gives information about both the diffusion coefficient of the fluorescent particles (expressed with the related correlation time τ_D) and their concentration. FCS has been applied to the study of ligand–receptor interaction, by virtue of its ability to distinguish between species, based on the speed of their diffusion. A free ligand molecule in the vicinity of the cell membrane will show fast 3D Brownian diffusion, at a rate corresponding to its molecular mass. If that ligand binds subsequently to a slow-moving membrane-localized receptor, its diffusion coefficient will be reduced substantially. Therefore, placing the FCS detection volume on the cell membrane will yield an autocorrelation function composed of a fast-moving component (representing the free ligand diffusion) and one or more slow-moving components (representing the membrane-bound ligand). Subtle and specific differences in receptor binding characteristics between different membrane areas from the same cell can be detected. Conversely, radioligand binding studies require large numbers of cells and thus give affinity and binding capacity values that are representative of the cell population as a whole [35]. In FCS, as fluorescent probes are used in nanomolar concentration, well defined fluorescent properties are required: high molecular brightness; low photobleaching; low percentage of triplet state, preventing any underestimation of diffusion time; insensitivity to solvent polarity and pH changes [8]. For instance, NBD, fluorescein and BODIPY FL are not recommended, because of the fluorescence quenching in aqueous solutions and the low photostability [36]. BODIPY 630/650 fluorophore has been successfully employed also because excitation at 630 nm wavelengths produces much lower levels of cellular autofluorescence than higher energy wavelengths, which may autocorrelate due to excitation of flavins and flavoproteins [37]. Other fluorophores being used in FCS are Alexa Fluor 532 [36] and Cy5 [38].

FCS, in combination with CLSM, can provide pharmacological information from defined subcellular compartments. For example, Middleton *et al.* [8] have reported the identification of fluorescent adenosine A_1 receptor agonists starting from adenosine and from the analogue 5′-*N*-ethyl carboxamide, NECA. Previous structure–activity relationship studies indicated that modifications at the N6-position could be well-tolerated. This allowed the insertion of N6-aminoalkyl spacers of various lengths to yield the corresponding functionalized congeners that were labelled with BODIPY 630/650. Despite a tripling of molecular weight compared to the parent agonist, all of the molecules displayed potent competitive agonism at the A_1 receptor. One of these agonists, that is ABA-BY630 (Figure 1c), was used to visualize, by CLSM, the A_1 receptor on CHO cells [37]. In FCS experiments, ABA-BY630 was used to quantify binding at the subcellular level. Positioning of the detection volume on the upper cell membrane two diffusing species of τ_{D2} = 8 ms and τ_{D3} = 233 ms were detected, in addition to fast-diffusing free ligand. Extracellular and intracellular FCS measurements confirmed that the above species were membrane-localized. Both τ_{D2} and τ_{D3} appeared to represent complexes of ABA-BY630 with the A_1 receptor, because they were both sensitive to the adenosine antagonist DPCPX. It was suggested that the faster component could represent receptor diffusion within a membrane domain, and the slower diffusion represented the movement of the membrane domain itself, in which the receptor resides.

Another fluorescent ligand reported by Middleton *et al.* [8] that is ABEA-X-BY630 (Figure 1c), was characterized as an adenosine A_3 receptor agonist [39]. This red-emitting probe was capable of stimulating inositol phospholipid hydrolysis. In this way, simultaneous measurement of ligand binding and of intracellular calcium levels was monitored in single cells by fluorescent methods. It was found that A_3 receptors were not uniformly distributed between individual cells. The calcium change produced in response to ABEA-X-BY630 was oscillatory in nature and not coordinated between neighbouring cells. The uncoordinated nature of the oscillatory calcium response to A_3 receptor stimulation, pointed to the importance of measurements at the single cell level. Furthermore, it was notable that some 28% of cells bound ABEA-X-BY630, but did not exhibit a calcium response. This suggested that the receptor could exist in different signaling complexes, even within the same cell. Real time visualization of ABEA-X-BY630 binding to cells by CLSM is available online [40].

FCS experiments provided evidence for two agonist-occupied A_3-receptor species with markedly different diffusional characteristics (τ_{D2} 5.9 ms and τ_{D3} 131 ms), with the slow-diffusing species representing the majority (75%) of the total binding of ABEA-X-BY630. In particular, ABEA-X-BY630 showed higher affinity for the slow diffusing species τ_{D3} than for the faster diffusing species τ_{D2}. The authors speculated that, according to the cubic ternary complex model, the slower diffusing species τ_{D3} could represent the agonist-R^* complex (where R^* indicates the activated conformation of the receptor), whereas the faster diffusing species τ_{D2} could be the agonist-R complex (where R is the receptor in resting conformation).

Fluorescence polarization (FP)

Fluorescent ligands provide an attractive alternative to radioligands in receptor studies, circumventing drawbacks associate with radioactivity such as high costs, potential health hazards, and waste disposal problems. Simple fluorescence intensity can be used when the biological interaction generates an increase or a decrease in fluorescence. Several analytical issues hampered the development of intensity-based assays. These assays are usually heterogeneous, requiring a filtration step for the separation of the bound and unbound fraction of fluorescent ligand. Also, intensity assays are relatively sensitive to interference from compound impurities or biological matrices.

FP has emerged as one of the best methods for quantifying molecular interactions in solution, including ligand–receptor interaction because of its homogeneous format, high sensitivity, and flexibility. This technique exploits the observation that fluorophores emit light when excited by plane-polarized light and the polarization of the emitted light depends on how far the fluorophore rotates during the lifetime of its excited state. If the fluorophore tumbles rapidly relative to its fluorescent lifetime then it will be randomly orientated prior to light emission and therefore will show a low polarization value. If, however, the fluorophore rotation is slowed down so that it tumbles slowly with respect to the fluorescent lifetime (e.g. by binding to a large receptor) it will not rotate much before light emission and will show a high polarization value. This change in FP allows highly

accurate determination of the affinities for receptors of both fluorescent small molecules and non-fluorescent small molecules that compete for binding with a fluorescent probe to a macromolecular target. On the other hand, if the fluorescent lifetime is much shorter than fluorophore rotation, the excited molecules will stay aligned during the process of emission and as a result the emission will be polarized even for the unbound fluorescent molecule.

The homogeneous format of FP and the high sensitivity of the fluorescence made this technique particularly attractive for high-throughput screening (HTS), because there is no need for separation of free from bound fluorescent probe. Moreover, polarization is a ratiometric measurement and, thus, the variation caused by fluctuations in lamp intensity or interferences caused by quenching of the fluorescence can be self-corrected [41]. Several fluorescent ligands for FP assays have been developed in the last years. Adamczyk *et al.* [42] described the synthesis and the characterization of a fluorescent probe for the estrogen receptor (ER) prepared by conjugating diethylstilbestrol with fluorescein. This modification did not affect the affinity of diethylstilbestrol for ER and the dynamic range of fluorescence polarization was determined in FP assays. Subsequently, the fluorescent ligand could be competitively displaced by non-fluorescent ligand(s), indicating the possibility of developing a high-throughput screening (HTS) assay for ER. Fluorescein has been used successfully to label PPARγ receptor ligands to be employed in a simple, homogeneous and highly reproducible binding assay [43,44]. It is important to note that the fluorescent probes must be constructed to give maximum polarization change upon binding to the target receptor. For this reason the linker between the fluorophore and the pharmacophore must be as short and rigid as possible. Fluorescein- and rhodamine-conjugated peptides have also been developed for the vasopressin and oxytocin receptors but these fluorescent analogues were not useful for HTS because of their low photostability. The stability of FP signal is important for testing multiple sample plates in a HTS format. Peterson *et al.* developed a HTS FP assay for VEGF receptor by using a BODIPY-conjugated peptide, which retained constant FP signal for 8 h [45]. Moreover, common interferences arising from biological or fluorescent impurities tend to be diminished in assays that employ red-shifted fluorophores instead of green-emitting fluorophores. Researchers at Pfizer prepared and studied a series of fluorescent probes for the h-ERG channel by tagging dofetilide with fluorophores having different spectroscopic properties. A cyanine-labelled probe was selected for further studies in an FP platform on the basis of its h-ERG affinity and molar extinction coefficient. The probe revealed to be suitable for FP assay and, consequently, the assay was adopted globally at Pfizer for the evaluation of compounds in the drug discovery process [46]. Similarly, Albizu *et al.* [47] designed a series of fluorescent ligands for the vasopressin and oxytocin receptors with the aim of developing FP binding assays. The ligands were labelled with various fluorophores (Alexa 488, 546, 647) and were able to bind selectively the target receptors with high affinity. Among these, only the Alexa 488-labelled ligand exhibited good specific polarization signal.

Conclusions

Over the years, information available by use of fluorescent ligands has constantly increased. Initially intended for staining receptors on cell surface, fluorescent probes are becoming powerful tools to study the receptor pharmacology at single cell level. Moreover, the potential of fluorescent probes as diagnostic agents in molecular imaging is actively investigated. It is believable that the value of fluorescent probes for receptors will constantly grow in parallel to fluorescence technology evolution. A 'bright' future for fluorescent probes for receptors is warranted.

Conflict of interest

None of the authors have conflict of interest related to the information described in this paper.

References

1 Anderson, M.J. and Cohen, M.W. (1974) Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J. Physiol.* 237, 385–400

2 Atlas, D. and Levitzki, A. (1977) Probing of beta-adrenergic receptors by novel fluorescent beta-adrenergic blockers. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5290–5294

3 Wang, S. *et al.* (1998) Evidence for hydrophobic interaction between galanin and the GalR1 galanin receptor and GalR1-mediated ligand internalization: fluorescent probing with a fluorescein-galanin. *Biochemistry* 37, 9528–9535

4 Zhong, Z.H. *et al.* (2001) Insulin binding monitored by fluorescence correlation spectroscopy. *Diabetologia* 44, 1184–1188

5 Yates, A.S. *et al.* (2005) Chemical modification of the naphthoyl 3-position of JWH-015:In search of a fluorescent probe to the cannabinoid CB2 receptor. *Bioorg. Med. Chem.* 15, 3758–3762

6 Balboni, G. *et al.* (2004) Highly selective fluorescent analogue of the potent delta-opioid receptor antagonist Dmt-Tic. *J. Med. Chem.* 47, 6541–6546

7 Bai, M. *et al.* (2008) MBC94, a conjugable ligand for cannabinoid CB2 receptor imaging. *Bioconjug. Chem.* 19, 988–992

8 Middleton, R.J. *et al.* (2007) New fluorescent adenosine A1-receptor agonists that allow quantification of ligand–receptor interactions in microdomains of single living cells. *J. Med. Chem.* 50, 782–793

9 Berque-Bestel, I. *et al.* (2003) Synthesis and characterization of the first fluorescent antagonists for human 5-HT4 receptors. *J. Med. Chem.* 46, 2606–2620

10 Kuder, K. and Kieć-Kononowicz, K. (2008) Fluorescent GPCR ligands as new tools in pharmacology. *Curr. Med. Chem.* 15, 2132–2143

11 Middleton, R.J. and Kellam, B. (2005) Fluorophore-tagged GPCR ligands. *Curr. Opin. Chem. Biol.* 9, 517–525

12 Höltke, C. *et al.* (2007) A fluorescent photoprobe for the imaging of endothelin receptors. *Bioconjug. Chem.* 18, 685–694

13 Zhou, M. *et al.* (2007) Peptide-labeled quantum dots for imaging GPCRs in whole cells and as single molecules. *Bioconj. Chem.* 18, 323–332

14 Correa, F.M. *et al.* (1980) Fluorescent probes of alpha- and beta-adrenergic and opiate receptors: biochemical and histochemical evaluation. *Neurosci. Lett.* 16, 47–53

15 Kolb, V.M. *et al.* (1983) Fluorescent probes for opioid receptors. *Life Sci.* 33, 423–426

16 Kolb, V.M. *et al.* (1985) Synthesis and pharmacological characterization of fluorescent opioid receptor probes. *Pharm. Res.* 2, 266–271

17 Koman, A. *et al.* (1986) Prolonged receptor blockade by opioid receptor probes. *Pharm. Res.* 3, 56–60

18 Korlipara, V. *et al.* (1997) Fluorescent *N*-benzylnatrindole analogues as potential opioid receptor probes. *Eur. J. Med. Chem.* 32, 171–174

19 Kshirsagar, T. *et al.* (1998) NTI4F: a non-peptide fluorescent probe selective for functional delta opioid receptors. *Neurosci. Lett.* 249, 83–86

20 Tahtaoui, C. *et al.* (2004) Fluorescent pirenzepine derivatives as potential bitopic ligands of the human M1 muscarinic receptor. *J. Med. Chem.* 47, 4300–4315

21 Dumont, Y. *et al.* (2005) BODIPY®-conjugated neuropeptide Y ligands: new fluorescent tools to tag Y1, Y2, Y4 and Y5 receptor subtypes. *Br. J. Pharm.* 146, 1069–1081

22 Vázquez, M.E. *et al.* (2006) 6-*N*,*N*-dimethylamino-2, 3-naphthalimide: a new environment-sensitive fluorescent probe in delta- and mu-selective opioid peptides. *J. Med. Chem.* 49, 3653–3658

23 Krieger, F. *et al.* (2008) Fluorescent agonists for the Torpedo nicotinic acetylcholine receptor. *Chembiochem* 9, 1146–11146

24 Jaiswal, J.K. and Simon, S.M. (2004) Potentials and pitfalls of fluorescent quantum dots for biological imaging. *Trends Cell Biol.* 14, 497–504

25 Edwards, B.W. *et al.* (2008) Agonist-antagonist dilemma in molecular imaging: evaluation of a monomolecular multimodal imaging agent for the somatostatin receptor. *Bioconjug. Chem.* 19, 192–200

26 Oheim, M. *et al.* (2006) Principles of two-photon excitation fluorescence microscopy and other nonlinear imaging approaches. *Adv. Drug Deliv. Rev.* 58, 767–769 by stimulated emission depletion microscopy. *Neuroscience*, **144**, 135–143

27 Xu, C. and Webb, W.W. (1996) Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J. Opt. Soc. Am. B* 13, 481–491

28 Cai, M. *et al.* (2004) Real time differentiation of G-protein coupled receptor (GPCR) agonist and antagonist by two photon fluorescence laser microscopy. *J. Am. Chem. Soc.* 126, 7160–7161

29 Zeng, C. *et al.* (2007) Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy. *Cancer Res.* 67, 6708–6716

30 Hell, S.W. (2003) Toward fluorescence nanoscopy. *Nat. Biotechnol.* 21, 1347–1355

31 Hell, S.W. (2009) Microscopy and its focal switch. *Nat. Methods* 6, 24–32

32 Kellner, R.R. *et al.* (2007) Nanoscale organization of nicotinic acetylcholine receptors revealed by stimulated emission depletion microscopy. *Neuroscience* 144, 135–143

33 Anon., . http://www.mpibpc.mpg.de/groups/hell/STED_Dyes.html

34 Boyarskiy, V.P. *et al.* (2008) Photostable, amino reactive and water-soluble fluorescent labels based on sulfonated rhodamine with a rigidized xanthene fragment. *Chemistry* 14, 1784–1792

35 Briddon, S.J. and Hill, S.J. (2007) Pharmacology under the microscope: the use of fluorescence correlation spectroscopy to determine the properties of ligand–receptor complexes. *Trends Pharmacol. Sci.* 28, 637–645

36 Hegener, O. *et al.* (2004) Dye-labeled benzodiazepines: development of small ligands for receptor binding studies using fluorescence correlation spectroscopy. *J. Med. Chem.* 47, 3600–3605

37 Briddon, S.J. *et al.* (2004) Application of fluorescence correlation spectroscopy to the measurement of agonist binding to a G-protein coupled receptor at the single cell level. *Faraday Discuss.* 126, 197–207

38 Pick, H. *et al.* (2003) Monitoring expression and clustering of the ionotropic $5HT_3$ receptor in plasma membranes of live biological cells. *Biochemistry* 42, 877–884

39 Cordeaux, Y. *et al.* (2008) Agonist-occupied A_3 adenosine receptors exist within heterogeneous complexes in membrane microdomains of individual living cells. *FASEB J.* 22, 850–860

40 Anon., . http://www.cellaura.com/page/what-cellAura-do/index.htm

41 Sportsman, J.R. and Leytes, L.J. (2000) Miniaturization of homogeneous assays using fluorescence polarization. *Drug Discov. Today: HTS Suppl.* 1, 27–32

42 Adamczyk, M. *et al.* (2002) Synthesis of a novel fluorescent probe for estrogen receptor. *Bioorg. Med. Chem. Lett.* 6, 1283–1285

43 DeGrazia, M.J. *et al.* (2003) Synthesis of a high-affinity fluorescent PPARγ ligand for high-throughput fluorescence polarization assays. *Bioorg. Med. Chem.* 11, 4325–4332

44 Ramakrishna, S. *et al.* (2007) A rapid, homogeneous, fluorescence polarization binding assay for peroxisome proliferator-activated receptors alpha and gamma using a fluorescein-tagged dual PPARα/γ activator. *Anal. Biochem.* 363, 263–274

45 Peterson, K.J. *et al.* (2008) A fluorescence polarization assay for identifying ligands that bind to vascularendothelial growth factor. *Anal. Biochem.* 378, 8–14

46 Singleton, D.H. *et al.* (2007) Fluorescently labeled analogues of dofetilide as high-affinity fluorescence polarization ligands for the human ether-a-go-go-related gene (hERG) channel. *J. Med. Chem.* 50, 2931–2941

47 Albizu, L. *et al.* (2007) Toward efficient drug screening by homogeneous assays based on the development of new fluorescent vasopressin and oxytocin receptor ligands. *J. Med. Chem.* 50, 4976–4985