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REVIEW

Heteromers of μ-δ opioid receptors: new pharmacology and novel therapeutic possibilities

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Several studies suggest that heteromerization between μ (MOP) and δ (DOP) opioid receptors modulates the signalling properties of the individual receptors. For example, whereas activation of MOP receptors by an agonist induces G protein-mediated signalling, the same agonist induces β-arrestin-mediated signalling in the context of the MOP-DOP receptor heteromer. Moreover, heteromer-mediated signalling is allosterically modulated by a combination of MOP and DOP receptor ligands. This has implications in analgesia given that morphine-induced antinociception can be potentiated by DOP receptor ligands. Recently reagents selectively targeting the MOP-DOP receptor heteromer such as bivalent ligands, antibodies or membrane permeable peptides have been generated; these reagents are enabling studies to elucidate the contribution of endogenously expressed heteromers to analgesia as well as to the development of side-effects associated with chronic opioid use. Recent advances in drug screening technology have led to the identification of a MOP-DOP receptor heteromer-biased agonist that activates both G protein-mediated and β-arrestin-mediated signalling. Moreover, this heteromer-biased agonist exhibits potent antinociceptive activity but with reduced side-effects, suggesting that ligands targeting the MOP-DOP receptor heteromer form a basis for the development of novel therapeutics for the treatment of pain. In this review, we summarize findings regarding the biological and functional characteristics of the MOP-DOP receptor heteromer and the *in vitro* and *in vivo* properties of heteromer-selective ligands.

LINKED ARTICLES

This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-2

Abbreviations

BRET, bioluminescence resonance energy transfer; DAMGO, [D-Ala^2-N-Me-Phe^4,Gly-ol^5]-enkephalin; DPDPE, D-penicillamine(2,5)-enkephalin; DSLET, [D-Ser^2, Leu5, Thr^6]-enkephalin; GFP, green fluorescent protein; HTS, high-throughput screening; IBS-D, diarrhoea-predominant irritable bowel syndrome; i.t., intrathecal; TIPP$_\Psi$, H-Tyr-Tic[CH_2NH]-Phe-Phe-OH; TM, transmembrane domain

Introduction

G protein-coupled receptors (GPCR) comprise a large and most diverse family of seven transmembrane proteins that are encoded by more than 800 genes in the human genome (Fredriksson *et al.*, 2003). Based on the guidelines of the International Union of Basic Clinical Pharmacology (IUPHAR), there are four main classes of GPCRs: class A rhodopsin-like receptors, class B secretin-like receptors, class C metabotropic glutamate/pheromone receptors and frizzled receptors (Venkatakrishnan *et al.*, 2013).

Opioid receptors are members of the class A GPCR family. Three types of opioid receptors have been identified: μ, κ and δ opioid (MOP, KOP and DOP) receptors respectively (Kieffer,

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1995; receptor nomenclature follows Alexander *et al*., 2013a). Opioid receptors are coupled to $G\alpha_{i/o}$ proteins, and their activation leads to inhibition of adenylyl cyclase activity and voltage-gated Ca^{2+} channels and to increases in MAPK phosphorylation and in the activity of inwardly rectifying K^+ channels (K_{ir} channels; nomenclature follows Alexander *et al*., 2013b) and phospholipase Cβ (Waldhoer *et al*., 2004). The signalling cascades initiated by the activation of opioid receptors induce the transcription of genes that regulate cellular differentiation, proliferation and survival (Chen *et al*., 2008). At the systems level, opioid receptor activation leads to a number of physiological responses including analgesia, feelings of euphoria and anxiety, respiratory depression, constipation, immunosuppression and changes in feeding and locomotor activity (Kieffer, 1995).

Over the last decade, several studies showed that GPCRs, including opioid receptors, can form homomers or heteromers (Satake and Sakai, 2008; Milligan, 2009; van Rijn *et al*., 2010; Smith and Milligan, 2010; Al-Hasani and Bruchas, 2011; Gonzalez-Maeso, 2011; Gomes *et al*., 2013a) and that these receptor–receptor interactions modulate ligand binding, receptor signalling and trafficking properties (Satake and Sakai, 2008; Gonzalez-Maeso, 2011; Gomes *et al*., 2013a; Petko *et al*., 2013). Heteromers involving opioid receptor types, particularly between KOP and DOP receptors and between MOP and DOP receptors, were reported in the early 2000s (Jordan and Devi, 1999; George *et al*., 2000; Gomes *et al*., 2000). Since then, heteromerization between MOP and DOP receptors has been extensively studied (Milligan, 2009; van Rijn *et al*., 2010; Al-Hasani and Bruchas, 2011; Costantino *et al*., 2012; Stockton and Devi, 2012) and found to expand the cellular responses of the MOP receptor protomer, resulting in an enhancement of morphine-induced antinociception (Gomes *et al*., 2004). MOP-DOP receptor heteromer has also been shown to play a role in the adverse side effects of opioids such as development of analgesic tolerance (Gupta *et al*., 2010; He *et al*., 2011). Ligands targeting the MOP-DOP receptor heteromer, such as bivalent ligands (Daniels *et al*., 2005; Lenard *et al*., 2007; Harvey *et al*., 2012) and a MOP-DOP receptor-biased agonist, have been reported (Gomes *et al*., 2013b); these are likely to help in the elucidation of the physiological effects of MOP-DOP receptor heteromers. This review will summarize what is presently known about MOP-DOP receptor heteromers and their selective ligands.

Evidence for the formation of MOP–DOP receptor heteromers

Computational analysis and crystal structure

Computational studies have been used to predict the transmembrane (TM) regions involved in the formation of opioid receptor heteromers (Filizola and Devi, 2012). An early study used a combination of three-dimensional (3D) homology modelling, based on the crystal structure of rhodopsin, and a subtractive correlated-mutation method to predict the involvement of TM1 of MOP receptors and TM4, TM5 and TM6 of DOP receptors in the heteromer interface (Filizola *et al*., 2002). Another study used a combination of 3D homology modelling, molecular dynamics simulations and analysis of protein–protein docking, cluster, shape complementarity and interaction energy to identify the possible MOP-DOP receptor heteromer interface (Liu *et al*., 2009). This analysis revealed that the most likely interface involved TM1 of MOP receptors and TM4 of DOP receptors and that the next likely interface involved TM6 of MOP receptors and TM4 of DOP receptors (Liu *et al*., 2009). Although both studies predict the involvement of TM1 of MOP receptors and TM4 of DOP receptors in the MOP-DOP receptor heteromeric interface, differences in the predictions for other likely interfaces could be due to the methodologies used. More recently, a study used energy transfer techniques and homology modelling methods to generate putative configurations for MOP-DOP receptor tetramers and predicted symmetric interactions of TM4, TM5 or TM1 at the MOP-DOP receptor heterodimeric interface (Golebiewska *et al*., 2011).

Recently, the crystal structures of the opioid receptor types revealed dimers and/or higher-order oligomers; for example, the MOP receptor crystal lattice comprises tightly packed parallel receptor dimers with an interface involving TM1, TM2 and helix 8 or TM5 and TM6 (Manglik *et al*., 2012). This would suggest that physiologically MOP receptors could be present as dimers or higher-order oligomers (Manglik *et al*., 2012). DOP receptors were shown to crystallize as an antiparallel arrangement of receptor proteins, suggesting the absence of physiologically relevant oligomeric contacts (Granier *et al*., 2012). However, examination of the amino acids involved in the MOP receptor dimer interface shows that they exhibit a high degree of homology with the corresponding amino acids in DOP receptors, suggesting that MOP-DOP receptors could share the same interface (Manglik *et al*., 2012). Taken together, both computational analysis and crystallization studies are consistent with the idea that opioid receptors could form dimeric, heteromeric and higher-order oligomeric complexes.

Anatomical evidence for MOP and DOP receptor heteromerization

Direct interactions between MOP and DOP receptors would require that both receptors be present not only in the same cell but also in the same subcellular compartment. Early electrophysiological studies examining the cell firing profile of single neurons using either MOP or DOP receptor agonists supported the presence of both receptors in the same neuron that was being investigated (Fields *et al*., 1980; Egan and North, 1981; Zieglgansberger *et al*., 1982). In addition, radioligand binding assays with commercially available neuroblastoma cell lines detected the presence of endogenous MOP and DOP receptors in these cells (Yu *et al*., 1986; Kazmi and Mishra, 1987; Baumhaker *et al*., 1993; Palazzi *et al*., 1996). In order to ascertain whether MOP-DOP receptor colocalization could be detected in brain and spinal cord, immunohistochemical studies using antibodies to endogenous receptors have been carried out. These studies detected MOP and DOP receptor colocalization to the same axonal terminals of the superficial dorsal horn (Arvidsson *et al*., 1995). Moreover, ultrastructural analysis carried out using a combination of electron microscopy and dual immunocytochemical labelling of MOP and DOP receptors indicated that the two receptors colocalized in the plasmalemma of dorsal horn neurons (Cheng *et al*., 1997) and in the dendrites or spines of the

striatum (Wang and Pickel, 2001). These data showing colocalization of MOP and DOP receptors in brain and spinal cord were challenged by a report that examined DOP receptor localization in mice expressing enhanced green fluorescent protein-tagged receptor (eGFP–DOP receptor knockin mice). This study showed that in dorsal root ganglion neurons, MOP and DOP receptors were segregated from each other. MOP receptors were expressed on small, peptidergic C fibres, while DOP receptors were preferentially localized to medium-sized, non-peptidergic primary afferents and on large myelinated neurons (Scherrer *et al.*, 2009). A limited colocalization of MOP with DOP receptors was found in <5% of the neurons, leading the authors to suggest that previous reports of colocalization between these two receptors were inaccurate due to the quality of the DOP receptor antibodies used in the studies (Scherrer *et al.*, 2009). Several factors could account for the discrepancies in the colocalization of these two receptors. Firstly, the intensity of the staining obtained with the GFP antibody is much stronger than that obtained with the MOP receptor antibody (Scherrer *et al.*, 2009). This could be due to an increase in *Oprd1* transcription in eGFP–DOP receptor knock-in mice leading to higher levels of DOP receptors and/or to differences in the binding affinities/avidity of the anti-GFP antibody compared to the anti-MOP receptor antibody. Either of these conditions would lead to an overestimation of DOP receptor and underestimation of MOP receptor abundance and therefore extremely low colocalization between these two receptors. Secondly, the eGFP tag at the *C*-terminus has been known to increase the cell surface localization of DOP receptors (Wang *et al.*, 2010), leading to different localization of endogenous DOP receptors and eGFP–DOP receptors (Wang *et al.*, 2010; Zhang and Bao, 2012). Thirdly, molecular and pharmacological chaperones have been shown to help target DOP receptors to the cell surface by stabilizing receptor precursors and facilitating their release from the stringent quality control of the endoplasmic reticulum (Leskela *et al.*, 2007; Decaillot *et al.*, 2008). In addition, *in vivo* interactions with other proteins facilitate surface expression of DOP receptors (see Cahill *et al.*, 2007) and decrease the expression of MOP receptors (Decaillot *et al.*, 2008). Fourthly, it is possible that under physiological conditions MOP-DOP receptor heteromer levels are low and that they increase under pathological conditions such as pain or development of tolerance to morphine (see Cahill *et al.*, 2007). Thus, although the eGFP-DOP receptor knock-in mouse is a good model for understanding the physiological role of DOP receptors, care has to be taken in the interpretation of the data, as the eGFP tag could disrupt interactions normally seen involving endogenous DOP receptors and other intracellular proteins.

A number of studies have tried to address the conflicting reports on the colocalization of MOP and DOP receptors in DRG neurons. These included studies that (i) used single-cell PCR in subsets of DRG neurons and reported the presence of MOP and DOP receptors in peptidergic small DRG neurons (Wang *et al.*, 2010); (ii) used *in situ* hybridization to demonstrate the presence of MOP receptor and DOP receptor mRNA in DRG neurons that also expressed the mRNA for preprotachykinin A (a marker for peptidergic small neurons) (Wang *et al.*, 2010); (iii) demonstrated the presence of DOP receptor immunoreactivity in peptidergic small DRG neurons using anti-DOP receptor antibodies that gave a signal with DRG neurons from wild-type but not from *Oprd1* exon 1-deleted mice – reporting, interestingly, that high antibody dilutions (1:30 000 to 1:60 000) were needed to prevent non-specific binding (Wang *et al.*, 2010); (iv) expressed *myc*-tagged DOP receptors and eGFP-tagged DOP receptors in small DRG neurons and, using antibodies to the epitope tags, showed that *myc*-DOP receptors localize to CGRP containing large dense-core vesicles, while eGFP–DOP receptors localize at the cell surface (Zhang and Bao, 2012); (v) used receptor-selective agonists to show that MOP and DOP receptors expressed by peptidergic nociceptors inhibited the release of substance P following formalin or capsaicin treatment and this could be blocked by receptor-selective antagonists (Beaudry *et al.*, 2011); and (vi) used MOP-DOP receptor heteromer-selective antibodies to demonstrate the presence of heteromers in DRG neurons as well as in neurons in the pain pathway (Gupta *et al.*, 2010). Taken together, these studies strongly support the presence of MOP and DOP receptors in peptidergic DRG neurons.

Behavioural evidence for MOP and DOP receptor heteromerization

Early behavioural studies provided indirect evidence for interactions between MOP and DOP receptors. One set of studies examined the effect of DOP receptor agonists on morphine-mediated antinociception and on the development of tolerance to this clinically used opioid. These studies showed that endogenous or synthetic DOP receptor agonists enhanced morphine-mediated analgesia as well as the development of tolerance to morphine (Vaught and Takemori, 1979a,b; Porreca *et al.*, 1987). Another set of studies found that potent and selective DOP receptor antagonists could block morphine-mediated antinociception as well as development of tolerance following chronic morphine administration (Abdelhamid *et al.*, 1991; Abul-Husn *et al.*, 2007; Ballesta *et al.*, 2012). More recently, a study examined the effect of DOP receptor ligands on antinociception mediated by [D-Ala^2-N-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) in naïve and morphine-tolerant mice; this study reported that the development of tolerance to morphine also led to tolerance to DAMGO, a MOP receptor-selective agonist (Szentirmay *et al.*, 2013). Moreover, in morphine-tolerant mice, treatment with selective DOP receptor antagonists restored the antinociceptive effects of DAMGO to the levels observed with naïve animals (Szentirmay *et al.*, 2013). Taken together, these studies indicated that DOP receptor could modulate MOP receptor-mediated antinociception and play a role in the development of tolerance following chronic administration of MOP receptor agonists.

The involvement of DOP receptors in MOP receptor-mediated antinociception has been examined using animal models with reduced DOP receptor levels or animals lacking individual receptors. For example, a study found that repeated i.c.v. injections with antisense oligonucleotides selective for DOP receptors reduced receptor levels in the brain and this, in turn, led to attenuation in the development of dependence on morphine (Sanchez-Blazquez *et al.*, 1997). Also, studies with mice lacking MOP receptors showed that they exhibited reduced antinociception to DOP receptor-selective agonists compared with wild-type controls (Matthes *et al.*, 1996;

1998). Examination of DOP receptor function in these mice showed that it was comparable to that of wild-type controls, thereby suggesting that optimal DOP receptor-mediated analgesia requires the presence of MOP receptors (Matthes *et al.*, 1998). In addition, studies with mice lacking DOP receptors demonstrated that they exhibited normal morphine-mediated antinociceptive responses, although they did not develop antinociceptive tolerance to morphine (Zhu *et al.*, 1999). Taken together, these studies support the notion that the interactions between MOP and DOP receptors could play a role in the development of tolerance to morphine.

Biochemical evidence for MOP and DOP receptor heteromerization

Biochemical evidence for the formation of MOP and DOP receptor complexes was first provided by studies in recombinant systems using co-immunoprecipitation methods and epitope-tagged (Flag or *myc*) receptors (George *et al.*, 2000; Gomes *et al.*, 2000). For example, cells transfected with either *myc*-DOP receptors, *Flag*-MOP receptors or a combination of both receptors were subjected to immunoprecipitation with antibodies to the *myc* epitope tag (to enable immunoprecipitation of DOP receptors). The immunoprecipitates containing DOP receptors were subjected to Western blot analysis with antibodies to the Flag epitope to detect the presence of MOP receptors. This strategy led to the detection of a distinct signal only in immunoprecipitates from cells co-expressing MOP and DOP receptors (George *et al.*, 2000; Gomes *et al.*, 2000). Interestingly, the level of interacting MOP-DOP receptor complexes decreased when using cells co-expressing *myc*-tagged MOP receptors along with a Flag-tagged *C*-terminally truncated DOP receptor (Fan *et al.*, 2005), suggesting that the *C*-terminal region might be involved in MOP-DOP receptor heteromerization. Co-immunoprecipitation studies carried out with spinal cord membranes from wild-type and DOP receptor knockout mice detected interacting complexes only in membranes from wild-type animals (Gomes *et al.*, 2004). While these co-immunoprecipitation studies imply that MOP and DOP receptors are in interacting complexes, they do not demonstrate direct interaction between the two receptors. Biophysical assays, such as proximity-based energy transfer assays, have been used to explore the possibility of interaction (i.e. if the two receptors are in close enough proximity to directly interact) in live cells. Studies using the bioluminescence resonance energy transfer (BRET) assay, where one of the receptors is *C*-terminally tagged with luciferase (Luc) and the partner receptor is *C*-terminally tagged with either yellow fluorescent protein or GFP, showed that MOP receptors and DOP receptors are within <10 nm in live cells and therefore are in close enough proximity to associate with each other (Gomes *et al.*, 2004; Wang *et al.*, 2005; Hasbi *et al.*, 2007). This was supported by another study involving fractionation of cells co-expressing MOP receptor-Luc and DOP receptor-GFP followed by BRET assays that showed that MOP and DOP receptors were in sufficiently close proximity to directly interact in the plasma membrane (Hasbi *et al.*, 2007). In addition, this study showed that MOP receptor-Luc interacted preferentially with $G\alpha_z$-GFP in the presence of DOP receptors and with $G\alpha_i$-GFP in its absence (Hasbi *et al.*, 2007). Taken together, these results show that in live cells MOP and DOP receptors are in sufficiently close proximity to interact and that this leads to a change in receptor associated G protein from $G\alpha_i$ to $G\alpha_z$.

The use of heteromer-selective antibodies (Gupta *et al.*, 2010) or agents that disrupt heteromer formation (He *et al.*, 2011; Kabli *et al.*, 2013) has provided further support for the presence of MOP-DOP receptor heteromers in interacting complexes. MOP-DOP receptor heteromer-selective antibodies (that do not recognize individual receptors) were instrumental in demonstrating the presence of native MOP-DOP receptor heteromers in brain regions involved in pain processing (Gupta *et al.*, 2010). Additional evidence for MOP-DOP receptor heteromeric interactions comes from the use of agents that disrupt these interactions, such as membrane-permeable TAT (YGRKKRRQRRR) peptides fused to either the peptide representing TM1 of MOP receptors or to the peptide representing the distal portion of the *C*-tail of DOP receptors (He *et al.*, 2011; Kabli *et al.*, 2013). The TAT peptide fused to TM1 of MOP receptors was found to disrupt MOP-DOP receptor heteromers both *in vitro* and *in vivo* (He *et al.*, 2011). Moreover, *in vivo* disruption of the MOP-DOP receptor heteromer was found to lead to an increase in morphine-mediated antinociception (He *et al.*, 2011). Disruption of this heteromer was also observed following substitutions of the G-G-G sequence in the carboxyl terminal tail of DOP receptors or of the S-V-R sequence in the third intracellular loop of MOP and DOP receptors (O'Dowd *et al.*, 2012) or by using a TAT peptide fused to the peptide corresponding to the distal *C*-tail of DOP receptors (Kabli *et al.*, 2013). While these studies suggest that disruption of the MOP-DOP receptor might be achieved by targeting different domains (TM1, C-terminal tail), collectively they provide direct evidence in support of the presence of MOP-DOP receptor heteromers in interacting complexes.

Modulation of MOP and DOP receptor properties by heteromerization

In the following sections we describe how the binding, signalling and trafficking properties of cells expressing MOP-DOP receptors differ from those expressing individual receptors. Although these differences could be due to receptor cross-talk, the data from these studies, taken together with co-immunoprecipitation and proximity-based assay studies, provide further support for MOP-DOP receptor heteromerization.

Pharmacological and signalling properties of MOP-DOP receptor heteromers

A number of studies have examined the changes in the binding properties of selective ligands in cells and/or tissues expressing MOP-DOP receptor heteromers. In a study comparing the binding of selective synthetic agonists and of endogenous opioid peptides at MOP-DOP receptor heteromers with that at individual receptor homomers, the authors noted that cells expressing such heteromers exhibited ~10-fold decrease in affinity for selective synthetic agonists and a 2–3-fold increase in affinity of endogenous opioid peptides compared with cells expressing individual receptors (George *et al.*, 2000). Another study examined the effect of low non-

Table 1

Ligands modulating MOP-DOP receptor heteromer signalling or trafficking

Ligand	Ligand binding and signalling	Trafficking	References
DAMGO	Induces prolonged ERK1/2 activation that is blocked by β-arrestin-2 siRNA[1] Activates Ca^{2+}-mediated signalling[2] Binding is increased in the presence of a DOP receptor antagonist[3]	Internalizes the MOP-DOP receptor heteromer and this is blocked by DOP receptor antagonists[4]	[1]Rozenfeld *et al.*, 2007 [2]Charles *et al.*, 2003 [3]Gomes *et al.*, 2000 [4]Milan-Lobo and Whistler, 2011
Methadone	Not reported	Induces internalization and degradation of MOP-DOP receptor heteromer, and this is blocked by DOP receptor antagonists	Milan-Lobo and Whistler, 2011
Deltorphin II	Induces β-arrestin recruitment, and this is blocked by MOP-DOP receptor heteromer-selective antibody[5]	Induces internalization of MOP-DOP receptor heteromers[6]	[5]Gomes *et al.*, 2013b [6]Hasbi *et al.*, 2007
DPDPE, DSLET	Not reported	Does not internalize MOP-DOP receptor heteromers	Hasbi *et al.*, 2007
Bivalent ligands (oxymorphone + ENIT, naltrexone+DM-SNC80)	These ligands show low affinity at DOP receptors and enhanced affinity at MOP-DOP receptor heteromers	Not reported	Harvey *et al.*, 2012
Bivalent ligand (MDAN21)	Not reported	Does not internalize MOP-DOP receptor heteromers, and this is reversed by naltrindole.	Yekkirala *et al.*, 2013
Monovalent ligands (MA19, DN20)	Not reported	Combination of these ligands facilitates internalization of MOP-DOP receptor heteromer.	Yekkirala *et al.*, 2013
Biased agonist (CYM51010)	CYM51010 induces both β-arrestin- and G protein-mediated signalling.	Not reported	Gomes *et al.*, 2013b

signalling doses of ligands to one receptor protomer on radiolabelled agonist binding to the partner protomer, and reported that selective ligands (binding to one of the protomers) could allosterically increase the radiolabelled agonist binding to the partner protomer by affecting the rate of dissociation of the radiolabelled ligand only in cells co-expressing MOP and DOP receptors (Gomes *et al.*, 2000; 2004; 2011). These changes in the pharmacological properties of MOP-DOP receptor heteromers compared with MOP or DOP receptor homomers suggested possible differences in signalling properties between heteromers and homomers.

When the intracellular signalling between MOP-DOP receptor heteromers and MOP or DOP receptor homomers was examined, interesting properties were revealed. The occupancy of one of the protomers (by low non-signalling doses of an agonist, antagonist or inverse agonist) in the MOP-DOP receptor heteromer was found to lead to an enhancement in the signalling activity of the partner protomer (Gomes *et al.*, 2000; 2004; 2011). A study examining the G proteins associated with the MOP-DOP receptor heteromer by carrying out [^{35}S]GTPγS binding found that the receptors were associated with *Pertussis* toxin-insensitive $G\alpha_z$ in cells co-expressing both receptors and with *Pertussis* toxin-sensitive $G\alpha_i$ in cells expressing individual receptors (George *et al.*, 2000; Fan *et al.*, 2005; Hasbi *et al.*, 2007). However, another study found that DAMGO inhibited Ca^{2+}-mediated signalling in cells expressing MOP receptors while increasing *Pertussis* toxin-dependent Ca^{2+} signalling in cells co-expressing MOP and DOP receptors (Charles *et al.*, 2003); this suggested an involvement of *Pertussis* toxin-sensitive G proteins in the heteromer-mediated effects. These differences could be due to differences in experimental conditions and the type of cells used in these studies. Together, these studies suggest that MOP-DOP receptor heteromerization leads to a switch in the G protein or signalling pathway associated with the receptor. Interestingly, a study examining the localization of β-arrestin in cells expressing the MOP-DOP receptor heteromer found that the latter was associated with and signalled via a β-arrestin-2-mediated pathway (Rozenfeld and Devi, 2007). Furthermore, the heteromer-mediated signalling was shown to lead to changes in the spatiotemporal dynamics of ERK1/2 phosphorylation, including cytosolic retention of ERK1/2 leading to phosphorylation of its cytosolic substrates and resulting in differential activation of transcription factors (Rozenfeld and Devi, 2007). Taken together, these studies suggest that MOP-DOP receptor heteromerization leads to a switch in signalling and activation of different signal transduction pathways, thereby increasing the repertoire of signalling of MOP and DOP receptors (Table 1).

Trafficking properties of MOP-DOP receptor heteromers

A number of studies have examined the trafficking properties of MOP-DOP receptor heteromers. Relative to the studies examining trafficking of the heteromers from the cell surface to the intracellular compartment, fewer studies have explored

the trafficking of the heteromers from an intracellular compartment to the cell surface. In the latter case there are conflicting reports about whether MOP-DOP receptor heteromers are present only at the cell surface or if they are pre-assembled in the endoplasmic reticulum prior to trafficking to the cell surface. One study used cells where MOP receptors were constitutively expressed and where DOP receptor expression could be induced and reported that MOP and DOP receptors heteromerized only at the cell surface and this required interactions with G proteins (Law *et al*., 2005). Another study used BRET in combination with cell fractionation to show that MOP-DOP receptor heteromerization could be detected in the endoplasmic reticulum, where the heteromers were associated with $G\alpha_z$ protein (Hasbi *et al*., 2007). These differences in detection of the site of heteromerization between MOP and DOP receptors could be due to the differences in the experimental conditions as the first study used a staggered receptor expression (inducing DOP receptors expression in cells already expressing MOP receptors) while the other study used co-expression of MOP receptor-luciferase and DOP receptor-GFP. Finally, a study examining the biosynthesis and maturation of MOP-DOP receptor heteromers reported the requirement for chaperone proteins for efficient cell surface expression of this heteromer. Thus, in cells co-expressing MOP and DOP receptors, a significant portion of the heteromer was found to localize to the Golgi apparatus; heteromer expression at the cell surface required the presence of receptor transport protein 4 (Decaillot *et al*., 2008). This chaperone was found to protect the heteromer from ubiquitination and proteasomal degradation during folding and maturation (Decaillot *et al*., 2008). It is not clear if this chaperone contributes to the unique binding and signalling properties of the MOP-DOP receptor heteromer.

A number of studies have examined the trafficking of MOP-DOP receptor heteromers from the cell surface to an intracellular compartment (endocytosis). One study found that the agonist to one receptor protomer promoted endocytosis of that protomer and not of the MOP-DOP receptor heteromer (Law *et al*., 2005). Other studies found that heteromer endocytosis is probe-selective, that is, some agonists (DAMGO, deltorphin II, SNC80, methadone) but not others (D-penicillamine(2,5)-enkephalin, DPDPE; [D-Ser^2, Leu5, Thr^6]-enkephalin, DSLET) induce endocytosis of MOP-DOP receptor heteromers (Law *et al*., 2005; Hasbi *et al*., 2007; Kabli *et al*., 2010; Milan-Lobo and Whistler, 2011). Moreover, endocytosis induced by select MOP receptor agonists was found to be blocked by DOP receptor-selective antagonists (Milan-Lobo and Whistler, 2011). Additionally, endocytosed MOP-DOP receptor heteromers were targeted for degradation, in contrast to MOP receptor homomers, which were found to be recycled back to the cell surface (Milan-Lobo and Whistler, 2011). These studies suggest that heteromerization leads to changes in receptor trafficking properties. Studies using MDAN21, a bivalent MOP-DOP receptor heteromer-selective ligand, found that it did not induce endocytosis of the heteromer, while a combination of individual monovalent pharmacophores (DN20 and MA19) did (Yekkirala *et al*., 2013). This led to the suggestion that the spacer arm in MDAN21 that joins the DN20 and MA19 pharmacophores helps in effective bridging of both protomers in the MOP-DOP receptor heteromer, thereby immobilizing it and preventing its internalization (Table 1). Additional studies are needed to characterize the underlying molecular mechanisms involved in the differential trafficking of MOP-DOP receptor heteromers.

Heteromer-biased ligands

Over the last several decades a number of ligands have been identified as being selective for one opioid receptor type versus the others. It is likely that some of these ligands exhibit differential selectivity towards the heteromer as compared to individual receptors. Studies evaluating the selectivity of some classical MOP receptor and DOP receptor ligands in the context of the MOP-DOP receptor heteromer as well as signalling pathways activated by these ligands have been initiated. In addition, compounds selective for MOP-DOP receptor heteromers have also been synthesized/identified. These are described below along with their *in vivo* antinociceptive effects and possible side-effects.

Classical MOP receptor agonists

Assays to measure intracellular calcium release via chimeric G proteins or GTPγS binding via the native G proteins have been used to examine the signalling properties of classical as well as of clinically used MOP receptor agonists (DAMGO, morphine, fentanyl and methadone) in cells stably expressing either homomeric or heteromeric opioid receptors (Yekkirala *et al*., 2010; 2012). These signalling assays showed that the potency of DAMGO, morphine, fentanyl and methadone was ~7–12 fold greater at MOP-DOP receptor heteromers than at homomeric MOP receptors while showing no activity with homomeric DOP receptors (Yekkirala *et al*., 2010; 2012). These results suggested that these MOP receptor agonists are more potent at inducing signalling at MOP-DOP receptor heteromers compared to MOP receptor homomers. Moreover, the DOP receptor-selective antagonist naltrindole antagonized the signalling mediated by morphine, fentanyl and methadone only in cells expressing MOP-DOP receptor heteromers, and it also antagonized the antinociception mediated by these drugs in monkeys (Yekkirala *et al*., 2012). These findings led the authors to suggest that MOP-DOP receptor heteromers are the primary targets for the antinociceptive effects of morphine, fentanyl and methadone as well as in the development of tolerance and dependence to these drugs.

Studies also show that selective ligands activate distinct signalling pathways in cells expressing MOP-DOP receptor heteromers compared with those expressing MOP receptor homomers. For example, in cells expressing MOP receptors alone, treatment with DAMGO activates Gαi/o-mediated signalling, whereas in cells expressing MOP-DOP receptor heteromers it activates β-arrestin-mediated signalling (Rozenfeld and Devi, 2007) (see 'Pharmacological and signalling properties of MOP-DOP receptor heteromers' above). Taken with the report suggesting the involvement of β-arrestin-mediated signalling in the development of tolerance, this would suggest that the MOP-DOP receptor heteromer plays a role in this process (Bohn *et al*., 1999). Indeed, *in vivo* studies have suggested the involvement of such heteromers in the modula-

Table 2

Evidence for physiological effect of ligands targeting MOP-DOP receptor heteromers

Ligand	Experimental model	Outcome	References
Morphine	Tail-immersion test (acute treatment)	Antinociception (i.m.) Morphine antinociception (ED_{50} of 2.67 mg/kg) is lowered by pretreatment with 3.2 mg/kg of naltrindole (ED_{50} of 15.48 mg/kg).	Yekkirala *et al.*, 2012
Fentanyl	Tail-immersion test (acute treatment)	Antinociception (i.m.) Fentanyl antinociception (ED_{50} of 0.011 mg/kg) is lowered by pretreatment with 3.2 mg/kg of naltrindole (ED_{50} of 0.048 mg/kg).	Yekkirala *et al.*, 2012
Methadone	Tail-immersion test (acute treatment)	Antinociception (i.m.) Methadone antinociception (ED_{50} of 1.79 mg/kg) is lowered by pretreatment with 3.2 mg/kg of naltrindole (ED_{50} of 4.35 mg/kg).	Yekkirala *et al.*, 2012
SNC80	Tail-flick test (acute treatment)	Antinociception (i.t.) SNC80 antinociception (ED_{50} of ~50 nmol) is lowered in the MOP receptor and DOP receptor knockout mice (ED_{50} of 131 nmol and 327 nmol, respectively).	Metcalf *et al.*, 2012
Bivalent ligand (MDAN)	Tail-flick test (acute treatment)	Antinociception (s.c., i.c.v., and i.t.) MDAN21 exhibits 100 times more potent antinociception (i.c.v., ED_{50} of 0.04 nmol) than morphine (i.c.v., ED_{50} of 4.1 nmol) without development of tolerance or dependence.	Daniels *et al.*, 2005
Biased agonist (CYM51010)	Tail-flick test (acute treatment)	CYM51010 (s.c.) exhibits equipotent antinociception to morphine with lesser antinociceptive tolerance.	Gomes *et al.*, 2013b
TAT-fused peptide (MOP receptor TM1–TAT)	Tail-flick test (chronic morphine treatment)	Disruption of MOP-DOP receptor interaction by TAT-fused peptide increases morphine antinociception and decreases the development of antinociceptive tolerance.	He *et al.*, 2011
TAT-fused peptide (DOP receptor carboxyl tail–TAT)	Forced swim test Novelty-induced hypophagia Elevated plus maze	Disruption of MOP-DOP receptor interaction by TAT-fused peptide inhibits UFP-512 antidepressant-like and anxiolytic-like effects.	Kabli *et al.*, 2013
MOP-DOP receptor heteromer selective antibody	IHC, ELISA (chronic morphine treatment)	Increase of MOP-DOP receptor heteromers in various brain regions after chronic morphine treatment.	Gupta *et al.*, 2010

i.m, intramuscularly; i.t, intrathecal; s.c., subcutaneously; i.c.v, intracerebroventricularly; IHC, immunohistochemistry.

tion of morphine-mediated antinociception; these include studies showing that the antinociceptive effect of morphine (acting primarily through μ opioid receptors) is enhanced by Leu-enkephalin, an endogenous DOP receptor agonist; by FK33824, a synthetic analogue of enkephalin; and by $TIPP_{\Psi}$ (H-Tyr-Tic[CH_2NH]-Phe-Phe-OH), a DOP receptor antagonist (Lee *et al.*, 1980; Gomes *et al.*, 2004). Other studies have supported the involvement of DOP receptors in the development of tolerance to morphine; these include the demonstration that naltrindole, a DOP receptor antagonist, can block the development of antinociceptive tolerance to morphine (Abdelhamid *et al.*, 1991) and that DOP receptor knockout mice do not develop antinociceptive tolerance to morphine (Zhu *et al.*, 1999; Nitsche *et al.*, 2002). More recently, in a study using heteromer-selective antibodies, an increase in MOP-DOP receptor heteromer levels in the brain and spinal cord after chronic morphine treatment has been reported (Gupta *et al.*, 2010). Finally, a study with the TAT peptide targeting TM1 of MOP receptors (which disrupts heteromer formation) showed that pretreatment with the peptide prevents the development of antinociceptive tolerance to morphine (He *et al.*, 2011). Taken together, these findings are consistent with the idea that the MOP-DOP receptor heteromeric complex plays a role in the development of antinociceptive tolerance to morphine (Table 2).

Classical DOP receptor agonists

The involvement of MOP-DOP receptor heteromers in the antinociceptive effects of SNC80, a DOP receptor-selective agonist, has also been investigated. Studies using cells co-expressing a chimeric G protein with either opioid receptor heteromers or individual receptor homomers found that SNC80 elicited a robust release in intracellular calcium only in cells expressing MOP-DOP receptor heteromers (Metcalf *et al.*, 2012). In addition, behavioural studies showed that the antinociceptive effect of SNC80 was abolished in animals lacking MOP receptors (Sora *et al.*, 1999; Metcalf *et al.*, 2012). Furthermore, the antinociceptive efficacy of SNC80 was right-shifted by approximately threefold and sixfold in MOP or DOP receptor knockout mice, respectively (Metcalf *et al.*, 2012). Taken together, these results indicate that the presence of both MOP and DOP receptors are necessary for

the antinociceptive activity of SNC80 (Table 2). One study used a combination of highly selective MOP receptor agonists with DOP receptor antagonists (and *vice versa*) to explore MOP-DOP receptor heteromer-mediated signalling. For example, the occupancy of the DOP receptor (by selective antagonist) was found to reverse MOP receptor-mediated signalling in cells co-expressing MOP-DOP receptor heteromers from β-arrestin-mediated to $G\alpha_{i/o}$-mediated; this occupancy of DOP receptors was also found to lead to an enhancement of morphine-mediated antinociception (Gomes *et al*., 2004; Rozenfeld and Devi, 2007). These results indicate that DOP receptor ligands could function as allosteric modulators of MOP receptor activity (within the MOP-DOP heteromer). Finally, using a TAT peptide fused to the peptide corresponding to the distal *C*-tail of DOP receptors to disrupt MOP-DOP receptor heteromers, Kabli and colleagues (Kabli *et al*., 2013) reported that this led to a loss of the antidepressant and anxiolytic effects of the DOP receptor agonist UFP-512, suggesting a potential role for this heteromer in anxiety and depression.

Bivalent ligands

Attempts to synthesize heteromer-selective ligands have led to the generation of bivalent ligands such as MDAN21. This compound consists of a DOP receptor antagonist pharmacophore, DN20, separated by a 21-atom spacer from the MOP receptor agonist pharmacophore MA19 (Daniels *et al*., 2005). MDAN21 was found to exhibit 100 times higher potency as compared with morphine without significant development of tolerance or dependence (Daniels *et al*., 2005). Moreover, MDAN21 did not induce receptor internalization in cells expressing MOP-DOP receptor heteromers, probably accomplishing this by bridging both protomers and effectively immobilizing the heteromer at the cell surface (Yekkirala *et al*., 2013). Other bivalent ligands consisting of a high-affinity mu agonist (oxymorphone) linked by a spacer arm to a low-affinity delta antagonist (ENTI) or a high-affinity mu antagonist (naltrexone) joined by a spacer arm to a low-affinity delta agonist (DM-SNC80) have also been generated (Harvey *et al*., 2012). However, the antinociceptive effects of these ligands and their side-effects have not been adequately evaluated. Taken together, these studies demonstrate that bivalent ligands provide critical tools to explore *in vitro* and *in vivo* properties of MOP-DOP receptor heteromers.

Screening for ligands targeting the MOP-DOP receptor heteromer and their pharmacology in pain regulation

From studies described above, the unique pharmacological and signalling properties of MOP-DOP receptor heteromers make them potential targets for the development of new therapeutics to treat pain with reduced side-effects. This would require high-throughput screening (HTS) of large libraries of drug-like compounds, which could lead to the identification of MOP-DOP receptor heteromer-selective/biased ligands. This would require suitable screening assays. Below we describe a few of the HTS assays suitable for the screening of heteromer-selective ligands.

High-throughput screening using calcium signalling

Several assays that measure G protein-mediated signalling, such as adenylyl cyclase/cAMP, phospholipase C/Ca^{2+} and Rho assays, can be used for HTS. Of these, HTS assays that measure the release of intracellular Ca^{2+} are commonly used to screen for ligands to receptors that are normally coupled to $G\alpha_q$. Moreover, measurement of intracellular Ca^{2+} release has also been used to screen for ligands for receptors that couple to $G\alpha_i$ or $G\alpha_s$, due to the development of promiscuous chimeric G proteins such as $G\alpha_{qs}$ or $G\alpha_{qi}$, which provide a calcium readout for these receptors that do not normally signal via the $G\alpha_q$ pathway (Harvey *et al*., 2013). Thus, the activation of opioid receptors that are co-expressed with chimeric $G\alpha_{qi}$ protein can be detected by monitoring the release of intracellular Ca^{2+}.

Recently, a screening assay for the detection of heteromer-mediated signalling has been described that makes use of carboxyl-terminally truncated GPCRs fused to chimeric $G\alpha_{qi}$ proteins. These receptors, upon agonist binding, do not induce intracellular Ca^{2+} release; Ca^{2+} release is observed only when these chimeric receptors are co-expressed with the wild-type receptor (van Rijn *et al*., 2013). This method allowing for the selective detection of Ca^{2+} release by MOP-DOP receptor heteromers was developed using wild-type MOP receptors and $G\alpha_{qi}$-fused DOP receptors (van Rijn *et al*., 2013). An important advantage of this method is that in cells expressing receptor heteromers, it detects only heteromer-mediated signalling. Using this assay, ADL5859 was found to be poor at eliciting a signal from MOP-DOP receptor heteromers, compared with DOP receptor homomers (van Rijn *et al*., 2013). Thus, this Ca^{2+} signalling-based assay could be useful in the identification of heteromer-selective compounds.

High-throughput screening using β-arrestin signalling

This assay makes use of receptor activation-mediated arrestin recruitment coupled to an enzyme-fragment complementation assay leading to reconstitution of β-galactosidase activity (Figure 1). For MOP-DOP receptor heteromer screening, a cell line expressing MOP receptors *C*-terminally tagged with a small fragment of β-galactosidase and β-arrestin tagged to a complementary β-galactosidase fragment and untagged DOP receptors was generated ($MOP^{\beta gal}$-DOP cells; DiscoveRx, Fremont, CA, USA). The basic premise of this assay is that activation of DOP receptors in $MOP^{\beta gal}$-DOP receptor cells will induce the recruitment of β-arrestin to MOP receptors and reconstitution of active β-galactosidase activity. Hence, deltorphin II, a DOP receptor agonist, would bind to DOP receptors within the MOP-DOP receptor heteromer, leading to an increase in recruitment of β-arrestin to MOP receptors. The extent to which the increase in recruitment represents activation of the heteromer is then tested using the MOP-DOP receptor heteromer-selective antibody (which selectively blocks the heteromer). Such an assay was developed and showed that a significant portion (~60%) of DOP receptor agonist-mediated recruitment could be blocked by the MOP-DOP receptor heteromer-selective antibody (Gomes *et al*., 2013b). As in this assay, activation of the MOP receptor will

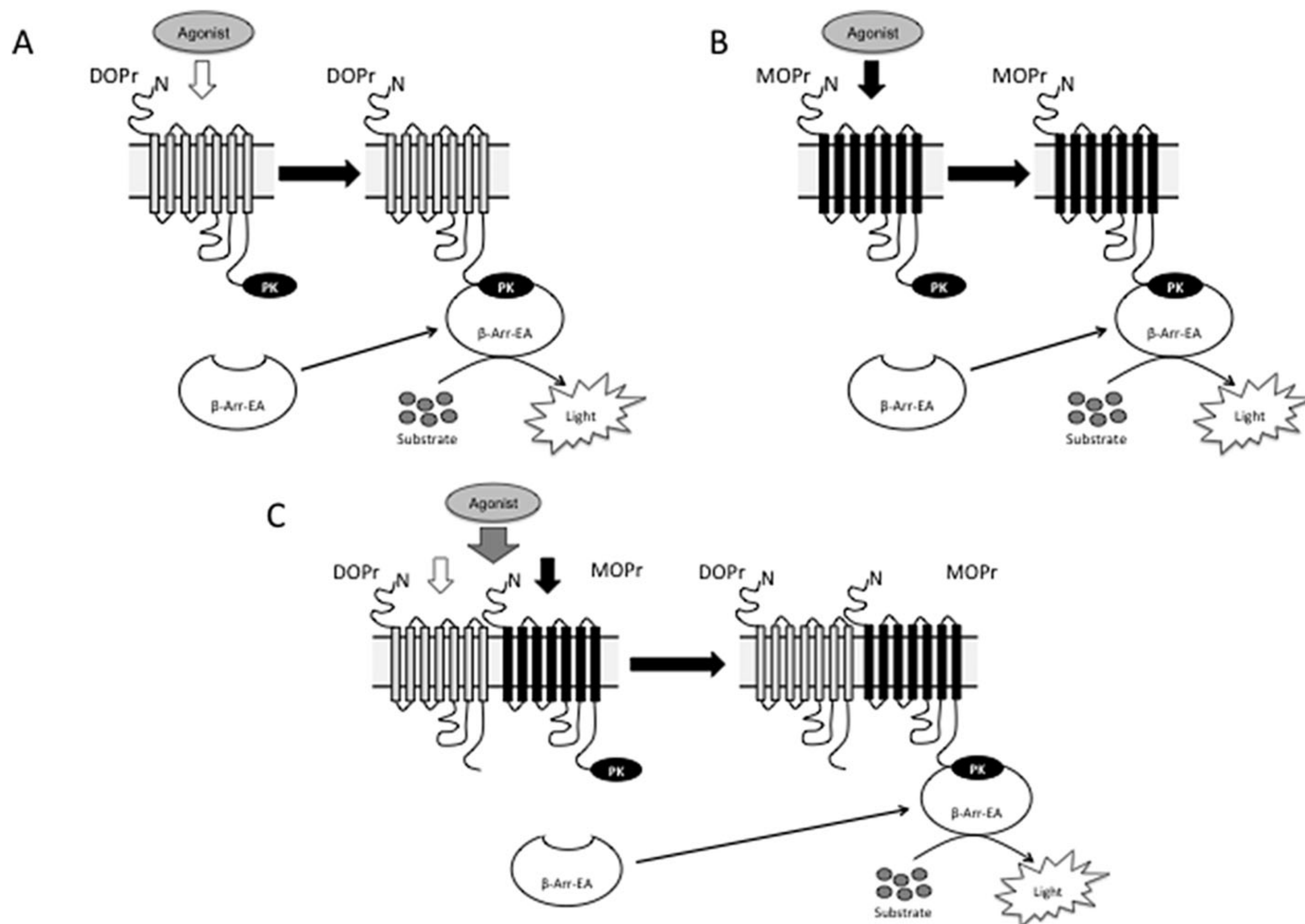

Figure 1

High-throughput screening assay for heteromer-selective ligands using cells expressing DOP^{βgal} receptor, MOP^{βgal} receptor or MOP^{βgal}-DOP receptor. (A & B) Schematics of monomer or homomer-mediated β-arrestin recruitment. Treatment of cells expressing either DOP receptor (DOPr) (A) or MOP receptor (MOPr) (B) tagged with ProLink/β-galactosidase donor (PK) (DOP^{βgal} receptor and MOP^{βgal} receptor, respectively) and β-arrestin (β–Arr-EA) tagged with a β-galactosidase activator (EA) with receptor-selective agonists leads to recruitment of β-arrestin to the receptor and reconstitution of a functionally active β-galactosidase whose activity can be measured by addition of an enzyme-specific substrate. (C) Schematic of heteromer-mediated β-arrestin recruitment. Treatment of cells expressing untagged DOP receptor and MOP receptor tagged with ProLink/β-galactosidase donor (PK) (MOP^{βgal}-DOP receptor) and β-arrestin tagged with a β-galactosidase activator (EA) with a DOP receptor-selective agonist leads to recruitment of β-arrestin to MOP receptor and reconstitution of a functionally active β-galactosidase whose activity can be measured by addition of an enzyme-specific substrate, only if both receptors form interacting complexes. Modified from Gomes *et al.* (2013b).

also lead to an increase in recruitment of β-arrestin to the receptor, all positive 'hits' need to be confirmed using counter-screens with cells expressing only MOP receptors (MOP^{βgal} receptor; DiscoveRx). A recent study used this assay to identify CYM51010 as a MOP-DOP receptor heteromer-biased agonist (Gomes *et al.*, 2013b).

The strategy used to identify CYM51010 as a MOP-DOP receptor heteromer-selective agonist involved the primary screening of a low MW library (~335 461 compounds) at a single concentration using either MOP^{βgal} receptor-DOP receptor or $5HT_{5A}^{βgal}$ cells (Pinello *et al.*, 2010; Gomes *et al.*, 2013b). Comparison of the hits between the two cell lines identified 885 hits as unique to MOP^{βgal}-DOP receptor cells; these were subjected to a secondary screen (single concentration in triplicate). This led to the identification of 346 hits as unique for MOP^{βgal}-DOP receptor cells, which were then subjected to a tertiary screen (10-point dilution series in triplicate) using cells expressing either MOP^{βgal}-DOP receptors, MOP^{βgal} receptors, DOP^{βgal} receptors or $5HT_{5A}^{βgal}$ receptors (Pinello *et al.*, 2010; Gomes *et al.*, 2013b). Comparison of the dose–response curves led to the identification of 94 compounds as potential MOP^{βgal}-DOP receptor-biased agonists; these were selected based on the criteria that they exhibited an EC_{50} of ≤10 μM with MOP^{βgal}-DOP receptor cells and a fivefold difference in EC_{50} between MOP^{βgal}-DOP receptor and either MOP^{βgal} receptor or DOP^{βgal} receptor cells (Gomes *et al.*, 2013b). For validation of the identified hits, 14 compounds selected based on their potency, efficacy and uniqueness of

structure compared with known opioid ligands were repurchased and tested in signalling assays. One of the tested compounds, CYM51010, exhibited a high intrinsic efficacy (1197 ± 31% over basal) for β-arrestin recruitment and for G protein-mediated signalling (via GTPγS binding assay; 168 ± 3% over basal) in $MOP^{\beta gal}$-DOP receptor cells compared with $MOP^{\beta gal}$ receptor or $DOP^{\beta gal}$ receptor cells (Gomes *et al.*, 2013b). Moreover, antinociception assays (tail-flick test) showed that CYM51010 exhibited antinociceptive activity similar to that of morphine and that chronic administration of this small molecule agonist resulted in less antinociceptive tolerance compared to morphine (Gomes *et al.*, 2013b). Also, the MOP-DOP receptor heteromer-selective antibody significantly, albeit partly, blocked CYM51010-induced β-arrestin recruitment, GTPγS binding and intrathecal antinociception (Gomes *et al.*, 2013b), indicating that CYM51010 exerted the majority of its effect via MOP-DOP receptor heteromer activation. The unique signalling properties of CYM51010 and its potent antinociceptive effects make it a novel candidate or a good lead compound for the development of new analgesics with lower antinociceptive tolerance compared with morphine.

Development of therapeutics: from the lab bench to clinical use

Drugs targeting MOP-DOP receptor heteromers could be novel therapeutics for the treatment of chronic pain and of mood disorders such as anxiety or depression. However, the considerable efforts to identify such novel therapeutics targeting heteromers raise the question as to when they will be clinically available. In general, drug development is a multi-year, multi-million-dollar proposition where the majority of promising compounds fail to reach the clinic. It has been reported that the average time from target identification to approval by the U.S. Food and Drug Administration (FDA) is 13.5 years (Paul *et al.*, 2010).

There are several stages in drug development, including (i) identification of a potential therapeutic drug; (ii) examination of its potential side-effects in animal models; (iii) determination of the absorption, distribution, metabolism and elimination (ADME) properties of the compound in animals; (iv) if data from (ii) and (iii) are unfavourable, examination of whether chemical modifications in the structure of the potential drug will lead to a compound with fewer side-effects and better ADME parameters; and (v) human clinical trials, which are generally carried out in three phases. Phase I trials involve testing of the drug in a small group of people to evaluate the safety and dosage range and to identify side-effects in humans. Phase II trials involve testing the drug in a larger group of people in order to test its effectiveness to treat a disease and further evaluate its safety. Phase III trials are generally conducted in large groups of people in different countries to confirm the effectiveness of the drug, monitor side-effects, compare its therapeutic effects with those of commonly used treatments and collect information that will allow the drug to be safely used. After the drug passes the clinical trials it needs to be approved by the FDA for clinical use.

The National Institute of Health RoadMap programme created the Molecular Libraries Initiative and the Molecular Libraries Screening Center Network to facilitate the establishment of translational and chemical screening programs at academic screening centers (http://www.slas.org/screeningFacilities/facilityList.cfm) (Macarron *et al.*, 2011). The use of these libraries in combination with HTS carried out using *in vitro* cell culture systems provides researchers with a rapid means to narrow down the search for potential hits. However, HTS-derived hits need to be optimized, characterized for their side-effect profile, and subjected to clinical trials and FDA approval. In general, the number of new drugs approved by the FDA is around 20 per year, and around 10 can be developed by large pharmaceutical companies, of which 20–30% would be considered 'first-class' medicines (Paul *et al.*, 2010).

In the case of drugs targeting MOP-DOP receptor heteromers, none are currently under clinical trials. A potential therapeutic for the treatment of diarrhea-predominant irritable bowel syndrome (IBS-D) was developed by Furiex Pharmaceuticals (Morrisville, NC, USA) (Breslin *et al.*, 2012; Wade *et al.*, 2012; Dove *et al.*, 2013) and is currently undergoing phase III clinical studies. This compound, named eluxadoline, is a locally active mixed MOP receptor agonist/DOP receptor antagonist with low oral bioavailability. However, the mechanism of action of this drug and whether it exerts its effect by activation of MOP receptors or of MOP-DOP receptor heteromers is not known. Further studies are needed to evaluate this. In animal models of altered gastrointestinal function, eluxadoline is able to normalize faecal output without completely blocking gastrointestinal transit, unlike the pure MOP receptor agonist loperamide (Wade *et al.*, 2012). This suggests that eluxadoline will exhibit weaker side-effects compared to loperamide (Wade *et al.*, 2012). The phase II clinical trials revealed that patients suffering from IBS-D, when given eluxadoline, showed an improvement of their symptoms based on decrease in abdominal pain and normal stool consistency (Dove *et al.*, 2013). These promising results suggest that further studies are needed to evaluate eluxadoline for the treatment of IBS-D.

Summary and perspective

Over the last decade, the heteromerization between MOP and DOP receptors has been extensively studied. These studies are helping us understand how heteromerization between these two receptor types modulates individual receptor pharmacology, signalling and trafficking properties. Most of these studies have used heterologous cells. Research using the recent generation of antibodies that selectively recognize endogenous MOP-DOP receptor heteromers and TAT peptides that disrupt MOP-DOP receptor heteromers is beginning to identify the physiological roles of these heteromers. Heteromer-selective antibodies have begun to detect the presence of MOP-DOP receptor heteromers in endogenous tissue as well as changes in the levels of these heteromers following drug administration or in a pathological condition such as development of antinociceptive tolerance, while TAT peptides are helping to elucidate the contribution of MOP-DOP receptor heteromers to morphine-mediated antinociception and development of tolerance to the drug. Moreover, efforts are being made towards the identification of MOP-DOP receptor heteromer-selective ligands. In this context, studies reveal that classic drugs such as morphine, fentanyl or metha-

done that were once thought to be conventional MOP receptor agonists in fact exhibit greater signalling potency in cells co-expressing MOP and DOP receptors, suggesting that they could be targeting heteromers. Thus studies are needed to rigorously evaluate classical MOP receptor- or DOP receptor-selective ligands with regard to their binding and signalling to receptor homomers and heteromers. It is interesting to note that binding of morphine to MOP-DOP receptor heteromers leads to β-arrestin-mediated signalling, and the latter has been implicated in the development of tolerance to morphine. In this context, the heteromer-selective ligands described in this review, both bivalent ligands and CYM51010, exhibited similar or greater antinociceptive activity compared with morphine but with lesser development of antinociceptive tolerance. Given that CYM51010 can activate both β-arrestin- and G protein-mediated signalling, this suggests that this compound is likely to be a good candidate for the development of drugs for treatment of pain and with reduced abuse liability.

Conflict of interest

None.

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