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Development and Biological Applications of Fluorescent Opioid Ligands

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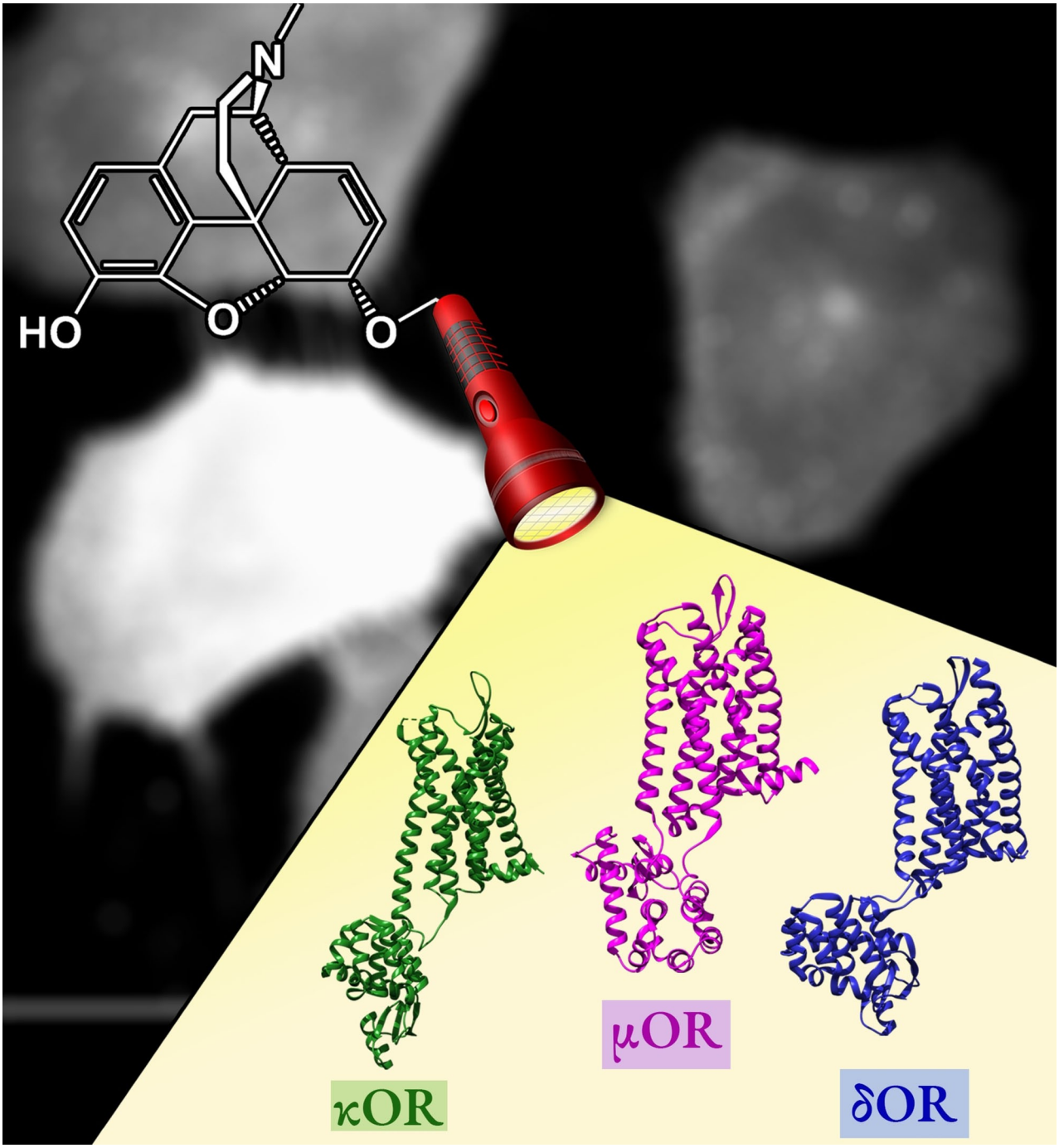
Dedicated to Professor John L. Neumeyer on the occasion of his 90th birthday



Opioid receptors (ORs) are classified among the oldest and best investigated drug targets due to their fundamental role in the treatment of pain and related disorders. ORs are divided in three conventional subtypes (μ, κ, δ) and the non-classical nociceptin receptor. All ORs are family A G protein-coupled receptors (GPCRs), and are located on the cell surface. Modern biophysical methods use light to investigate physiological processes at organismal, cellular and subcellular level. Many of these methods rely on fluorescent ligands, thus highlighting their importance. This review addresses the advancements in the development of opioid fluorescent ligands and their use in biological, pharmacological and imaging applications.

1. Introduction

Opium is a natural product medicament processed from the dried latex obtained from the unripe seed pods of the plant *Papaver somniferum L. Papaveraceae*. The most highlighted pharmacological properties of opium include analgesia, sedation, euphoria and respiratory depression. In addition, opium is highly addictive, leading to tolerance and dependence, which is associated with abuse and increased mortality.[1–4] Opium has been used for therapeutic and recreational purposes since the Neolithic Age.[5,6] Despite their severe side effects, opioids remain one of the most commonly prescribed pain relievers and broad spectrum analgesics.[7,8]

The pharmacological actions of opioids are produced by their binding to specific receptors which are located at the surface of neurons. Opioid receptors (ORs)[9] are divided in three conventional subtypes: μ, δ, κ[10–13] and the nociceptin receptor[14]. Their genes were identified and cloned during the 1990s[9], revealing a sequence similarity of 60–70%, while their structures were recently elucidated through crystallography.[15–20] All opioid receptor subtypes are G protein-coupled receptors (GPCRs); as such they are situated in the cell membrane and consist of an extracellular N-terminus, seven transmembrane (TM) domains, three extracellular loops, three intracellular loops and an intracellular C-terminus. They belong to the family A (Rhodopsin family) of GPCRs and interact mainly with G proteins of the G_i/G_o family.[21,22]

Since the discovery of morphine as the main active constituent of opium by Friedrich Wilhelm Adam Sertürner in the early 1800s, several synthetic opioid analogs have been developed, some of which are hundred or thousand times more potent than morphine. Furthermore, partial agonists and antagonists were discovered, as well as compounds which are more suitable than morphine for oral administration and/or have distinct pharmacokinetic profiles, with duration of action that ranges from short- to long-term. Prime examples include oxymorphone, etorphine as well as the opioid antagonists naloxone, naltrexone and naltrindole (Figure 1). Groups of endogenous opioid peptides with subtype specificity were discovered in the 1970s, i.e. enkephalins, endorphins, endomorphins, dynorphins and nociceptin. In addition to opioid peptides produced in the human body, there are opioid peptides produced in other organisms named exorphins, i.e. exogenous opioid peptides. These are opioid peptides which derive from proteins of exogenous origin, such as milk or plants.

2. An overview on the biological applications of fluorescent probes

In its early stages, the development of fluorescent ligands for GPCRs was aiming towards exploring receptor localization in the body as a means to identify the binding targets of pharmacologically active compounds (as potential alternatives to radioligands). Increased fluorescence and permeability of the bound ligand was not an objective from this perspective. Low detector sensitivity as well as lack of appropriate, readily available fluorescent dyes proved important obstacles.[23]

As fluorescent techniques became more sensitive and sophisticated, the value of fluorescent ligands grew. The development of brighter and more photostable fluorophores which became commercially available, the improvement of fluorescence instrumentation and the advancement of data analysis methods were instrumental for these developments. Starting from flow cytometry and confocal microscopy, fluorescent techniques progressively became more elaborate and could address more complicated biological questions. Biophysical techniques such as fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET),

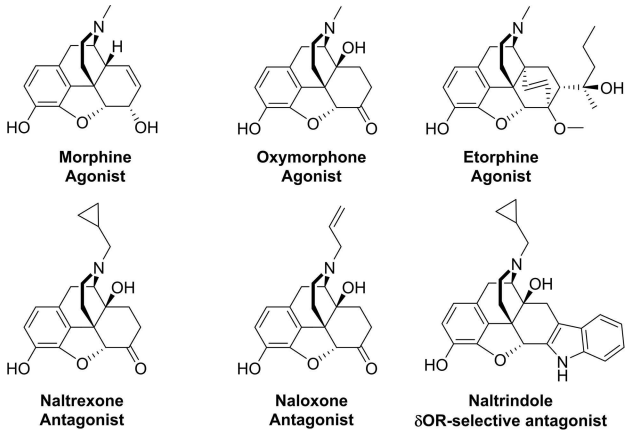


Figure 1. Selection of opioid ligands with various intrinsic activities.

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fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) allow investigating biological processes directly in living cells and with high spatiotemporal resolution[24,25] The application of such biophysical methods contributed decisively to our understanding of various aspects of GPCR function, including receptor dynamics, oligomerization, trafficking and internalization, as well as the mechanisms of G protein coupling or arrestin recruitment. In addition, fluorescent probes have been successfully used *in vivo* in animal models and their potential use for *in vivo* diagnostics in humans is a topic of intense investigation.[23–27]

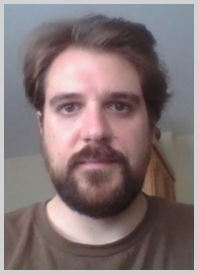
More recently, the introduction of single-molecule superresolution techniques, such as single-particle tracking, stochastic optical reconstruction microscopy (STORM) or photoactivation localization microscopy (PALM), allowed researchers to study the organization and dynamics of individual receptors in real time and with a resolution of approximately 10–30 nm, which is at least 10 times better than with conventional microscopy. This breakthrough has been made possible by the development of new imaging methods with high signal-to-noise ratio -mostly due to very sensitive cameras- and novel fluorophores. Contrary to classical biochemical and imaging methods, which provide only average information about the molecules under investigation ("ensemble methods"), single molecule methods enable the direct monitoring of the behavior of individual receptors, with very high spatiotemporal resolution. Thus, single molecule methods provide detailed information on complex receptor populations, including their spatial distribution, diffusion on the plasma membrane or association in supramolecular complexes. Moreover, single molecule microscopy (SMM) enables a direct investigation of the kinetics of biological events that take place in mixed and non-synchronized populations[26,28–31]

3. Opioid fluorescent probes

3.1. General aspects of fluorescent probe design

The development of fluorescent probes aiming at a specific biological target is generally achieved via attaching a chemical moiety with fluorescent properties to a ligand with known affinity towards that target (Figure 2). This conjugation can be direct or occur with the intervention of a linker-like alkylene moiety or amino acid chain. The parent ligand is often referred to as the "pharmacophore", because it represents the part of the molecule responsible for receptor recognition. However, a pharmacophore by definition would be the ensemble of steric and electronic features which result to optimal supramolecular interactions with a specific biological target. Thus, according to the above, only certain residues/functional groups in the correct positions of a ligand constitute a pharmacophore, while in the probe design approach the whole parent ligand is named "pharmacophore". The resulting compound has a bifunctional character (Figure 2), since one part of the molecule binds the biological target, while another part of the molecule emits fluorescence without a scope of interfering with the target (thus it is often referred to as "fluorophore"). Although this design is in principle straightforward, there are several practical challenges:

- The parent ligand should have well-studied pharmacological properties. Since addition of a fluorescent probe usually reduces binding affinity and selectivity (more scarcely intrinsic activity) compared to the parent ligand, parent ligands with very high affinity and exceptional pharmacological profiles are the cornerstone of fluorescent probe development.
- The attachment of the fluorophore or linker moiety on the parent ligand should take place on an easy-to-handle position with minimal consequences on its pharmacological profile (affinity, selectivity etc.). The conjugation requires the presence or introduction of a reactive functional group (e.g. amine, hydroxyl, alkynyl, carboxyl) on a topology of the ligand which will not temper the binding. This underlines the



Antonios Drakopoulos received his Diploma (2011) and M.Phil. (2014) from the Faculty of Pharmacy, University of Athens. In 2014 he joined the research group of Prof. Michael Decker in the Institute of Pharmacy of the University of Würzburg, Germany, becoming a member of the ENB International Doctoral Program "Receptor Dynamics". His investigation focuses on the design, synthesis and characterization of subtype-selective fluorescent probes for the study of opioid receptor oligomerization via single-molecule microscopy studies. He conducted these studies in the research group of Prof. Davide Calebiro at the University of Birmingham.

Michael Decker studied chemistry at Bonn and Cambridge University. He received his Ph.D. in pharmaceutical chemistry from Bonn University in 2001, and pursued his postdoctoral research qualification („habilitation") in pharmacy at Jena University. After that, he worked as a visiting scientist at McLean Hospital of Harvard Medical School as a Leopoldina Scholar. He was appointed a lecturer at Queen's University Belfast, followed by a position at Regensburg University. Since 2012 he is a professor of pharmaceutical and medicinal chemistry at Würzburg University, where his group works on various GPCRs and enzymes developing hybrid molecules, PET radiotracers, fluorescent and photoswitchable probes.

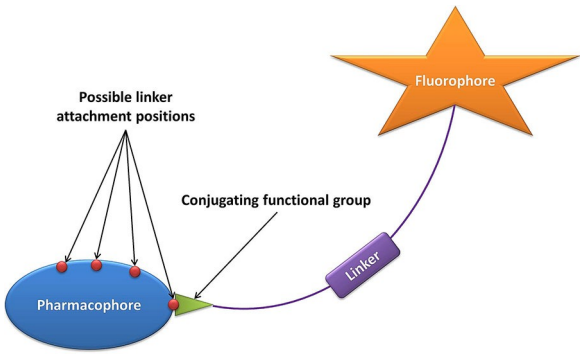


Figure 2. Fluorescent ligand-design rationale.

importance of deep knowledge on the SAR and binding mode of the parent ligand.

- As a rule of thumb, it is considered that the further away the fluorophore is situated with regards to the binding site (therefore to the pharmacophoric part), the less it will modify the established pharmacological profile, as it becomes more unlikely to interact with the binding pocket. As a result, the length, flexibility and physical chemical properties of the linker are of great significance.
- The selection of the fluorescent dye is also crucial. There are many factors which should be considered, including its excitation wavelength, photostability, pH sensitivity, environment-dependent fluorescence quenching, depending on the biological problem to be investigated and the biophysical method to be applied. Furthermore, the fine tuning of the physical chemical properties in the target compound plays also an important part in the selection, due to the size and functional groups of the fluorophore.
- The fluorescent probe is often larger and of a significantly higher molecular weight than the parent ligand and may include additional functional groups. These modifications can have an impact on the lipophilicity, solubility, polar surface area and charge of the probe. Besides its affinity and selectivity, this can also affect its binding kinetics. A good fluorescent probe is not only expected to bind potently and selectively to its target, but also to remain bound long enough until the assay of choice is concluded. An altered pharmacokinetic profile and ADME properties compared to the parent ligand are thus expected *in vivo*. Also, the kinetics with regard to the biological target *in vitro*, such as k_{on}, k_{off} and residence time, can be significantly altered.

From the above, it becomes obvious that although the fluorescent probe comprises mainly of two parts with studied and known properties (i.e. the ligand and the dye), it is a new chemical entity and thus it is likely that it exhibits distinct properties, rather than representing a mere combination of the parent compound properties.

In addition, it is derived from the above that the SAR and linker properties obtained by relevant bivalent ligands (i.e. ligands which comprise from the selected pharmacophore linked to another pharmacophore) can prove extremely useful in the development of fluorescent ligands, since they both belong to the broad category of bifunctional ligands.[32–34]

The pharmacophore part of a fluorescent probe may be either a small organic molecule or peptide-based. In peptides, the fluorophore or linker is usually conjugated to their C- or N-terminus, or on a side-chain. In general, the position of conjugation should be selected based on SARs of fluorescent peptides labelled in different positions. Furthermore, native peptides are generally not used directly for fluorescent probe development due to excessive residues (e.g. the side chains of aminoacids not participating in binding SAR), low selectivity and steady metabolism -opposed to the metabolism of artificial peptide-based compounds bearing groups and residues which are not found on native peptides. The basic principle of peptide-based fluorescent probe design is to keep only the essential pharmacophoric domain of the peptide and reduce the rest of the chain as much as possible.

Currently, small molecule-based fluorescent ligands have become important molecular tools for receptor investigations and even surpassed peptide-based probes for *in vivo* imaging.[35] This is mainly due to their superiority in terms of stability, solubility, cell permeability, selectivity and applicability in high-throughput screening assays. However, their synthesis is often very challenging.[35–37]

3.2. Non-selective fluorescent probes

There have been several early attempts to develop fluorescent probes starting from unselective opioid ligands, both agonist and antagonist. In several cases, the affinity of the probes was not tested against all opioid receptor subtypes. A general assumption was that since the fluorescent probes were developed using non-selective ligands, they should in principle retain the non-selective pharmacological profile of their parent ligand. Thus, showing binding to one or more subtypes (usually of particular interest for the specific study) was often deemed sufficient to prove their activity against the whole OR family. Nonetheless, this approach potentially underestimates the effect of fluorophore conjugation on the specificity of the probe. Furthermore, this practice might hamper not only fluorescent probe development for the ORs, but OR bifunctional ligand development in general, since it deprives us of potentially important SAR. Therefore, it seems appropriate to determine the affinity and selectivity for every new OR fluorescent probe at least for the three classic OR subtypes (μ, δ, κ) and ideally for the nociceptin receptor as well.

In the 1980s, Kolb et al. coupled the antagonists naloxone and naltrexone as well as the agonist oxymorphone with the fluorescent dyes fluoresceine and tetramethylrhodamine B. Although subtype selectivity was not measured, these fluorescent probes achieved very good affinities and were active also *in vivo*, while Madsen et al. used the naloxone probe successfully for confocal microscopy.[39–41] In 1992, Archer et al. labeled the antagonist β-naltrexamine and an etorphine-like derivative with a nitrobenzoxadiazole (NBD) dye. Both compounds exhibited good potencies with the latter showing a μOR

selectivity while the former retaining a non-selective profile.[42] Lawrence et al. had previously also labelled β-naltrexamine with fluoresceine isothiocyanate-conjugated (FITC) dye yielding a potent, non-selective probe. However, it did not exhibit a strong enough fluorescence for direct staining and therefore amplification with phycoerythrin-labelled anti-fluorescein antibodies was applied.[43] Naltrexamine coupled with BODIPY yielded very good results in a study by Emmerson et al. in 1997.[44] In 2000, Le Bourdonnec et al. developed a "reporter affinity label" compound based on β-funaltrexamine, by adding a fluorogenic *o*-phthalaldehyde moiety. The latter can cross-link covalently with a Lys and a Cys residue, resulting in a fluorescent isoindole moiety irreversibly bound to the receptor. The resulting fluorescence is suitable for flow cytometry and this approach has proven effective *in vitro* and *in vivo*.[45] A second generation of this ligand was developed by McCurdy et al. in 2002 by replacing the *o*-pthalaldehyde with a naphthalene dicarboxaldehyde, which resulted in highly improved fluorogenic properties.[46] In a more recent approach, Lam et al. successfully coupled sulfo-Cy5 to the non-selective OR agonist morphine (Figure 3) and showed that it has a very similar profile to the parent ligand in transfected cell assays (Figure 4)[38]. However, OR subtype selectivity measurements were not conducted for this compound, probably due to the low selectivity of the parent ligand (morphine $K_i = 2$ nM (μOR), 150 nM (δOR), and 50 nM (κOR), respectively).[47]

A new approach in receptor fluorescent labeling termed "traceless affinity labeling", was very recently presented for ORs. Arttamangkul et al. developed a fluorescent probe based on naltrexamine bearing an acylimidazole moiety on the linker and an Alexa 594 dye (Figure 5). The pharmacophoric part naltrexamine is used as a "guide moiety" for a transfer reaction of an acylimidazole moiety embedded in the linker with the receptor. After ligand binding, a nucleophilic attack from a Lys, Ser, Tyr or Thr residue in the extracelluar loop 2 or 3 of the receptor takes place on the acylimidazole, leading to the formation of a covalent bond of the acylimidazole-fluorophore part of the linker with the receptor, while naltrexamine is released and leaves the binding site. This technique permitted the detection of opioid-sensitive neurons in rat and mouse brains, without loss of function of the labeled receptors (Figure 6).[48]

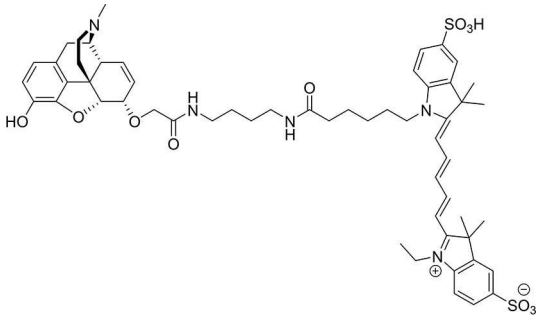


Figure 3. Morphine-derived fluorescent probe with fluorophore sulfo-Cy5, developed by Lam et al.[38]

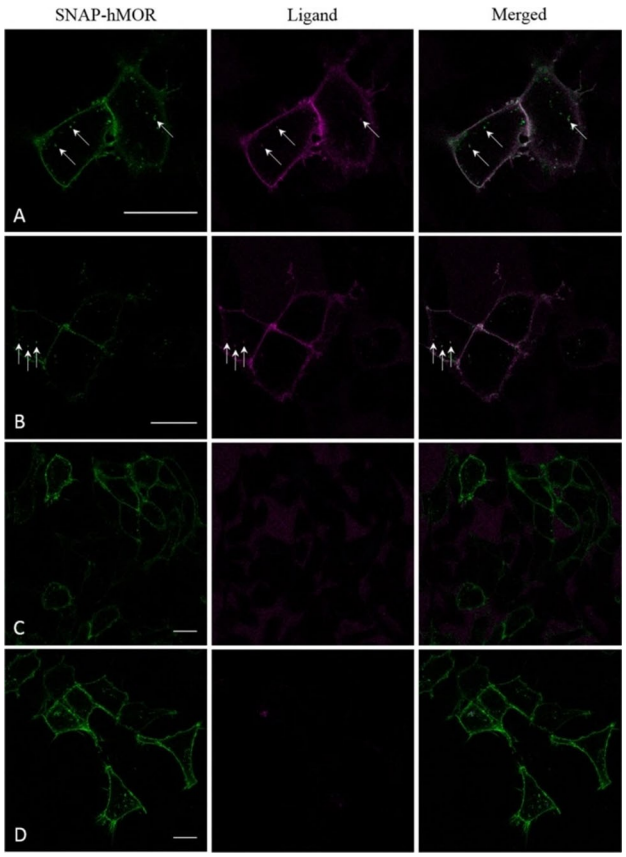


Figure 4. Live cell confocal imaging of a morphine-derived fluorescent ligand in cells expressing SNAP-human μOR, by Lam et al.[38] (A,B) Confocal microscopy results; the white arrows indicate areas where co-localization of intracellular receptor populations with the fluorescent ligand was observed. (C) Treatment with fluorescent ligand following preincubation with naloxone, indicating specific binding of the probe to μOR. (D) Loss of ligand signal following a postincubation wash. This is typical of non-peptide opioids, which tend to have a fast k_{off} rate. Scale bars, 20 μm. Reproduced from reference [38] with permission from the American Chemical Society.

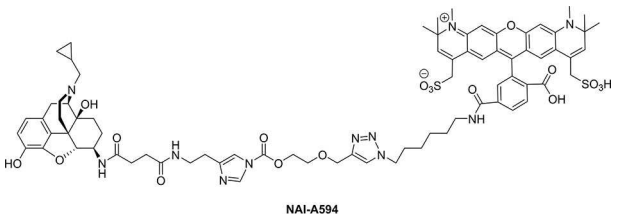


Figure 5. Naltrexamine-derived "traceless affinity labeling" probe (NAI-A594) with the fluorophore AFDye 594, developed by Arttamangkul et al.[48]

3.3. μ opioid receptor-selective fluorescent probes

Due to the high interest associated with the role of the μOR in pain management, the synthesis of μOR fluorescent probes has received the greatest attention. Enkephalins were among the first opioids to be employed as templates for the development of fluorescent probes already from the late 1970s, yielding good results with high affinities both for the μOR and δOR (Figure 7). Using these probes it was shown that μORs are organized as clusters on the surface of living neuroblastoma

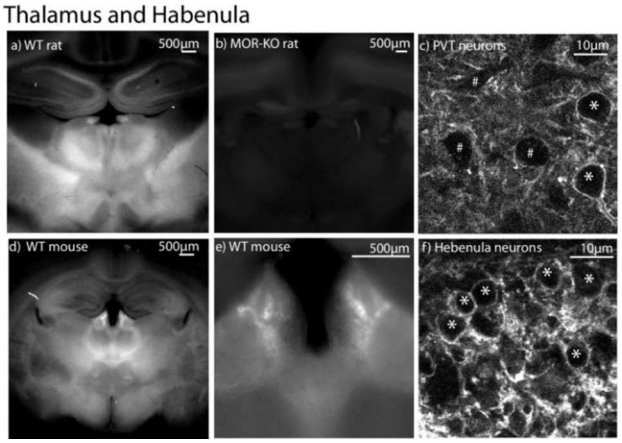


Figure 6. Localization of labeling in the thalamus and nearby structures of rat brain coronal slices induced by NAI-A594, by Arttamangkul et al. (a) A wild-type rat coronal brain slice after incubation with NAI-A594 showing strong fluorescence at low magnification. (b) A similar slice from a μOR knockout rat, showing no fluorescence. (c) A high magnification image of the paraventricular thalamus showing two labeled cells and three unlabeled cells. (d–f) a series beginning with low magnification images in the area of the habenula (d,e) and ending with an image of labeled cells in the habenula (f). * = Labeled and # = non labeled neurons.[48]

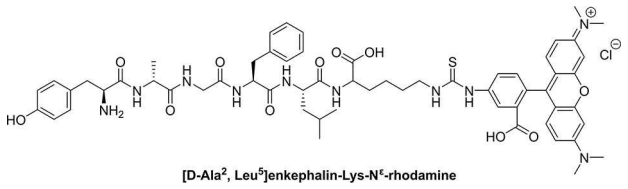


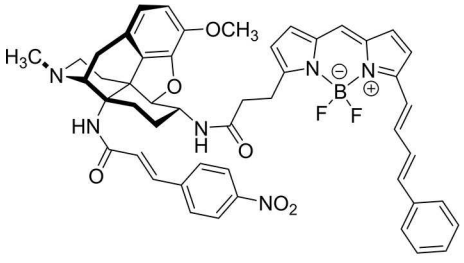
Figure 7. Enkephalin-based fluorescent probe with the fluorophore rhodamine, developed by Hazum et al.[49,50]

cells.[49–55] Endomorphins were also used as templates, albeit resulting in low to moderate μOR affinities.[56,57] In a recent study, endomorphin-1 was coupled with the fluorescent dye tetramethylrhodamine (TAMRA) to study the internalization and trafficking of μORs, yielding good optical and pharmacological properties and successfully detecting μORs in keratinocytes.[58]

Dermorphin is a μOR-selective agonistic exorphin, produced in the skin of the frog *Phylomedusa sauvagei*. It was used by Gaudriault et al. in 1997 for the development of fluorescent probes using BODIPY dyes, although achieving only low selectivity against the δOR.[59] Arttamankgul et al. returned on the issue, accomplishing highly potent dermorphin probes with BODIPY and Alexa dyes with a high selectivity against the δOR, which were used to study real-time dynamics of ligand binding and receptor internalization, in an interesting combination of microscopy and electrophysiology.[57] Other peptide-based approaches include Berezowska et al. (DALDA coupled with dansyl- and anthraniloyl-based dyes), Lukowiak et al. (biphalin coupled with a coumarin-based dye) and Li et al. (cyclic pentapeptide coupled with an anthraniloyl-based dye).[60–62]

As noted previously, a thebaine-derived fluorescent probe using the dye NBD was developed in 1992 by Archer et al.[42] A notable non-peptide fluorescent probe with high μOR selectivity is WA-III-62 (Figure 8), and it was developed by Emmerson et al.[44] via coupling CACO[63] with BODIPY 581/591 dye via a short alkylene linker. This compound exhibited a wash-resistant profile, which was suggested to derive from the formation of a covalent bond of the *p*-nitrocinnamoyl group with the receptor through a Michael reaction, albeit no further investigation followed to prove this. WA-III-62 achieved an EC_{50} of 24 nM (radioligand binding assay) for the μOR, while for the other subtypes the respective values were reported to be larger than 1000 nM.

Following up on this study, Gentzsch, Seier et al. replaced the fluorophore with sulfo-Cy3 and sulfo-Cy5 and employed a tetraglycine linker moiety to achieve a highly potent and selective pair of partial agonist probes (Figure 9), which were then employed to study μOR diffusion and homodimerization using Single Molecule Microscopy (SMM) techniques. Cells transiently transfected to express μORs at low density on the plasma membrane (close to physiological levels) were labeled with a mixture of sulfo-Cy3 and sulfo-Cy5 fluorescent ligands, as well as with only sulfo-Cy3. This was followed by fast two-color and one-color TIRFM imaging, respectively; while unspecific binding to the glass-coverslip was kept low in both settings. The results show that 5% of the receptors form transient dimers lasting approximately 1–2 seconds (Figure 10).[64] In another recent investigation, Schembri et al. synthesized buprenorphine-based probes using a variety of fluorophores (Cy5, BODIPY, tetrazine, naphthalimide) achieving very good affinities to the μOR but poor selectivity against κOR. The Cy5-bearing fluorescent probe was successfully used in a fluorescence-based competition binding assay representing a radiation-free alternative to radioligand binding studies.[65]



WA-III-62

Figure 8. μOR-selective fluorescent probe with the fluorophore BODIPY 581/591, developed by Emmerson et al.[44]

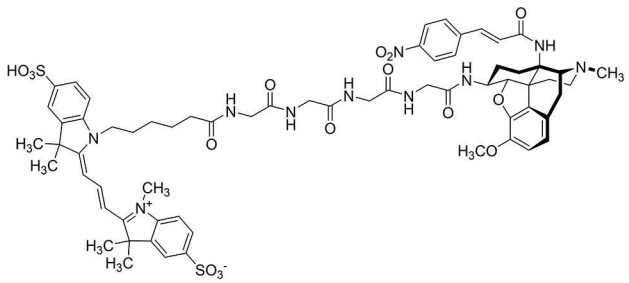


Figure 9. μOR-selective fluorescent probe with the fluorophore sulfo-Cy3, developed by Gentzsch, Seier et al.[64]

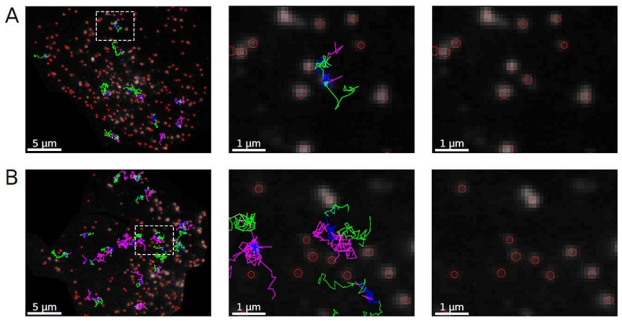


Figure 10. Single-molecule imaging of transient μOR interactions in relationship to the location of clathrin coated pits (CCPs) via TIRF microscopy, by Gentzsch, Seier et al. Transiently transfected CHO cells with wild-type μOR and GFP-clathrin were incubated with a sulfo-Cy3 fluorescent ligand and its sulfo-Cy5 analogue. The receptors were tracked, followed by acquisition of a CCP image. Left: Images of a representative cell with the obtained trajectories of receptors (green and magenta, respectively) undergoing interactions (blue) overlaid on the CCP image (grey). Red circles correspond to individual CCPs. Middle: Enlarged views of the regions delimited by the dashed boxes. Right: Same regions without trajectories. A) interactions occurring within or near CCPs. B) interactions occurring outside CCPs.[64]

3.4. δ opioid receptor-selective fluorescent probes

Research aiming at δOR-selective fluorescent probe development has yielded interesting and important results. Deltorphin is an exorphin with agonistic properties isolated from the skin of the frog *Phylomedusa bicolor* which exhibits very high affinity and selectivity for the δOR. Gaudriault et al.[59] successfully used it as parent ligand for the development of fluorescent probes linked to BODIPY analogs characterized by good selectivity for the μOR. The probes were employed to study receptor internalization in δOR-transfected, μOR-δOR co-transfected, and also in primary cells. A time- and temperature-dependent mode of internalization was revealed, as well as partly distinct endocytic pathways for the two receptor subtypes in the co-transfected cells.[59,66] A similar approach by Arttamangkul et al.[57] further enhanced selectivity, employing Alexa dyes in deltrophin and the antagonist δOR-selective peptide TIPP (Tyr-Tic-Phe-Phe). The probes were used to study real-time internalization and binding dynamics, as noted previously. Deltorphin was also used for the development of water-soluble quantum dots for imaging and SMM. These bioconjugates were tested in δOR-transfected cells for their imaging and biochemical properties, while purified δORs were incorporated in artificial lipid bilayers to perform SMM.[67] Handl et al. in 2005 employed enkephalin and deltorphin derivatives for developing fluorescent probes with Eu-chelated complexes. A potent probe was accomplished and successfully used in competitive binding assays for the δOR, but no selectivity measurements were reported.[68]

A number of fluorescent probes with excellent affinity and selectivity were prepared from 2004 to 2016 as antagonist derivatives of the potent peptide Dmt-Tic using a variety of fluorophores (fluorescein, Cy5, Eu-chelated complexes, 6-*N,N*-(dimethylamino)-2,3-naphthalimide, Li-cor IR800CW).[56,69–72] For example, in their 2004 study Balboni et al. developed a H-Dmt-Tic-Glu-NH_2 analogue containing fluorescein linked to the peptide C-terminus through a pentamethylene spacer which achieved high selectivity over the μOR and an irreversible antagonist profile.[71] Based on these results, in 2006 Vazquez et al. replaced the fluorophore with 6-*N,N*-(dimethylamino)-2,3-naphthalimide and produced two probes, both with and without the alkylene linker, which retained the irreversible binding profile and also exhibited high selectivity over the μOR.[56]

In 2009, Josan et al. synthesized two fluorescent probes using Dmt-Tic-Lys(R) as pharmacophoric part, 3-mercaptopropionyl (Mpr) and 8-amino-3,6-dioxaoctanyl (Ado) moieties as linkers and fluorophores sulfo-Cy5 (Figure 11) and Eu-DOTA. The antagonist probe bearing the lipophilic linker and sulfo-Cy5 exhibited high affinity and selectivity for the δOR, and was successfully used *in vivo* in mice for imaging of tumor xenografts, as it has been reported that certain peripheral tumors overexpress δORs (e.g. lung, liver, breast).[69] This probe was further studied by Huynh et al. in 2016 with respect to its pharmacokinetic profile, cellular uptake, biodistribution, clearance, and *in vivo* tumor imaging in a xenograft mouse model, demonstrating that its ADME profile is suitable for *in vivo* imaging. It should be noted that due to photobleaching of the probe, keeping the animals in a dark chamber after injection permitted lowering the dosage more than 10 fold (50 nmol/kg to 4.5 nmol/kg).[70] Also in 2016, Cohen et al. substituted the fluorophore of the aforementioned compound with the longer emission wavelength dye Li-cor IR800CW, and used it successfully to image δOR endogenously expressed in lung tumors in a mouse model (Figure 12).[72] Furthermore, a book chapter published by Josan et al. summarizes the synthesis and application of enkephalin-based and Dmt-Tic-Lys-based fluorescent and luminescent probes (with organic dyes or lanthanide-complex dyes) with heterobivalent functionality (i.e. ligands with two pharmacophoric parts targeting two different μOR and δOR receptors simultaneously).[73]

The Portoghese group developed morphinane-based δOR-selective fluorescent probes using the highly potent and selective antagonist naltrindole and the dye fluorescein. In 1997, an initial approach by Korlipara et al., yielded potent but moderately selective analogs, which also exhibited unspecific binding to the lipid membrane.[75] Shortly afterwards, Kshirsagar et al. managed to fine tune the ligand properties by changing the linking position from position 1′ to position 7′, enlarging the spacer and incorporating a tetraglycine moiety in the latter, resulting to an excellent fluorescent probe for the δOR (Fig-

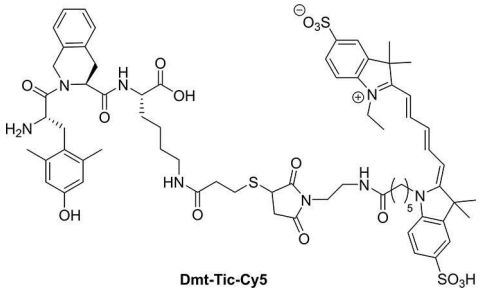


Figure 11. δOR-selective probe with the fluorophore sulfo-Cy5, developed by Josan et al.[69,70]

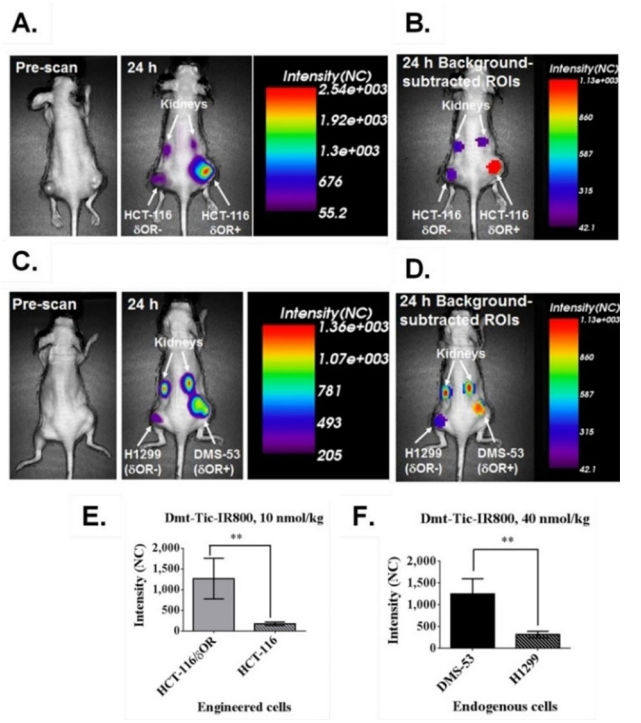


Figure 12. Fluorescent ligand Dmt-Tic-IR800 shows δOR selectivity *in vivo* allowing tumor imaging, by Cohen et al.[72] Imaging with Dmt-Tic-IR800 in engineered cells (A,B,E) and endogenous lung cancer cells (C,D,F). (A) and (C) Images acquired before and 24 h after administrating the probe. (A) 10 nmol/kg and (C) 40 nmol/kg fluorescent probe were injected to mice with (A) bilateral HCT-116 (δOR−) and HCT-116/δOR (δOR+) and (C) bilateral H1299 (δOR−) and DMS-53 (δOR+) tumors in the left and right flanks, respectively. (B and D) The same 24 h acquisitions shown in (A and C), but with background-subtracted fluorescence signal obtained from regions of interest drawn around the tumors and kidneys. (E and F) Graphs of mean normalized fluorescence intensities obtained from (E) the HCT-116 (δOR-) and HCT-116/δOR (δOR+) tumors and (F) the H1299 (δOR-) and DMS-53 (δOR+) tumors. Reproduced from reference [72] with permission from the American Chemical Society.

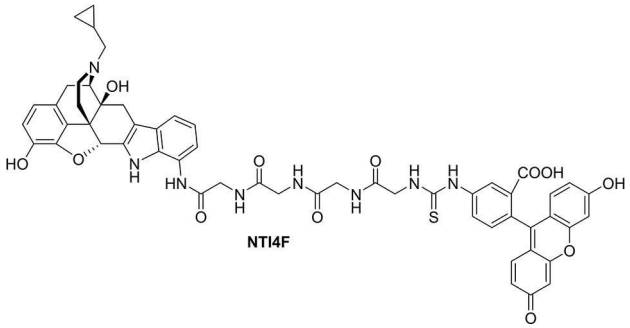


Figure 13. δOR-selective fluorescent probe with the fluorophore fluorescein, developed by Kshirsagar et al.[74]

ure 13).[74] Furthermore, in 2001 Le Bourdonnec et al. linked a fluorogenic *o*-phthalaldehyde to position 7′ of naltrindole, affording a δOR-selective "reporter affinity label" suitable for flow cytometry, following up their previous approach.[76] Our group, based on the aforementioned SAR on naltrindole-derived probes, synthesized respective analogues using the fluorophores sulfo-Cy3 and sulfo-Cy5, also yielding fluorescent ligands which exhibited excellent optical properties, high affinity and selectivity (unpublished data).

3.5. κ opioid receptor-selective fluorescent probes

Only a scarce number of investigations addressing the development of κOR-selective fluorescent probes have been reported so far. To our knowledge, most of the few highly κOR-selective fluorescent probes currently described in literature are peptide or peptide-mimetic agonist ligands.

In 1995, the first fluorescent probes using the κOR-selective arylacetamide agonist ICI-199,441[78] and fluorescein dye were developed. A high-affinity probe was achieved and it was successfully employed in detecting κORs in mouse lymphocytes. However, its selectivity over δOR was low and also its fluorescent signal was not sufficient for the direct detection of κORs. Therefore, fluorescence amplification with phycoerythrin-labelled anti-fluorescein antibodies was performed.[79–81] Shortly afterwards, based on these findings, the Portoghese group developed analogs that incorporated glycine linker moieties between ICI-199,441 and fluorescein. These efforts resulted in the first highly potent ($K_i = 0.91$ nM) and selective (700-fold over μOR, 300-fold over δOR) κOR fluorescent probe (Figure 14), which enabled direct κOR staining of mouse microglial cells.[77]

In a recent publication, Drakopoulos et al. presented the design, synthesis and biological application of the first set of antagonistic and morphinane-based selective fluorescent ligands for the κOR, using the extensively studied κOR-selective antagonist/inverse agonist 5′-GNTI as a pharmacophore in combination with the fluorophores sulfo-Cy3 and sulfo-Cy5 (Figure 15). A fitting pair of these fluorescent ligands was employed in SMM experiments using TIRFM to investigate the spontaneous homodimerization of inactive κORs at low/physio-

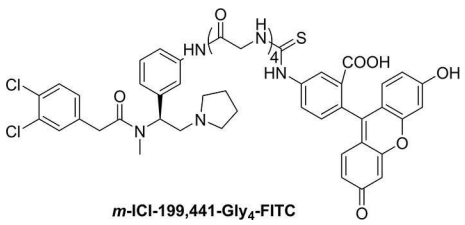


Figure 14. κOR-selective arylacetimide-derived fluorescent probe with the fluorophore fluorescein, developed by the Portoghese group.[77]

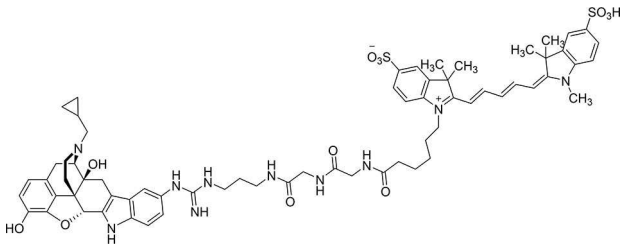


Figure 15. κOR-selective morphinane-based fluorescent probe with the fluorophore sulfo-Cy5, developed by Drakopoulos et al.[34]

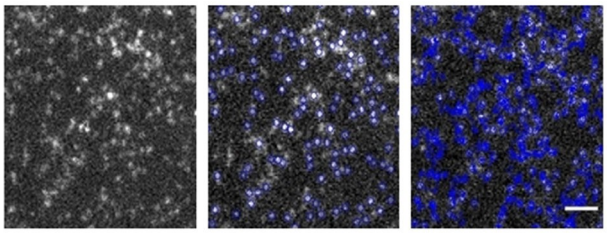


Figure 16. SMM images of KORs labelled with a selective sulfo-Cy3 fluorescent ligand, by Drakopoulos et al.[34] The CHO cells were transiently transfected with human κOR and treated with 100 nM of fluorescent ligand for 20 min, followed by a quick washing step. Left: A representative cell. Middle: The blue circles indicate all automatically detected particles. Right: Representative trajectories of the moving particles. Scale bar: 2 μm. Reproduced from reference [34] with permission from the American Chemical Society.

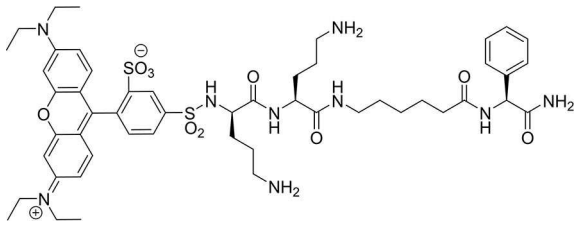


Figure 17. κOR-selective peptide-derived fluorescent probe with the fluorophore sulforhodamine B, developed by Houghten et al.[82]

logical expression levels - similar to the work of Gentzsch, Seier et al. presented above. Interestingly, in this study no dimer formation was observed and the results suggest that most κORs bound to the new fluorescent ligands are present as apparently freely diffusing monomers on the surface of transiently transfected CHO cells (Figure 16).[34]

The fluorescent probes with the highest selectivity for κOR reported so far (5000-fold against μOR, >10000 against δOR) and K_i values ranging from 6–80 nM were developed by Houghten et al. via a *de novo* approach in 2004. More specifically, a combinatorial tetrapeptide positional scanning library was synthesized, in which every tetrapeptide was labelled on its *N*-terminal amine with the dye sulforhodamine B (Figure 17). Testing of the highest affinity compounds showed partial agonist intrinsic activity. Interestingly, the same tetrapeptides without the *N*-terminal sulforhodamine B did not exhibit significant affinity at the κOR.[82]

4. Summary and Outlook

The vast majority of fluorescent probes developed for the ORs were mostly employed in simple biochemical and optical characterization assays –such as OR detection in transfected or naturally expressing cell lines, as well as fluorescence-based competition binding assays. Nonetheless, the technological progress in the field of microscopy introduced novel instruments and techniques, while the development of new, optimized, commercially available fluorescent dyes yielded a wide variety of choices for the development of fluorescent probes. The combination of both advances in technology and chemical development now results in fluorescent probes which could be used to study receptor oligomerization, trafficking, internalization, for *in vivo* imaging or to be employed as vehicles for receptor labelling. As presented herein, although the efforts in chemistry have been tremendous on developing fluorescent ligands suitable for high-end techniques, their application to the degree of todays' biophysical potential is still pending. Several fluorescent probes have been developed up to now, but most of them did not quite go beyond the level of proof-of-principle biological/biophysical/biochemical/pharmacological applications.

A plausible reason explaining the vast usage of OR fluorescent probes mostly on proof-of-principle biological applications can be traced at the booming of chemical biology as a distinct scientific discipline since roughly the early to mid 00s. A distinctive aspect of chemical biology as a field, is the development of chemical entities with the sole purpose of using them as molecular tools for several biological assays and applications.[83] On the other hand, traditional medicinal chemistry proceeds with developing compounds as therapeutic or diagnostic drug candidates, and the aforementioned proof-of-principle biological applications serve only to show that there is a potential for this target. Nowadays though, synthetic medicinal chemists also aim at applying drug discovery efforts to the development of molecular tools of high quality. Therefore, they need to 1) redeem the importance of chemical biology and related applications, 2) investigate and sort out which compounds from the past can be useful today, 3) define what kind of compounds are required to meet current needs in state-of-the-art applications.

The most current developments in OR imaging from the pharmacological point of view have been reviewed at the same time as this article.[84]

As illustrated in the presentation of the OR fluorescent probes already developed, in the past there have been some oversights that –in the long run- may hamper progress in the field of OR fluorescent probe development. In addition to the necessity of investigating OR subtype affinity and selectivity, we highlight the importance of studying intrinsic activity for OR subtypes, as well as the dissociation kinetics from the OR subtype of highest affinity and we advocate addressing all the above properties in every study focusing on the development of new OR fluorescent probes. That is because, the challenges concerning the development of new fluorescent probes lie mostly in achieving simultaneously very high subtype selectivity, high affinity levels, slow dissociation kinetics, excellent optical properties, while ideally also having good pharmacokinetic and ADME properties, such as retaining their ability to pass the BBB.

The process of trying to overcome the aforementioned difficulties will surely yield novel molecules which can be used as tools to investigate several OR-related unanswered questions, such as the possibility of *in vivo* heteromer formation, details concerning the post-internalization course of the receptor (e.g. potentially signaling) etc; thus providing insights

which could prospectively lead to better drugs with less side effects. The development of fluorescent probes for ORs also possesses model character for discovery and development of fluorescent probes for other GPCRs and other molecular targets alike. Such chemical molecular tool compounds will surely help cellular biology, receptor research and other disciplines to elucidate unanswered questions, e.g. with regard to oligomerization, location and so on.

Note on the frontispiece

The graphics of μOR, δOR, κOR, were depicted using the respective crystal structures with RCSB Protein Data Bank IDs: 4DKL,[15] 4EJ4,[16] 4DJH.[17] Molecular graphics were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).[85]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biophysics · fluorescent ligands · imaging · microscopy · opioid receptors

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