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Jolien De Neve[a], Thomas M. A. Barlow[a], Dirk Tourwé[a], Frédéric Bihel[b*], Frédéric Simonin[c*], Steven Ballet[a*]

[a] Research Group of Organic Chemistry, Departments of Chemistry and Bioengineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium; [b] Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, UMR 7200, CNRS Université de Strasbourg, Illkrich, France; [c] Biotechnologie et Signalisation Cellulaire, UMR 7242, CNRS, Université de Strasbourg, Illkirch, France

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Abbreviations

6'-GNTI, 6'-guanidinonaltrindole; 7-HMG, 7-hydroxymitragynine; AC, adenyl cyclase; Aib, 2-aminoisobutyric acid; AIM, abnormal involuntary movement; ATP, adenosine-5'-triphosphate; BBB, blood-brain barrier; BNTX, 7-benzylidenenaltrexone; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; CBM, cyclobutylmethyl; CDP, chlordiazepoxide; CHO, Chinese hamster ovary; Cl, clearance; CNS, central nervous system; CPA, conditioned place aversion; CPM, cyclopropylmethyl; CPP, conditioned place preference; DALCE, [D-Ala^2,Leu^5,Cys^6]enkephalin; DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DAG, diacylglycerol; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; DMSO, dimethylsulfoxide; Dmt, 2,6-dimethyl-tyrosine; DOR, δ-opioid receptor; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; Dyn, dynorphin ; ED_{50}, half maximal effective dose; EC_{50}, half maximal effective concentration; ECG, electrocardiogram; EFC, enzyme fragment complementation; EM, endomorphin; E_{max}, efficacy; ENS, enteric nervous system; ERK, extracellular-signaling regulated kinase; F, bioavailability; FDA, Food and Drug Administration; FRET, fluorescence resonance energy transfer; GDP, guanosine-5'-diphosphate; GEF, guanosine nucleotide exchange factor; GI, gastrointestinal tract; GPCR, G protein-coupled receptor; GPI, guinea pig ileum; GRK, G protein-coupled receptor kinase; GTP, guanosine-5'-triphosphate; GTPγS, guanosine-5'-O-[γ-thio]triphosphate; HEK, human embryonic kidney; HTS, high throughput screening; i.c.v., intracerebroventricular; i.p., intraperitoneal i.t., intrathecal; i.v., intravenous; IC_{50}, half maximal inhibitory concentration; IP_3, inositol trisphosphate; KO, knock out; KOR, κ-opioid receptor; MAPK, mitogen-activated protein kinase; MOR, μ-opioid receptor; MPE, maximal possible effect; MV, minute volume; MVD, mouse vas deferens; N/OFQ, nociceptin; NalBzOH, benzoylhydrazone; NFEPP, *N*-(3-fluoro-1-phenethylpiperidin-4-yl)-*N*-phenylpropionamide; NMDA, *N*-methyl D-aspartate; NOP receptor, nociceptin/orphanin FQ peptide receptor; NSAIDs, nonsteroidal anti-inflammatory drugs; NTB, naltriben; NTII, 5'-cyanate-naltrindole; ORL1, opioid-like orphan receptor; PAG, periaqueductal gray; PAM, positive allosteric modulator; PCT, patent cooperation treaty; PD, pharmacodynamics; Pen, penicillamine; PK, pharmacokinetics; PNS, peripheral nervous system; PWT, peptide welding technology; s.c., subcutaneous; Sal A, salvinorin A; SAR, structure-activity relationship; SBDD, structure-based drug discovery; SUD, substance use disorder; Tic, tetrahydro-3-isoquinoline carboxylic acid; V_c, volume of distribution

Abstract

One of the main challenges in contemporary medicinal chemistry is the development of safer analgesics, used in the treatment of pain. Currently, moderate to severe pain is still treated with the "gold standard" opioids whose long-term often leads to severe side effects. With the discovery of biased agonism, the importance of this area of pharmacology has grown exponentially over the past decade. Of these side effects, tolerance, opioid misuse, physical dependence and substance use disorder (SUD) stand out, since these have led to many deaths over the past decades in both USA and Europe. New therapeutic molecules that induce a biased response at the opioid receptors (MOR, DOR, KOR and NOP receptor) are able to circumvent these side effects and, consequently, serve as more advantageous therapies with great promise. The concept of biased signaling extends far beyond the already sizeable field of GPCR pharmacology and covering everything would be vastly outside the scope of this review which consequently covers the biased ligands acting at the opioid family of receptors. The limitation of quantifying bias, however, makes this a controversial subject, where it is dependent on the reference ligand, the equation or the assay used for the quantification. Hence, the major issue in the field of biased ligands remains the translation of the *in vitro* profiles of biased signaling, with corresponding bias factors to *in vivo* profiles showing the presence or the lack of specific side effects. This review comprises a comprehensive overview of biased ligands in addition to their bias factors at individual members of the opioid family of receptors, as well as bifunctional ligands.

I. Introduction

Over the past decades, G protein-coupled receptors (GPCRs) have proven to be important in drug discovery, due to human pathophysiology and their pharmacological tractability. Not surprisingly, GPCRs comprise more than 30% of all prescription drugs and nearly 40% of all FDA-approved therapeutics.[1] An important subfamily of GPCRs is the opioid receptor family (belonging to the GPCR family A: rhodopsin-like receptors).[2-3] The opioid receptors were named after the opium poppy plant (*Papaver somniferum*), from which the first opioids, i.e. morphine (1803-1806)[4-5] and codeine (1832), were extracted.[4] The pharmacological actions of opioids are mediated through three 'true' opioid and one opioid-like receptors, with the former being composed of the μ-, δ- and the κ-opioid receptors (MOR, DOR and KOR respectively),[6-10] and the latter being the nociceptin/orphanin FQ peptide receptor (NOP), also sometimes referred to as the opioid receptor-like orphan receptor (ORL1).[3, 11] The high sequence homology of NOP receptor with the other opioid receptors (>60%) places it within the 'opioid receptor family'.[12-13]

The opioid receptors are present and located in high quantity in the central nervous system (CNS), mostly expressed on prejunctional neurons. The CNS is responsible for the transmission and processing of pain-related nerve impulses, rather than mere participation in sensory perception of pain.[14-15] Since the opioid receptors occur in the midbrain, limbic and cortical structures, they may be involved in the regulation of other functions, such as stress response and memory.[16] Furthermore, they are involved in desensitization and internalization, which can account for the development of tolerance as a consequence of β-arrestin recruitment (*vide infra*), in addition to analgesic responses.[17] Alongside their presence in the CNS, opioid receptors can also be found in the peripheral nervous system (PNS). The administration of centrally acting opioid analgesics and/or nonsteroidal anti-inflammatory drugs (NSAIDS) can provide pain relief, but also produces adverse effects. The avoidance of these adverse effects through a peripheral mode of action offers an attractive means to circumvent this,[18] since peripheral opioids are not capable of crossing the blood-brain barrier (BBB), thereby avoiding CNS side effects.[19] As an example, the prevention of desensitization in the PNS, where recycling of the peripheral opioid receptors avoids the development of tolerance to opioids. This being one of the major side effects occurring with systemic administration of opioids, peripheral administration could prevent this problem.[20] Opioid receptors and their ligands also play an important role in the gastrointestinal (GI) tract, since neuronal excitability is primarily affected by opioids through interaction with neurotransmitters in the enteric nervous system (ENS).[16]

A. GPCR signaling

We will briefly turn our attention to the mechanisms by which the opioid receptors carry out their signaling, i.e. GPCR signaling processes. GPCR signaling, in general, is constitutionally controlled by three protein families: G proteins, G protein-coupled receptor kinases (GRKs) and β-arrestins (Figure 1A).[21] The intracellular heterotrimeric G protein consists of three subunits, G_α, G_β, and G_γ, and is bound to GDP in a 'rest' state or inactive state of the heterotrimer. Upon extracellular agonist binding, the active conformation of the receptor is stabilized by intracellular binding of the G protein,[22-23] followed by the exchange of GDP for GTP, catalyzed by a GEF,[21] leading to the dissociation of the G_α subunit, which is bound to GTP, and the $G_{\beta\gamma}$ subunit. Both subunits are involved in the activation and formation of downstream second messengers, e.g. cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP_3), and diacylglycerol (DAG).[21-24] Upon stimulation

of the receptor, followed by G protein activation, the receptor can be phosphorylated by GRKs on the intracellular side of the receptor, most commonly at the *C*-terminus (Figure 1B). Phosphorylation brings about the recruitment of β-arrestins, which, in turn, is responsible for the desensitization and internalization of the receptor[21-22] β-arrestins induce internalization via interaction with clathrin-coated pits, and signaling via downstream effectors, e.g. MAPKs.[21-22, 25] Initially, it was thought that G proteins were unable to interact with the receptor due to steric blocking by β-arrestins, but this was later countered by the fact that both G protein and β-arrestin can bind simultaneously to the receptor. Cryo-EM and bioluminescence resonance energy transfer (BRET) demonstrated this latter fact in a "megaplex" conformation, capable of activating G protein-signaling.[26]

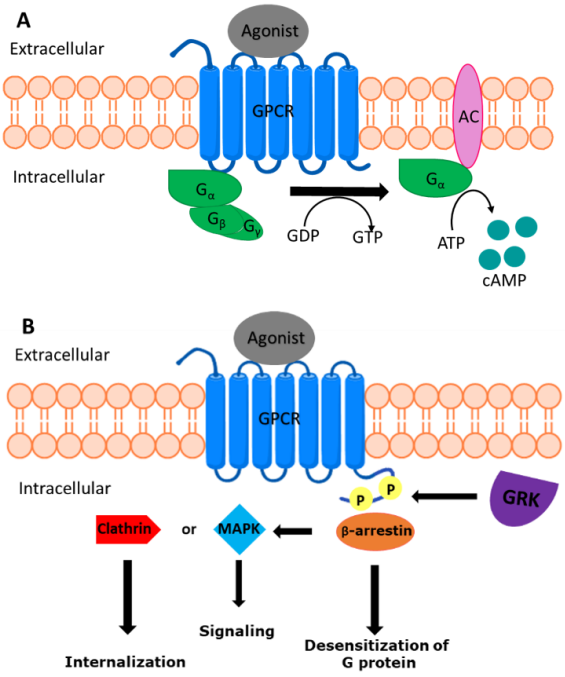


Figure 1: Signaling pathways of GPCRs. A) The G protein pathway B) The β-arrestin pathway

Within the opioid receptor subfamily, the desired analgesic effects are mediated through G protein-mediated signaling whereas adverse effects are linked to β-arrestin-2 recruitment.[27] The binding of a non-biased agonist at MOR results in an analgesic effect, along with detrimental effects such as respiratory depression, nausea, constipation, tolerance, and physical dependence.[19, 27] Analgesia is also induced at DOR, but to a lesser extent than for MOR, and DOR activation also causes severe side effects, e.g. respiratory depression,[28] anxiety,[29] convulsion, depressant effects,[19, 30] constipation and addictive liability.[31] Stimulation of KOR leads to antinociceptive, antipruritic and antiaddictive effects, but also anhedonia/dysphoria, sedation[19, 32] and anxiety,[27, 33] as well as reduced motor skills and reduced motivation.[34] NOP receptor has the most complex signaling profile and can either induce or block the analgesia depending on the method by which the ligand is administered,[13, 35] but it can also cause antidepressant-like effects.[36] Non-biased MOR agonists give way to the strongest analgesic effect, when compared to the other opioid receptors, but also the strongest side effects.

B. The μ-opioid receptor (MOR)

MOR was first cloned from rat brain cDNA by Chen *et al.* in 1993[37-39] and can be classified into μ_1, μ_2,[40] and μ_3[41]-subtypes. This classification, not only for MOR, but also for the other opioid receptors, essentially originate from classical pharmacology experiments.[40] The μ_1-subtypes are involved in

various opioid effects, such as supraspinal analgesia, decrease in acetylcholine turnover, the induction of catalepsy and prolactin release. The other subtypes, μ_2, are involved in respiratory depression, the delayed GI tract transit induced by opioids and decreased dopamine turnover.[42-43] Additionally, the presence of μ_3-subtypes in endothelial cells has been described by Stefano *et al.* who demonstrated the good binding affinities of this subtype for alkaloids (K_i < 50 nM) , but not for peptide-based ligands (K_i > 1000 nM).[41]

MOR ligands, comprising mainly opioids, are used in the clinic to treat pain. One of the most commonly known MOR ligands is morphine, which is still currently used in clinic as a drug to treat pain, both acute and chronic pain. Unfortunately, morphine also leads to constipation, tolerance, and physical dependence.[44] A milestone within the field of G protein-biased MOR ligands was the discovery of **TRV130** (*vide infra*) which progressed as far as phase III clinical trials due to its G protein-biased activity showing fewer side effects, e.g. respiratory depression[45] and gastrointestinal inhibition,[46] comparable to that of morphine.[47] Ultimately, however, the FDA declined the compound owing to concerns about safety profiles,[48] but subsequently in August 2020, **TRV130** was approved marketed as OLINVYK™, for the treatment of severe acute pain through intravenous administration. Nevertheless, there is still a lack of better pain killers, especially for the treatment of chronic pain.

C. The δ-opioid receptor (DOR)

DOR was the first opioid receptor to be cloned from mouse cDNA and monkey kidney COS cells (by Evans *et al.* and Kieffer *et al.* in 1992).[38-39, 49-50]As for MOR, so too can DOR be classified into different subtypes: δ_1 and δ_2. The δ_1-subtypes is activated by **DPDPE** and blocked by DALCE and BNTX whereas the δ_2-subtypes are activated by deltorphin II and blocked by NTB and NTII. It follows, then, that δ_1 and δ_2 differ not only in the signaling pathways to which they are coupled, but also in their structure.[51] Both subtypes are capable of inducing analgesia, but the δ_1-subtype is located in the brain and periphery, whereas the δ_2-subtype is located in the brain and spinal cord.[43] Furthermore, upon increase in DOR cell surface expression, an increase in DOR function was observed in periaqueductal gray (PAG), caudate and accumbens nuclei when testing physiologic stressor, e.g. stress-induced by forced swim test.[51-52] For chronic stress, increased DOR function was observed in the ventral tegmental area. Additionally, DORs are located as such to modulate nociceptive transmission, since they are present on the dendrites and soma of intrinsic neurons as well as on primary afferent terminals of sensory neurons. Additionally, an improved antihyperalgesic effect increasing the DOR function was induced by chronic inflammation associated with tissue injury. Furthermore, it's unclear whether DOR activation does produce rewarding effects, considering many studies report conflicting results in outcome measurements of reward and additive behaviors.[51] DOR also plays an important role in modulating different types of memory processes and hippocampal- and striatal-dependent learning, as well as motor function, motivation, and reward, with major implications for the control of cognitive performance and motor function under healthy and pathological conditions.[53]

In contrast to MOR, DOR ligands are involved in regulating anxiety and other mood disorders as well as analgesia.[51] It has been shown that the anxiolytic and antidepressant-like effects can be separated from other behavioral effects, e.g. convulsions.[30] This makes DOR ligands highly desirable for a number of therapeutic applications that differ significantly from MOR,[51] even though clinical

candidates **ADL5747** and **ADL5859** failed in phase II, since the primary endpoint (pain reduction) was not met. For this reason, further investigation was aborted.[54]

D. The κ-opioid receptor (KOR)

Another opioid receptor, KOR, was first cloned from mouse brain cDNA in the same year as MOR by Yasuda *et al.*[38-39, 55] As well as both MOR and DOR, different subtypes were also discovered for KOR based on receptor binding studies. Two variants, κ_1 and κ_2, were first described in rat and guinea pig brain. The κ_1-subtypes are discriminated by **U69,593** (*vide infra*),[56] whereas the κ_2-subtypes, at least two in both rat and human brain, are differentiated via ligand selectivity, which differs from that observed in guinea pigs.[57] In addition to κ_1 and κ_2, κ_3-subtypes have been suggested by Clark *et al.*, showing a high affinity towards naloxone benzoylhydrazone (NalBzOH), but no affinity towards **U50,488** (*vide infra*).[42-43, 58] KOR is well distributed throughout the CNS, as well as the PNS.[19] Furthermore, the presence of high levels of KOR in the nucleus accumbens shell and core, claustrum, ventral pallidum, medial habenula, caudate putamen, endoperiform nucleus, bed nucleus of the stria terminals, and amygdala was demonstrated by immunohistochemical and autoradiographic studies.[59-60] After the production of KOR in the ventral tegmental area, the receptors are transported to the nucleus accumbens and caudate putamen. Here, they are expressed on presynaptic terminals and additionally control the release of dopamine.[61-62] KOR agonists can induce antipruritic effects, due to their expression not only in the CNS, but also in the skin, since pruritus can be treated via peripherally-acting KOR agonists. Additionally, in the epidermis of atopic dermatitis and itchy psoriasis patients KOR immunostaining is downregulated.[63] KOR is widely expressed in the PNS.[19, 64] The need to cross the BBB is therefore avoided, alongside any possible CNS side effects.[19]

KOR also seems to be involved in sedation and diuresis. Interestingly, upon administration of different KOR agonists, e.g. bremazocine, ethylketazocine, tifluadom, and **U50,488** (*vide infra*) in rhesus monkeys, an increased urine output was observed, whereas this was not the case for morphine, but could be antagonized by naltrexone, **MR2266**, and quadazocine.[65] Additionally, in the pathophysiology of depression and anxiety disorders, dynorphin and KOR are present throughout limbic brain areas.[66] KOR agonists can exhibit hallucinogenic effects, as is the case with salvinorin A (*vide infra*),[67] in addition to antipruritic and analgesic effects. Furthermore, KOR agonists are able to induce anhedonia, dysphoria and anxiety. A well-known natural product still used today is menthol, an antipruritic/analgesic compound activated through the central κ-opioid system [68] used in ointment form, to treat abrasions.

E. The nociceptin/orphanin FQ peptide receptor (NOP receptor)

The last opioid receptor, NOP receptor, was discovered many years after the three 'classical' opioid receptors. Mollereau *et al.* cloned the receptor with high homology towards the other opioid receptors.[69] In mice CNS, NOP receptor transcripts are mainly expressed in the limbic areas, hypothalamus, brainstem and spinal cord, meaning they are potentially endowed in different central functions. Mollereau and coworkers suggested that NOP receptor could regulate neuroendocrine secretion in the hypothalamo-pituitary axis, together with regulation of nociception in the central gray and dorsal horn of the spinal cord in addition with emotions, behaviors and memory in the limbic areas.[69] The effects of the administration of the endogenous peptide nociceptin (**N/OFQ**) on nociception were summarized by Mogil and Pasternak, who highlighted a number of different phenomena, e.g. anxiolytic effects, hypotension, induction of withdrawal symptoms, controversial

pain effects, inhibition of bronchoconstriction, etc. Moreover, these effects suggest a wide range of application at NOP receptor.[70]

NOP receptor has some interesting properties of its own that, again, are very different from what is seen in the classical opioid receptors. NOP receptor agonists have been investigated for their activity against pain, abuse, anxiety and cough.[71] NOP receptor can either induce or block analgesic effects depending on the route of administration of its agonists.[13, 35] Under conditions of opioid-induced analgesia, **N/OFQ** can block opioid-analgesic effects, or regulate the analgesic effect by antagonizing the μ-opioid-induced analgesia resulting in a reduction of hyperalgesia.[35, 72] The first clinical evidence for this was proven by the fact that increased nociceptin levels, **N/OFQ** at NOP receptor, were found in the cerebrospinal fluid of parkinsonian patients.[73-74] Furthermore, administration of NOP receptor antagonists, just before administration of morphine, increased tail flick latency, which illustrated the blocking of tolerance.[71] In mice, upon **N/OFQ** administration, tail flick latency was not decreased and blocked intracerebroventricular injection-induced analgesia.[71] Sadly, upon chronic administration of NOP receptor agonists, attenuation of anti-allodynic and analgesic effects occured.[75] On the other hand, NOP receptor antagonists have been examined for their activity towards depression, and additionally motor symptoms in Parkinson's disease.[74]

F. Biased agonism

As a result of these adverse effects, the search for better and safer opioid analgesics has been expanded over the past decades, in the form of biased agonists.[21, 26-27, 76-78] Nowadays, biased agonism has gained serious interest in modern drug discovery, as fine-tuned GPCR ligands have the potential to improve existing therapies through, for example, the exclusion of side effects.[76] GPCR ligands have been classified based on their efficacies or potencies for activation of G proteins dependent on their ability to provoke a receptor response.[24] These ligands are able to engage distinct motifs in the GPCR structure to stabilize one of a number of discrete active conformations which favor the activation of one signaling pathway over the other.[79] The word "bias" implies an inherent inequality and therefore needs to be applied to a pleiotropically linked receptor.[80] The term "biased agonism" describes the ability to selectively activate one cell signaling pathway of the receptor over another (Figure 2).[24, 26, 76-77] This can also be referred to as "functional selectivity".[24]

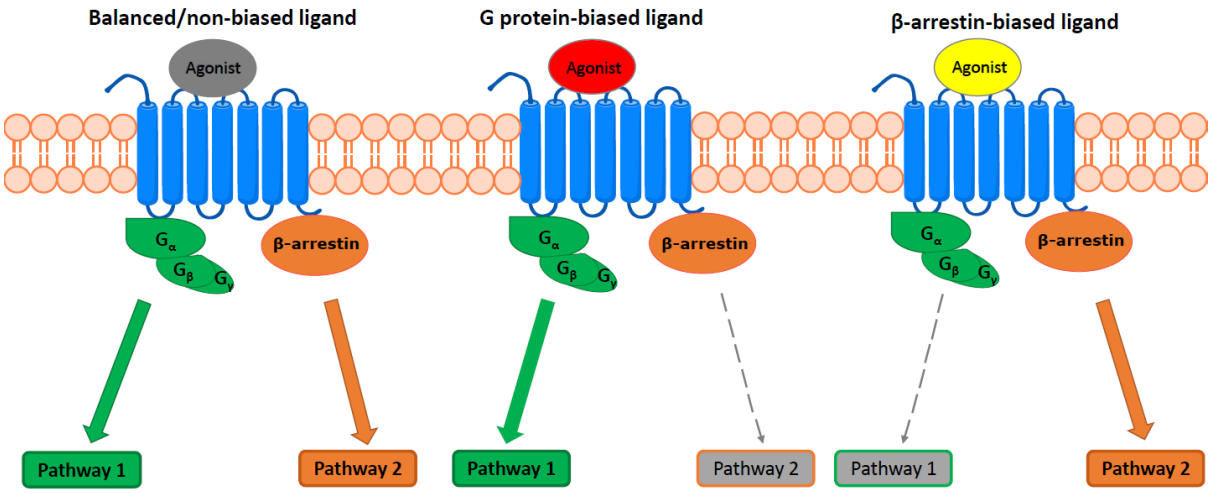


Figure 2: Signaling of balanced agonists versus biased agonists, adapted from Conibear & Kelly[81]

The very first arguments about biased signaling came from the idea of developing more effective and selective therapeutics, in particular antipsychotics.[80] This led to the development of aripiprazole, an agonist of the dopamine D_2 receptor. While eliciting the desired response, aripiprazole did not promote internalization[82] and was therefore classified as a biased agonist.[80] Depending on the GPCR, a specific pathway – for instance the G protein or the β-arrestin pathway (Figure 2) – is preferred for a biological response. For example, carvedilol is a non-selective $\beta_{1/2}$-adrenoceptor antagonist used for congestive heart failure. It stimulates phosphorylation and internalization of the receptor and β-arrestin translocation, while it fails to activate G protein and thus serves as a β-arrestin-biased ligand, since this is the pathway with the wanted biological responses.[83-84] The ability of biased ligands to discriminate between G protein and β-arrestin-mediated responses at receptor level should ease selective commitment of a group of signals from a specific GPCR. To determine the therapeutic potential of the different pathways, i.e. G protein and β-arrestin, the biggest obstacle is the lack of knowledge concerning the roles of the specific pathways in terms of signaling for both health and disease.[85] For opioid receptors specifically, the G protein pathway is preferred, since it leads to the analgesic responses, whereas the β-arrestin pathway leads to the undesired effects (*vide supra*).[27] Despite many advantages, a number of questions remain: the first among these is whether the observed biased responses originate from the partial agonism of the ligand or from an actual inherent bias. Importantly, recent reports have questioned the importance of β-arrestin-2 in the development of side effects associated with the administration of opioids.[86-90] In one study, Kliewer *et al.* demonstrated an increase in analgesia, and decrease in tolerance, but at the same time worsening the other opioid side effects in phosphorylation-deficient G protein-biased MOR.[90] In addition, the authors were unable to replicate the original data regarding the results obtained in β-arrestin-2 KO mice with morphine.[89] In 2020, Gillis *et al.* showed that opioids with improved side effect profiles can be obtained by low intrinsic efficacy for G protein activation, rather than from a bias itself.[86] Furthermore, the role of biased agonism in GPCR drug discovery is taking an increasingly prominent role, but is accompanied by additional complexities in the search for safer drugs.[24] Importantly, to date, a major complication in this process has been the translation of *in vitro* profiles of biased signaling into *in vivo* systems, which is still lacking an efficient link. This is due, in part, to the many differences in physiological systems upon measuring bias. It remains an fundamental challenge to deconvolute *in vivo* biological responses towards the GPCR signaling pathways.[24, 80, 91] Besides a bias towards G protein and β-arrestin, and since opioid receptors can couple to multiple G proteins, the possibility of an intra-G protein bias also exists. However, since a treatment of this topic goes beyond the scope of this review, no further details on this will be discussed. Interested readers are, however, directed to work of Tso and colleagues dealing with this issue.[92]

In recent years it has also become clear that alongside spatial and qualitative parameters at play in dynamic signaling events, there is also a temporal dimension to be considered. A number of discoveries have shown that cells are able to use ligand residence times, kinetic scaffolding and oscillatory phenomena (among others) to introduce a time-encoded dimension into their signaling. These dynamics are still being fully elucidated and will not feature in the present discussion. Interested readers are, however, referred to the illuminating review of Grundmann and Kostenis.[93] In this review, biased ligands of all four opioid receptors, in addition to bifunctional biased opioid ligands, will be discussed. Even though G protein-biased ligands at the opioid receptors are favored, a number of β-arrestin-biased ligands are provided, all of which will be compared to a given reference ligand for the specific receptor.

II. Measuring bias *in vitro* and calculation of bias

When considering biased agonism or biased responses, three components contribute to the overall effect, namely biased ligands, biased receptors, and system bias. Biased ligands are compounds that selectively enhance one signaling pathway versus others, compared to a reference ligand. Biased receptors, on the other hand, are capable of producing bias in their signaling profiles by differences in receptor structure or conformation compared with the 'wild-type' receptor. In contrast, a system bias means biased signaling directed by the relative expression of receptor transducers, such as increased expression of the G protein, GRKs or β-arrestins.[21, 94] Biased agonism can be determined via *in vitro* measurements of specific ligands. Via different read-outs, such as quantification of GTPγS or cAMP levels, G protein-signaling can be determined, while for β-arrestin signaling, the GRKs expression or β-arrestin recruitment is measurable.[33] To assign *in vitro* bias, it is necessary to quantify the ligand bias which can be calculated with one of the following equations:

Equiactive comparison, analogous to the method of Furchgott (1966): [24, 95-96]

$$1) \quad \beta = \log\left(\frac{RA_{12,\text{lig}}}{RA_{12,ref}}\right) = \log\left(\left(\frac{E_{max,1}}{EC_{50,1}}\,\frac{EC_{50,2}}{E_{max,2}}\right)_{lig} x \left(\frac{E_{max,2}}{EC_{50,2}}\,\frac{EC_{50,1}}{E_{max,1}}\right)_{ref}\right)$$

Equation 1: The equiactive comparison with β: bias factor; RA: relative activity; 1 & 2 are pathways; E_{max}: efficacy; EC_{50}: potency; lig: ligand; ref: reference ligand

Operational model based on Black and Leff model (1983):[24, 91, 97-99]

$$2) \quad \beta = \Delta\Delta \log\left(\frac{\tau}{K_A}\right) = \left(log\left(\frac{\tau}{K_A}\right)_{lig} - \left(\frac{\tau}{K_A}\right)_{ref}\right)_{pathway1} - \left(log\left(\frac{\tau}{K_A}\right)_{lig} - \left(\frac{\tau}{K_A}\right)_{ref}\right)_{pathway2}$$

Equation 2: Operational model with β: bias factor; τ: efficacy; K_A: equilibrium dissociation constant; (τ/K_A): transduction coefficient; lig: ligand; ref: reference ligand

Of note, the antilog of the bias factor β (10^{β}) of equation 2 has also been described as a measure of bias.[98] In cases where the bias factor β is greater than zero (Table 1), the ligand is biased towards pathway 1. When β is smaller than zero, on the other hand, the ligand is biased towards pathway 2. In terms of the antilog, however, this either gives values greater than one or between zero and one respectively. For the purposes of this review, the biased values provided, correspond to the β values for equations 1 or 2.

Table 1: The bias towards a certain pathway is dependent on the sign of the bias factor β

Bias factor β	G protein pathway	β-arrestin pathway
Smaller than zero	/	**Unfavored bias**
Greater than zero	**Favored bias**	/

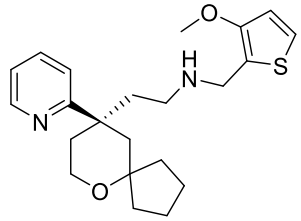
It should be noted that equation 1 is more accessible, since only E_{max} and EC_{50} values are needed, whereas in equation 2 a wider range of data (e.g. binding affinity data) is required.[98] Importantly, bias factors can differ drastically when changing between the two equations, when switching reference ligand or when using a different assay. This will be shown throughout the review upon discussion of the various ligands as well as in the extensive table in the supporting information.

In the next paragraphs, biased ligands with their bias factors will be discussed using equations 1 and 2. Additionally, an extensive overview of the bias factors of their discussed ligands are tabulated in the supporting information.

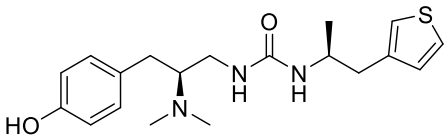
III. Biased ligands

A. Biased μ-opioid receptor ligands

Though the μ-opioid receptor induces the most and strongest adverse effects, it also induces the most powerful analgesic effects. The development of G protein-biased μ-opioid receptor ligands is therefore of great therapeutic importance as the G protein pathway is involved in antinociception, whereas the β-arrestin pathway is involved for the undesired side effects, e.g. tolerance, physical dependence, nausea, constipation and respiratory depression.[19] To determine the biased activity of MOR ligands, reference ligands are used to compare functional activities. These reference ligands for MOR in the literature are generally morphine, oxycodone, fentanyl, and mainly **DAMGO**. Different biased μ-opioid receptor ligands are listed and discussed briefly.



TRV130



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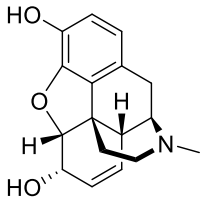
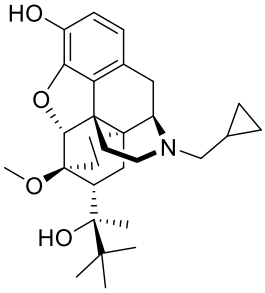


Morphine



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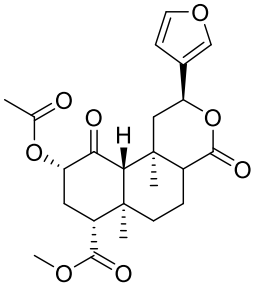

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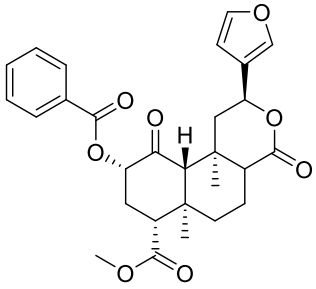
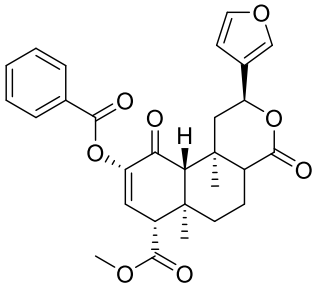
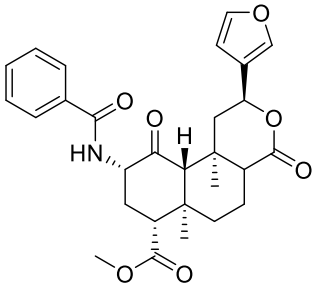


Herkinorin



Kurkinorin



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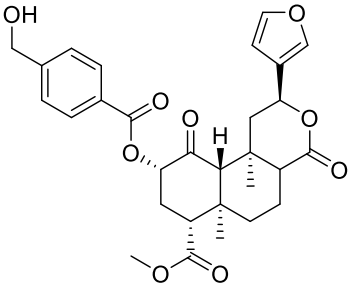

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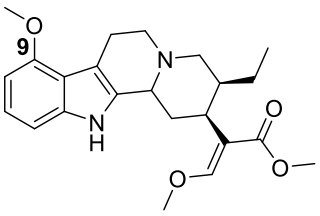
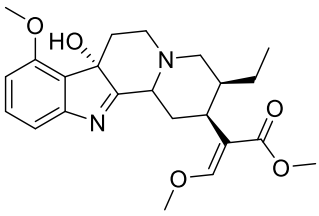
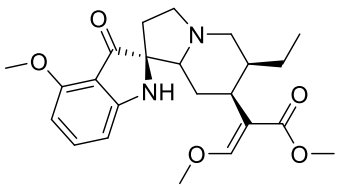


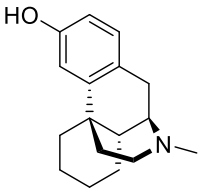
Mitragynine



7-HMG



Mitragynine pseudoindoxyl



Levorphanol

Figure 3: Structures of biased MOR ligands and reference ligands – part 1

1. Oliceridine (**TRV130**) and **TRV734**

Oliceridine ((*R*)-**TRV130**, Figure 3) is a small molecule G protein-biased MOR agonist which induces very little β-arrestin-2 recruitment in rodent models.[100] It was discovered by Chen and coworkers via structure-activity relationship (SAR) studies and later developed by Trevena. The *S*-enantiomer of **TRV130** showed a strong bias towards G protein-signaling, as it failed to recruit any of the β-arrestins, but unfortunately, the G protein activation by the *S*-isomer was also 90-fold lower than for the *R*-enantiomer. The stereochemistry is therefore of great importance for the binding kinetics.[101] In other studies, **TRV130** showed an EC_{50} of 8 nM and an E_{max} of 83% for G protein coupling using a cAMP accumulation assay and an E_{max} of 14% for β-arrestin-2 recruitment in hMOR compared to morphine (EC_{50} ≈ 50 nM). *In vivo* studies indicated that **TRV130** reduced the effects of respiratory depression and constipation in rats relative to morphine, upon rat blood gas and glass bead colonic motility assays respectively. Furthermore, morphine caused a statistically significant increase in pCO_2, whereas **TRV130** did not induce this effect even at 8-fold higher equianalgesic doses. The quantification of bias was performed using the equiactive comparison equation (Equation 1) resulting in a bias factor of 3 for **TRV130**; that is to say, **TRV130** is 3-fold more biased towards the G protein pathway than morphine.[45] Later studies by Burgueno *et al.* calculated a bias factor using the operational model of Black & Leff (Equation 2), providing a value of 1.64, when compared to morphine.[102] From this can be concluded that using both equations can give a sizeable difference in bias factors, even when using the same reference ligand. Additionally, Altarifi and coworkers published further proof of reduced side effect profile of **TRV130**. The authors demonstrated antinociception upon repeated administration, along with gastrointestinal inhibition and an abuse liability similar to morphine.[46] In the same study, the authors also demonstrated the inactivity of the (*S*)-isomer.[46] Despite the promising *in vitro* and *in vivo* studies,[45-46] the first clinical trials showing favorable pharmacokinetics (PK), pharmacodynamics (PD), safety, and tolerability results and favorable side effects profiles,[103-104] and successfully completing phase IIa and IIb,[105-106] the results of phase III clinical studies with patients suffering from moderate-to-severe post-operative pain were less gratifying, as no statistical significance was obtained in terms of analgesia compared to morphine, but a safety and tolerability profile was observed with regard to respiratory and gastrointestinal adverse effects compared to morphine.[47, 107] However, due to concerns about potential cardiac side effects (QT interval prolongation on the electrocardiogram (ECG)), the FDA advisory committee did not approve **TRV130**.[48] Supplementary safety data were then provided by Trevena, and in August 2020, **TRV130** was approved by the FDA, marketed as OLINVYK™, as a new chemical entity approved in adults for the management of acute pain severe enough to require an intravenous opioid administration.

TRV734, a close analog of **TRV130**, is an orally bioavailable G protein-biased μ-opioid receptor agonist developed by Trevena and currently in Phase I trials. The results of the first-in-human trials were published in early 2020 by James et al.[108] **TRV734** was shown to be safe and well-tolerated at single doses of 2 to 250 mg. Within this range, acceptable PK were demonstrated with a minimal effect of food on its absorption. The preliminary PD data indicate that concentrations after single doses of >80 mg may be effective for pain relief.[108]

2. **PZM21**

PZM21 (Figure 3) is a small molecule, reported by Manglik *et al* from the authors' own structure-based drug discovery (SBDD) efforts.[109] A docking campaign consisting of more than 3 million commercially available lead-like molecules from the ZINC database[110] docked into the orthosteric

pocket of the inactive MOR, led to a refined subset of 2500 compounds from which 23 highest-scoring molecules were selected. The compound with the highest potency was then optimized, resulting, ultimately, in **PZM21**, which showed affinities towards MOR (K_i = 1.1 nM), DOR (K_i = 506 nM), and KOR (K_i = 18 nM). In mice, **PZM21** produced a level of analgesia with a maximal possible effect (MPE) of 87% in a hotplate test, reached 15 min after administration of 40 mg/kg. This result was similar to **TRV130** and morphine (but used at lower doses of 1.2 mg/kg and 10 mg/kg respectively). **PZM21** showed no analgesia in the tail-flick assay compared to morphine, an unprecedented distinction among opioid analgesics. Respiratory depression was explored by measuring the respiration by whole-body mouse plethysmography, (which measures changes in the volume of the body due to differing amounts of air in the lungs). While the respiratory frequency decreased 20 min after administration of morphine, an equianalgesic dose of **PZM21** led to no effect on the respiration versus the vehicle. Weak β-arrestin recruitment, which was not quantifiable, was observed using a BRET assay, even with overexpressed GRK2 which resulted in an E_{max} of 32% of β-arrestin recruitment. Additionally, a minimal level of the MOR internalization with an E_{max} of 8% was obtained relative to **DAMGO** and morphine (E_{max} of 100% and 42% respectively) and comparable to **TRV130** (E_{max} of 9%).[109] However, these findings were countered by Hill *et al.* who reported that **PZM21** had low efficacy on G protein coupling (E_{max} of 39%) in comparison to **DAMGO** and morphine (E_{max} of 100% and 55% respectively) using a BRET assay.[111] Nevertheless, **PZM21** did produce antinociception upon administration of a 40 mg/kg dose using a hot-plate test, but alongside prolonged respiratory depression was reached after 10-15 min upon subcutaneous (s.c.) administration by measuring minute volume (MV) of breathing air, in the same way as morphine at equianalgesic doses. Moreover, it was noted that tolerance to antinociception by **PZM21** was developed in male mice upon receiving twice-daily doses for four days similar to morphine. After two days, the MPE was less than 40% and after the fourth day less than 10%.[111] In addition, a more recent study demonstrated that **PZM21**, but also **TRV130** and buprenorphine (*vide infra*), generate less respiratory depression at equiactive doses as compared to morphine and fentanyl, using the same test – i.e. the whole-body mouse plethysmography – as described by Manglik *et al*.[86] The discrepancies seen in the different studies above reflect the difficulties inherent to the interpretation of *in vivo* biased signaling profiles of *in vivo* profiles.

3. Morphine and morphine-like compounds

Morphine (Figure 3) is undoubtedly the most famous naturally occurring opioid, extracted from the opium poppy plant by Friedrich Sertürner at the beginning of the 19th century[4-5] and is still ubiquitously found in clinical settings over the world. It is used to treat severe pain or for anesthetic purposes, even though its therapeutic use is accompanied by several severe side effects including respiratory depression and physical dependence.[13] The role of β-arrestins in the occurrence of these side effects at MOR was first discovered in β-arrestin-2 knock out (KO) mice, which experienced less respiratory depression and constipation upon acute morphine administration. On the other hand, chronic administration of morphine to these KO-mice led to desensitization and tolerance as compared to wild-type mice.[44, 112-114] Two groups independently reported a contradictory bias of morphine towards both G protein and β-arrestin-2 recruitment.[76, 99] Thompson and coworkers quantified a bias factor of -0.99 towards β-arrestin-2 recruitment using a GTPγS assay, whereas the group of Schmid *et al.* quantified a bias factor of 0.11 towards G protein using the same type of assay, but a bias factor of -0.21 towards β-arrestin-2 recruitment was obtained when adopting the

cAMP assay. These bias factors were each determined on hMOR, with **DAMGO** as control and each using the operational model of Black and Leff (Equation 2) for the calculation.[76, 99]

Buprenorphine (Figure 3) is a semi-synthetic derivative of the naturally occurring alkaloids thebaine and morphine, and it serves as a mixed opioid acting at both MOR and NOP receptor,[115] but it also shows affinity towards KOR and DOR.[116] Buprenorphine is currently used in the clinic to treat opioid dependence.[117] The pharmacology of buprenorphine continues to be widely discussed within the research community, with some of the opinion that buprenorphine acts as a partial MOR agonist compared to morphine,[45] or describing buprenorphine as a mixed MOR/NOP receptor partial agonist,[118] whilst others report buprenorphine-mediated biased agonism. Burgueno and coworkers have demonstrated the G protein-biased agonism at MOR, relative to morphine, with subsequent quantification of the bias factor of 1.84, using the operational model (Equation 2).[102] More recently, buprenorphine was also defined as a G protein-biased agonist as it failed to recruit a significant amount of β-arrestins.[101] As a result of this, no bias factor could be calculated.[101] With no clear view on whether buprenorphine is a biased or a mixed partial agonist, it is difficult to understand whether the pharmacologic profile derives from the partial agonism or from an actual bias.[81]

Levorphanol (Figure 3) is a potent analgesic with agonist activity not only at MOR but also at DOR and KOR. Levorphanol shows NMDA antagonism, and because of its underutilization has been called 'the forgotten opioid'.[119-120] It was first approved for clinical use in the USA in 1953 as a treatment for moderate to severe pain.[121] This morphine-like compound was reported as a G protein-biased agonist for two 6 transmembrane MOR splice variants of mice, relative to **DAMGO**, together with reduced respiratory depression and incomplete cross-tolerance with both morphine and oxycodone. These splice variants were obtained by 5' splicing of the *Oprm1* gene, the gene that encodes the synthesis of the MOR protein. The bias factors obtained for splice variants MOR-1E and MOR-1O were 1.2 and 9.4 respectively, using the operational model (Equation 2). More importantly, levorphanol acts also as a β-arrestin-biased agonist at the normal 7 transmembrane MOR with a bias factor of -2.6.[122]

Consideration of the structures of the compounds mentioned above shows quite clearly that even small structural changes can lead to ligands with preferred signaling, i.e. β-arrestin-biased or unpreferred ligands. Additionally, even previously described MOR ligands ultimately appear as biased ligands, hence their pharmacology has to be reinvestigated at the light of this information.

4. Herkinorin and herkinorin-like compounds

Herkinorin (Figure 3) is derived from the selective KOR Salvinorin A (**Sal A**), which is a naturally occurring active ingredient from the hallucinogenic plant *Salvia divinorum*.[67, 123-125] Herkinorin was the first non-nitrogenous μ-opioid agonist discovered and has a greater affinity for MOR than KOR (μ/κ = 0.13-fold).[124] In 2007, Groer *et al.* reported the biased activity of herkinorin. The fact that herkinorin causes activation of G protein coupling and ERK1/2 phosphorylation in a naloxone-reversible manner yet does not cause β-arrestin recruitment and internalization suggests that herkinorin is a G protein-biased MOR agonist.[123, 126] Previous *in vivo* studies by Lamb *et al.* showed that upon treatment with herkinorin in morphine-tolerant rats, antinociceptive efficacy was still observed.[127] Another study demonstrated that herkinorin activated MOR receptor without recruiting β-arrestin-2 in primary sensory neurons.[128] Nevertheless, Manglik *et al.* have more recently reported the β-arrestin recruitment of herkinorin in a set of studies in which it and **TRV130** are compared to the effects of **PZM21**, **DAMGO**, and morphine. The results of these studies (E_{max} of 112 % and 104 %

in overexpressing GRK2 BRET assay) pointed to full agonistic activity of herkinorin with similar efficacy as **DAMGO** (E_{max} of 100 % in both cases).[109] On the other hand, another study has shown that herkinorin is a partial agonist of MOR.[129] As the latter authors themselves point out, the most likely explanation for the different result is that the different assays used to assess G protein activity have different sensitivities and dynamic ranges.

Kurkinorin (Figure 3) is a herkinorin analog, also derived from the KOR-selective ligand **Sal A**, with only a double bond of differing in their structures. It showed high potency (EC_{50} = 1.2 nM) and was reported as a selective MOR agonist over DOR and KOR (δ/μ = 63-fold and κ/μ > 8000-fold). In addition to this selectivity, kurkinorin was described as a G protein-biased ligand for MOR with a corresponding bias factor of 0.57 compared to **DAMGO** using the equiactive equation (Equation 1). From *in vivo* experiments, it was demonstrated that kurkinorin exhibited reduced tolerance, sedation and rewarding effects compared to morphine. These observations are interesting and counterintuitive, since kurkinorin recruited more β-arrestin-2 than morphine.[130]

Herkamide (Figure 3) is the benzamido-derivative of herkinorin, synthesized by Tidgewell *et al.* in 2008. They reported the high affinity of herkamide (K_i = 3.1 nM) towards MOR over DOR and KOR (δ/μ = 261-fold and κ/μ = 2397-fold), in addition with a 4-fold higher affinity towards MOR than herkinorin.[131] The bias factor of herkamide was calculated using the equiactive equation (Equation 1) with kurkinorin, also compared to **DAMGO** and led to the corresponding value of 0.32, making herkamide a G protein-biased ligand for MOR.[130]

Recently, Crowley *et al.* reported a series of kurkinorin derived compounds. The most promising compound developed was **25** (Figure 3), containing a 4-hydroxymethyl benzoate group. **25** demonstrated the best potency of all analogs (EC_{50} = 0.03 nM) for MOR, being 100 times more potent than for KOR. Additionally, it proved to be five times more potent to MOR than fentanyl (*vide infra*). When compared to **DAMGO**, **25** displayed a bias towards the G protein pathway, represented with a bias factor of 0.14, calculated using the equiactive equation (Equation 1). Consequently, *in vivo* studies proved the potent analgesic effects, as well as the lack of significant tolerance.[132]

It is noteworthy that all of the herkinorin-like compounds described above lack a basic nitrogen which is present in many other opioid ligand classes.

5. Mitragynine and mitragynine-like compounds

Mitragynine (Figure 3) was the first isolated alkaloid from the medicinal plant *Mitragyna* (also known as kratom).[133] Kratom can be used as a stimulant and produces opioid-like analgesic effects.[134] A total of 25 different alkaloids have been found in kratom leaves, all of which are analogs of mitragynine, which itself is the most abundant (comprising around 60% of the isolate).[135] A number of pharmacological studies showed that mitragynine exhibits mixed μ-agonist/δ-antagonist activity.[136-137] Murine models showed slow development of tolerance and a marked decrease of physical dependence as well as the inability to recruit β-arrestin-2 (in fact, because of the weak response during β-arrestin recruitment experiments, the authors were not able to calculate the bias factor[138]). These results are particularly noteworthy because the authors undertook more rigorous testing of tolerance than is commonly seen in the literature i.e. over a much-extended timeframe, yet still found significant reduction in antinociceptive tolerance. How exactly this mixed receptor activity contributes to the beneficial pharmacological profile of the molecule is not fully elucidated and remains under investigation.

Mitragynine's chemical structure represents an excellent springboard for further diversification efforts, and, to this end, the authors' own SAR studies revealed that substitution at the C-9 position has the most dramatic effects being able to switch between the partial-agonistic and antagonistic activities at MOR but also being able to module activity at DOR.

The second most abundant alkaloid extracted from the kratom plant is 7-hydroxymitragynine (**7-HMG**; Figure 3) – a selective and full agonist at MOR.[135] It showed a 46- and 13-fold higher potency than mitragynine and morphine respectively.[134] Similar to mitragynine, **7-HMG** demonstrated slow tolerance development, a decrease of physical dependence, and did not recruit β-arrestin-2.[138] Compared to morphine, **7-HMG** was 5-fold more potent in the antinociceptive effect.[136] In addition, this study focused on an oxidized rearrangement product of mitragynine, viz. mitragynine pseudoindoxyl (Figure 3). Complementary to mitragynine and **7-HMG**, mitragynine pseudoindoxyl failed to recruit β-arrestin-2, produced tolerance in a slower rate than morphine, together with limited respiratory depression, constipation and physical dependence, while still showing potency in a GTPγS assay at MOR with an EC_{50} of 1.7 nM and E_{max} of 122% compared to **DAMGO**.[136]

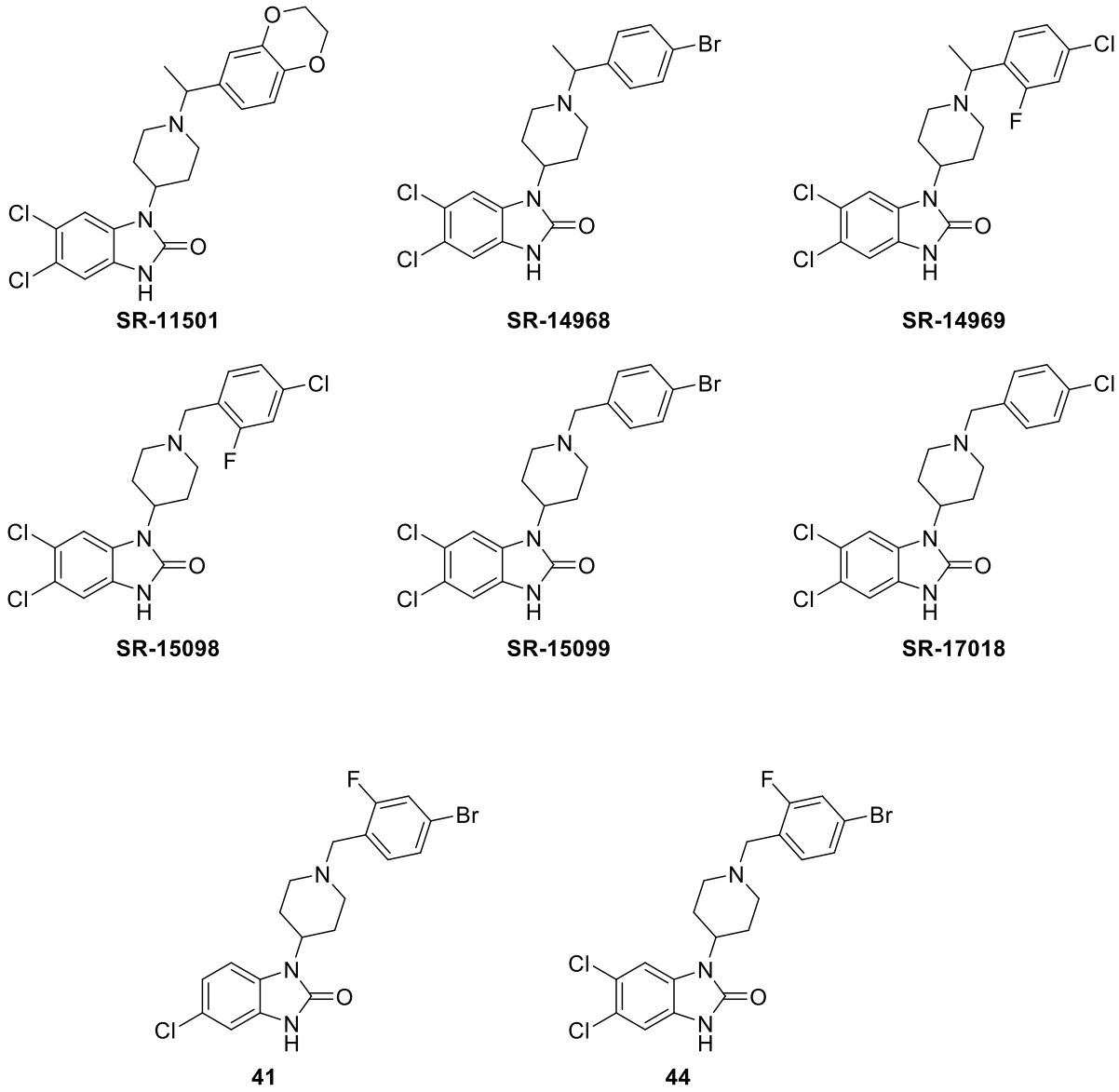


Figure 4: Structures of biased MOR ligands: SR-compounds– part 2

6. SR-compounds

The SR-compounds (Figure 4), developed by Schmid *et al.*, are a series of piperidine-based molecules,[99] bearing a slight relation to bezitramide, itself an opioid analgesic used to treat severe, chronic pain.[139] The SR-compounds, with the exception of **SR-11501**, were described as G protein-biased ligands. Notably they show reduced respiratory depression whilst still inducing antinociception in rodent models relative to **DAMGO**, fentanyl, and morphine. The authors quantified the bias factor of these compounds using the operational model (Equation 2) both on hMOR and mMOR using GTPγS and cAMP assays, which are reproduced in

Table 2.[99]

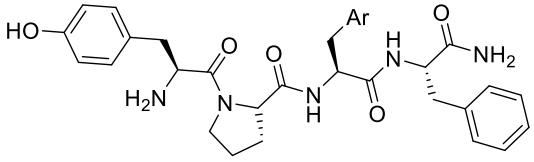
From

Table 2, a dramatic change can be seen in bias factor upon switching from hMOR to mMOR , or from GTPγS to cAMP cellular assays. Additionally, the bias factor increased in favor of the biased pathway when performing the cellular assay on a different cell type; in this case, the switch from Chinese hamster ovary (CHO) to mice brainstem cells. **SR-17018** showed the highest bias factor and consequently has the highest preference for the G protein-signaling pathway. It should be noted that one compound, **SR-11501**, is biased with favored β-arrestin-2 recruitment, resulting in a negative bias factor. This compound showed a decrease in plasma levels over time, whilst the plasma levels of the other SR-compounds remained elevated up to 6 hours after intraperitoneal (i.p.) injection. Additionally, **SR-11501** proved to be the least potent with an EC_{50} of 396 ± 68 nM in a GTPγS (brain) assay on mMOR.[99] Based on their chemical structures, **SR-11501** is the only compound lacking a halogen at the *para*-position of the phenyl group and is it the only ligand among the authors' compounds that acts as a β-arrestin-biased ligand. The authors ascribe this to favorable conformations imposed on MOR by halogen substitution at a number of positions – such as is seen in **SR-11501** – that promote the binding of GTPγS thereby limiting signaling through β-arrestin-2. Later, a chronic study on the most promising compound **SR-17018** was performed. This study demonstrated less antinociceptive tolerance in a hot plate test on mice relative to morphine and oxycodone. Interestingly, morphine sensitivity was restored within three days when morphine-tolerant mice were treated with **SR-17018**. Furthermore, upon chronic administration of **SR-17018**, no MOR desensitization was produced in periaqueductal gray (PAG). The authors suggest that **SR-17018** can stabilize MOR in a way where it could restore G protein signaling and could serve as a ligand to reestablish efficacy in tolerant systems.[140] This latter suggestion was countered in the authors' latest publication. In a warm water tail immersion test, **SR-17018** demonstrates tolerance which is in contrast with the hot plate test. Even though **SR-17018** showed G protein-biased signaling *in vitro*, the authors claim that the lack of β-arrestin-2 is directly linked to a decrease in tolerance.[141] Nevertheless, whether or not the *in vitro* biased profiles can be linked to *in vivo* systems remains to be fully elucidated.

*Table 2: Bias factors of SR-compounds at hMOR and mMOR in CHO and brain cells using **DAMGO** as a reference ligand*

Agonist	hMOR		mMOR	
	(GTPγS (CHO)/ β-arr-2)	(cAMP (CHO)/ β-arr-2)	(GTPγS (CHO)/ β-arr-2)	(GTPγS (brain)/ β-arr-2)
SR-11501	-0.39	-0.09	-0.91	-0.64
SR-14968	1.55	0.71	0.83	1.54
SR-14969	1.03	0.40	0.46	0.93
SR-15098	1.47	1.28	1.03	1.74
SR-15099	1.68	1.44	1.07	1.74
SR-17018	1.93	1.60	1.47	2.01

In addition to the previous results, the same research group published another series of SR-compounds. They screened for other and more halogens on the phenyl ring, and pendant groups such as halogens, -OMe, -OCF_3, -SO_2Me, -CN and -Me on the benzimidazolone as relates the calculation of their bias factors. The two best compounds obtained from this, were **41** and **44** (Figure 4), with a corresponding bias factors of 1.36 and 1.75 using the operational model (Equation 2), making them both biased towards the G protein pathway compared to **DAMGO**. Interestingly, the BBB penetration was determined after i.p. administration of 6 mg/kg in mice and brain levels were measured after 1 h. Both **41** and **44** were still present in the brain with a concentration of 17 µM and 4.6 µM respectively.[142] This latter fact makes both compounds very interesting as a consequence of MOR's brain localization.



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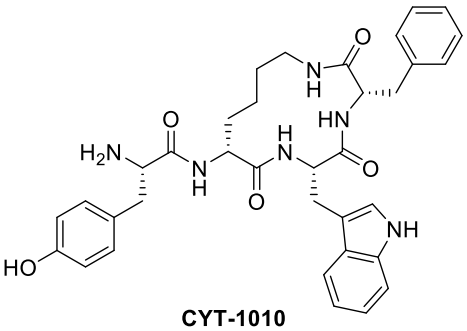
EM-2: Ar = phenyl



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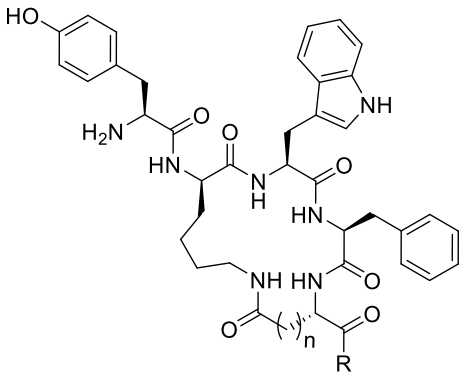

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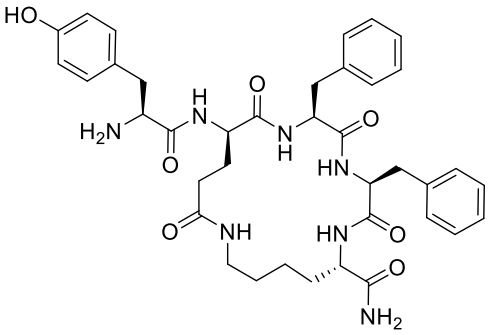

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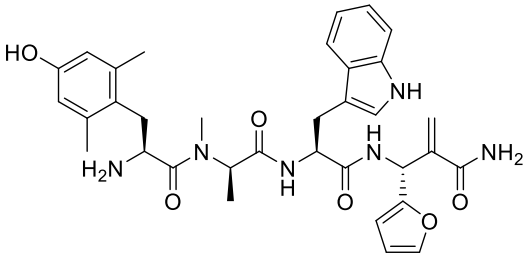
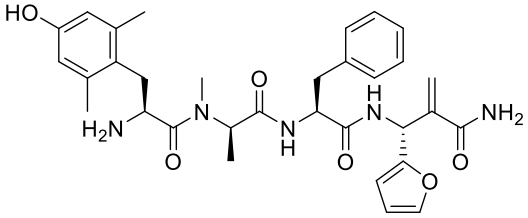
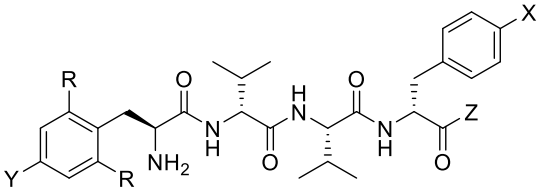


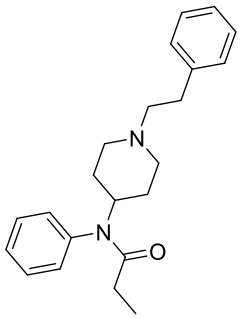
MEL-N1606



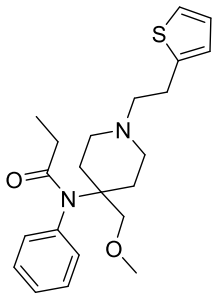
MEL-N1608



X	Y	Z	R
H	H	OH	H = **bilaid A**
OH	H	OH	H = **bilaid B**
H	H	OH	H = **bilaid C**
H	OH	NH_2	Me = **bilorphin**
H	OH	L-Ser(beta-Lac)-NH_2	Me = **bilactorphin**



Fentanyl



Sufentanil

Figure 5: Structures of biased MOR ligands and reference ligands – part 3

7. Endomorphin-1 & 2 and derivatives

Endomorphin-1 & 2 (**EM1** & **EM2**) (Figure 5) are endogenous tetrapeptides[5, 40] displaying high affinities towards MOR (K_i = 0.36 and 0.69 nM respectively) and a great binding selectivity over DOR and KOR (δ/μ = 4183-fold and κ/μ = 15077-fold for **EM1** and δ/μ = 13381-fold and κ/μ = 7594-fold for **EM2**).[143] Goldberg *et al.* reported the affinity of **EM1** and **EM2** on two splice variants of MOR in mouse brain homogenates resulting in K_i values of 0.67 and 3.2 nM for **EM1** and 0.43 and 4.0 nM for **EM2** respectively.[144] Both peptides have been reported as β-arrestin-biased ligands in different studies in contrast to most of the MOR ligands described above,[76, 102, 145-146] being the unfavored bias for MOR. The quantified bias for both **EM1** and **EM2** was reported by Thompson and coworkers using the operational model (Equation 2) with a GTPγS cellular assay for the G protein pathway versus β-arrestin-2 recruitment, which resulted in bias factors of -1.22 and -0.563 relative to **DAMGO** for **EM1** and **EM2** respectively.[76]

CYT-1010 (Figure 5) is a synthetic analogue of endomorphin-1 containing a D-Lys in the second position which is cyclized through the *C*-terminus of the peptide. It has a higher affinity for hMOR (K_i = 0.25 nM) than both **EM1** and **EM2** (K_i = 13.9 and 12.5 nM respectively).[147] The latter affinities of **EM1** and **EM2** for hMOR were found not as good as those described initially.[143] Preclinical data showed a reduced abuse potential, since it lacked rewarding behavior in rodents models in conditioned place preference (CPP) test, in addition with higher analgesic potency in a tail-flick test after both intravenous (i.v.) and oral administration relative to morphine. Results of phase I clinical trials showed that **CYT-1010** gave way to significant analgesia and no respiratory depression since over the first three hours after dosing, no significant decrease in plasma oxygen saturation or change in respiratory rate was observed. In light of this latter fact, **CYT-1010** has progressed to phase II clinical trials.[147]

In 2016, Zadina *et al.* described four cyclic endomorphin analogs (Figure 5): three of **EM1** (**ZH809**, **ZH850**, **ZH853**) and one of **EM2** (**ZH831**). They have all demonstrated a higher receptor selectivity for MOR over DOR and KOR (Table 3). All four showed drastically improved antinociception-vs-side effect ratios. Relative to morphine, in rodent models the analogs demonstrated a reduction of the most common side effects associated with opioids (*vide supra*), a profile potentially linked to a bias towards the G protein pathway, although this has not been validated experimentally. **ZH853** reduced or showed absence of six critical side effects, e.g. tolerance, hyperalgesia, respiratory depression, abuse liability, motor impairment, and glial activation, making it the most promising drug candidate of the four.[148]

Table 3: Selectivity of the ZH compounds at MOR

Ligand	δ/μ	κ/μ
ZH809	169	102
ZH850	132	453
ZH853	188	7584
ZH831	86	253

Another series of novel endomorphin analogs – the **MEL-N16** series – were developed in 2017 to find compounds with a biased activity, and thus a more favorable side effect profile. The whole series showed an great affinity and selectivity for the MOR. On top of that, the authors observed an

increase in stability and BBB permeability. Of these analogs, however, only two, **MEL-N1606** and **MEL-N1608** (Figure 5) were reported to be biased agonists towards the G protein-signaling pathway. More specifically, **MEL-N1606** produced less constipation, motor impairment, and drug-seeking behavior, as compared with morphine. Additionally, upon repeated administration, no significant decrease in analgesic effect was found, indicating the lack of tolerance development.[149]

8. Bilactorphin

Recently, three tetrapeptides, bilaids A, B, and C (Figure 5), were extracted from the Australian estuarine-derived *Penicillium* sp. MST-MF667.[150] Notably, they all contain the L,D,L,D stereochemical pattern. SAR studies proved that this L,D-alternation at positions 1 and 2 is necessary to maintain opioid activity. Following optimization studies, the authors obtained bilorphin (Figure 5), which showed a bias towards G protein-signaling to a similar extent as **TRV130**.[150] Contrary to the results obtained from intrathecal (i.t.) administration, studies demonstrated no antinociception after s.c. or i.v. administration. Further optimization led to the development of bilactorphin (Figure 5), a pentapeptide, with enhanced BBB permeability, still biased towards G protein-signaling. Interestingly, bilactorphin is orally available with similar potency to morphine *in vivo*.[150]

9. Fentanyl & Sufentanil

Fentanyl (Figure 5) is a synthetically developed potent MOR agonist, first synthesized by Janssen in 1960,[151] and followed by the discovery of sufentanil (Figure 5) in 1974.[152] Schmid *et al.* have previously described both fentanyl and sufentanil as ligands biased towards β-arrestin-2 recruitment, in comparison with **DAMGO**, with bias factors of -0.75 and -0.78 respectively using the operational model (Equation 2) with GTPγS assay at hMOR.[99] However, when comparing fentanyl to morphine, a bias towards G protein recruitment was observed (bias factor of 0.96 using the operational model with cAMP assay at hMOR).[102] Hence, the bias of a ligand is dependent upon the reference ligand and the type of assay employed.

B. Biased δ-opioid receptor ligands

Even though the μ-opioid receptor is the most common target in clinical research for new and/or improved opioid analgesics, the δ-opioid receptor (DOR) still has proven itself capable of exerting strong antinociception with fewer side effects.[153] Since these side effects could be respiratory depression,[28] anxiety,[29] convulsion, depressive effects,[19, 30] constipation, and addictive liability,[31] the development of G protein-biased δ-opioid receptor ligands are still profitable. In fact, DOR agonists can induce anxiolytic- and antidepressant-like effects, together with effective analgesia, which makes them significantly different from MOR and highly desirable in therapeutic applications. Additionally, this latter fact is also important on account of its relation to chronic pain, which in turn is associated with anxiety and mood disorders,[51] though it's worth pointing out that the clinical candidates **ADL5747** and **ADL5859** failed in phase II due to lack of efficacy.[54] Biased DOR agonists could offer an approach to by-pass the adverse effects, such as convulsion, seen during the administration of normal DOR agonists.[154] To determine the biased activity of DOR ligands, reference ligands are used to compare its activity, namely Leu-enkephalin, **BW373U86**, **DPDPE**, and **DADLE** but mostly **SNC-80**. Several different biased δ-opioid receptor ligands are listed and discussed here.

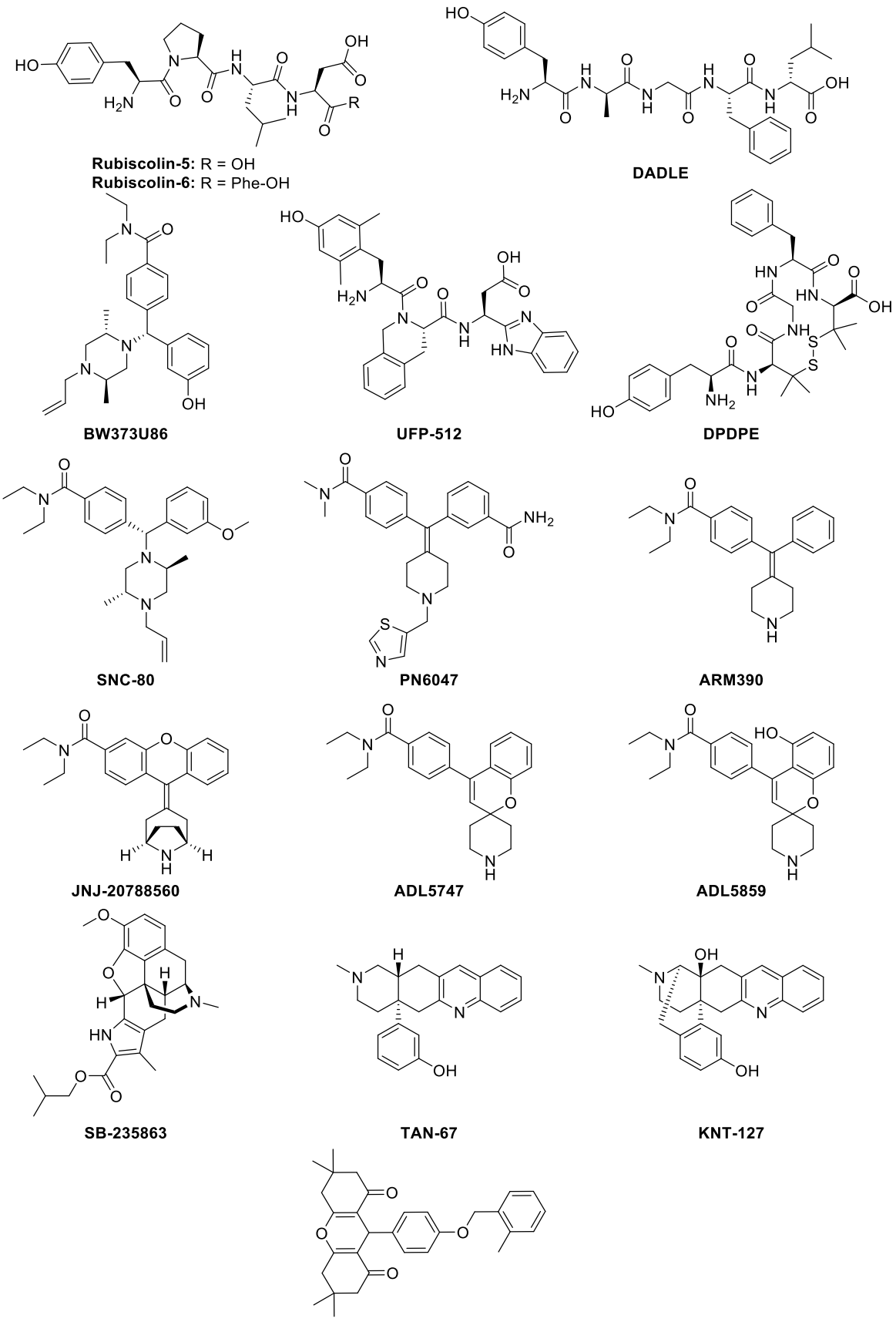


Figure 6: Structure of biased DOR ligands and reference ligands

1. Rubiscolin-5 & rubiscolin-6 (rubixyl)

Rubiscolin-5 and rubiscolin-6 (Figure 6) are hexapeptides first isolated from the spinach *Rubisco* plant.[155] Both demonstrated opioid activity with a high selectivity for DOR over MOR (μ/δ = > 500-fold and >2000-fold respectively), inducing antinociception even by oral administration.[155] In later studies, rubiscolin-6 was found to inhibit the internalization of DOR.[156] Additionally, Cassell *et al.* reported that both rubiscolin-5 and rubiscolin-6 are G protein-biased agonists, since they could not induce β-arrestin-1 recruitment. As a result of this latter fact, no bias factor could be calculated. As regards G protein-signaling vs. β-arrestin-2 recruitment , the bias factors were 0.31 and -0.28 for rubiscolin-5 and rubiscolin-6 respectively (which is in comparison to Leu-enkephalin in a cAMP assay), making rubiscolin-5 more G protein-biased than Leu-enkephalin, and rubiscolin-6 less G protein-biased or β-arrestin-2-biased.[157] This suggests that the addition of only one amino acid can make a huge difference to the extent to which signaling pathways can be biased. The additional phenylalanine, which contains a bulky phenyl-group, could interact in the binding pocket of DOR, potentially involving π-π stacking interactions, which, in turn, could be capable of switching its biased activity towards the unfavored pathway.

2. **DADLE** ([D-Ala², D-Leu⁵]-enkephalin)

DADLE (Figure 6) is a DOR-selective pentapeptide reported by Conibear *et al.* as a G protein-biased agonist over the recruitment of both β-arrestin-1 and β-arrestin-2 relative to **SNC-80** (*vide infra*). The bias factors were calculated at 1.5 and 1.15 respectively, using the operational model (Equation 2).[158] Upon mutation of DOR, **DADLE** was shown to be biased towards G protein-signaling compared to **BW373U86** (Figure 6), which is a selective DOR agonist. Mutation of Arg^{314} to Ala, led to no β-arrestin activation, whereas the G protein activation only decreased a bit (E_{max} of 79%) as compared to the wild-type DOR (E_{max} of 42% for β-arrestin activation and 102% for G protein activation) taking **BW373U86** as a reference ligand with E_{max} of 100% in all cases.[159]

3. **UFP-512**

UFP-512 (Figure 6) was developed in 2002 as a potent peptide-based DOR agonist,[160] and was proven to prevent tolerance when studying the antidepressant-like effects. After 7 days of daily administration of **UFP-512**, similar antidepressant-like effects as obtained after acute administration were observed.[161] Another study also demonstrated the antidepressive effects in addition to anxiolytic-like effects *in vivo*.[162] Furthermore, Charfi *et al.* demonstrated the biased behavior of **UFP-512** when comparing cAMP inhibition assay with internalization assay (an indication of β-arrestin recruitment). The authors obtained a bias factor of 2.12 using the operational model (Equation 2), relative to **DPDPE** (Figure 6).[163-164]

4. **SNC-80** and derivatives

SNC-80 (Figure 6) is a non-peptidic DOR agonist, chemically derived from **BW373U86**[165] and capable of selectively activating the heteromeric μ-δ opioid receptor.[166] Prior studies showed that **SNC-80** interacts with the δ-protomer, activating the complex *in vivo*.[166] In HEK293 cells stably expressing Flag-DOR, **SNC-80** demonstrated, in the same way as **UFP-512**, a biased character, when looking at cAMP inhibition versus internalization, displaying a bias factor of 1.70, relative to **DPDPE** as a reference ligand and using the operational model (Equation 2).[163-164] In rodent models, Saitoh *et al.* have reported the antidepressant- and anxiolytic-like effects of **SNC-80** upon activation of DOR,[167] whereas in a nitroglycerin-induced thermal hyperalgesia assay in 'wild-type' mice, as described by Dripps and coworkers, **SNC-80** did produce antihyperalgesia.[30, 168] As described above, **DADLE** was

reported to be G protein-biased as compared to **SNC-80**, thereby making **SNC-80** less G protein-biased, i.e. recruiting more β-arrestin than **DADLE**,[158] though **SNC-80** is more G protein-biased as compared to **DPDPE**.[163-164] As a consequence, the bias of **SNC-80** or of any other ligand is wholly dependent on the reference ligand.[21]

PN6047 (Figure 6) is a compound developed by PharmNovo AB in 2012. **PN6047** is a potent and selective DOR agonist, chemically derived from **SNC-80**. During the second half of 2018, the pre-clinical studies on **PN6047** were completed, showing high potency and efficacy in chronic pain models and no indications of undesired side effects. The first in-human clinical trials are planned between 2019 and 2021.[169-170] The bias factor of **PN6047** for G protein over β-arrestin-1 and for G protein over β-arrestin-2 signaling was quantified by Conibear *et al.* in 2020, using **SNC-80** as a reference ligand, giving values of 1.17 and 0.89 respectively using the operational model (Equation 2). This means that **PN6047** is a G protein-biased DOR agonist, with an additional layer of selectivity being a high selectivity for DOR over MOR and KOR.[158]

ARM390 (Figure 6) is a DOR-selective agonist, also chemically derived from **SNC-80** and developed by Wei *et al.* in 2000.[171] It exhibited very high selectivity over MOR and KOR (μ/δ = 4370-fold and κ/δ = 8590-fold), with an IC_{50} of 0.87 nM. **ARM390** also showed excellent oral bioavailability (F = 90-100 %) in rats.[171] While **SNC-80** caused DOR internalization, this was not significant in *in vivo* studies with **ARM390** when analyzing tolerance.[172-173] In addition, no behavioral desensitization after acute administration of **ARM390** was observed. Whereas chronic **SNC-80** administration led to complete loss of all DOR behavioral responses, including analgesia, **ARM390** did neither change the receptor number, the receptor internalization, cell membrane localization and G protein coupling. Although tolerance was developed to the analgesic effects of DOR agonists, other behavioral responses remained intact.[154, 174] Noteworthy, the potency and efficacy for G protein activation and analgesic ability are similar for both **SNC-80** and **ARM390**.[31] More recently, a bias factor of 0.55 was calculated for **ARM390** towards G protein-signaling with **SNC-80** as a reference ligand using the operational model (Equation 2). Consequently, **ARM390** is a G protein-biased ligand for DOR.[158]

JNJ-20788560 (Figure 6) is an orally bioavailable DOR-selective agonist, structurally derived from **SNC-80** and synthesized by Johnson & Johnson.[175] It showed a high affinity and potency towards DOR (K_i = 2.0 nM and EC_{50} of 5.6 nM), in addition to a high selectivity over MOR and KOR, e.g. 600-fold and 500-fold respectively. In preclinical models, **JNJ-20788560** demonstrated antihyperalgesia and produced a similar level of analgesia as **SNC-80**, **ARM390** (*vide supra*), **ADL5859**, **TAN-67**, and **SB-235863** (*vide infra)*. In rodent models, no tolerance was observed towards antinociceptive effects and antihyperalgesia. Moreover, in contrast to the NSAID ibuprofen, **JNJ-2075560** did not induce GI erosion and it also did not display respiratory depression compared to morphine. Subsequently, **JNJ-20788560** provides a useful profile for the treatment of different types of pain.[175]

ADL5747 (Figure 6) is a compound resulting from SAR exploration and optimization of the potent, selective and orally bioavailable DOR agonist **ADL5859** (Figure 6), which is 50-fold less potent than **ADL5747**. Both are chemically derived from **SNC80** and the synthesis of **ADL5747** was previously described by Le Bourdonnec *et al.*. The authors determined the half-life of both ADL-compounds, which were respectively 12.2 h and 5.1 h in canine models.[176] Later studies performed by Nozaki *et al.* described the analgesic, locomotive and receptor internalization effects of **ADL5747** and **ADL5859**. Neither compound induced receptor internalization or hyperlocomotion *in vivo* (relative to **SNC-80**), suggesting its biased activity for G protein-signaling at the receptor. In addition, both

ADL5747 and **ADL5859** reduced chronic pain in mice after nerve injury and tissue inflammation and displayed a longer mode of action.[177] The promising preclinical data justified the entry of both compounds into clinical development. **ADL5859** was well tolerated and showed good oral absorption and was subsequently investigated in phase II trials. A single dose administration of 200 mg of **ADL5859** demonstrated no analgesic effect. Sadly, after advancing to phase II, administration of **ADL5747** showed no difference compared to placebo. For these latter reasons, further investigation on both compounds was cancelled.[54]

All **SNC-80** derivatives provide a G protein-biased signaling pathway. Despite their structural similarity, all of the pharmacological data provided are different and different tests were performed, making it difficult to compare them.

5. TRV250

TRV250[178], which currently finished phase I clinical trials, is a G protein-biased DOR agonist that preferentially activates the G protein pathway showing reduced hyperalgesia in rodent models. In these studies, **TRV250** is developed for the treatment of acute migraine and was shown to have a quick absorption of 0.5 to 2 hours upon s.c. administration, which increased by up to 3 hours upon oral administration and by up to 6 hours in conjunction with a high-fat meal. The relative bioavailability of **TRV250** in the fed state was 19%, which was higher than in the fasted state (14%). **TRV250** showed mild side effects, such as headache and injection-site reactions, which were not dose-related and was proven to be well tolerated by the lack of serious adverse effects, like nausea.[179]

6. SB-235863

SB-235863 (Figure 6) is a morphine-like compound, developed by Petrillo *et al.*, demonstrating a high affinity for DOR (K_i = 4.81 nM) and selectivity over MOR and KOR (189-fold and 52-fold respectively).[180] Even though **SB-235863** was inactive in tail-flick and hot-plate tests in rodent models for acute pain, it exhibited potent thermal antihyperalgesia upon oral administration. Additionally, **SB-235863** lacked some opioid side effects, like slowing the GI tract and motor incoordination, up to 70 mg/kg after oral administration. **SR-235863** is therefore a DOR ligand with a favorable side effect profile.[180]

7. TAN-67

TAN-67 (Figure 6) was discovered in 1998 by Nagase *et al.* based on the 'message-address' concept as a DOR agonist. The morphinan moiety (message part) interacts with the anionic part of the receptor, in addition with π-π stacking and hydrogen bonding with the 3-hydroxy group. It has a high affinity for DOR over MOR and KOR (μ/δ = 2070-fold and κ/δ = 1600-fold).[181] **TAN-67** was capable of stimulating G protein binding, but it also gave way to a reduced rate of phosphorylation at DOR, leading to less β-arrestin-2 recruitment and less internalization.[182] Additionally, van Rijn and Whistler suggested that **TAN-67** acted on DOR/MOR heterodimers.[183] Moreover, **TAN-67** showed anxiolytic-like effects in ethanol-withdrawn mice, yet no decrease in anxiety-like behavior was observed in native mice.[184] Subsequently, **TAN-67** was found to be G protein-biased compared to **DPDPE**, since it recruits less β-arrestin-2 (E_{max} = 41 %).[185] The bias factor of **TAN-67** was calculated by Robins *et al.* using the equiactive comparison (Equation 1), resulting in a value of -1.4 relative to Leu-enkephalin, thereby showing a bias in favor of G protein-signaling. The authors opined that a negative bias factor

indicates a bias towards cAMP activity – i.e. G protein-signaling – and not towards β-arrestin recruitment, which is adopted in most papers.[186]

8. KNT-127

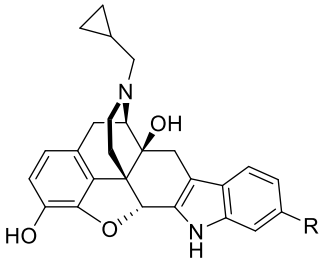
KNT-127 (Figure 6) was synthesized by Nagase *et al.* in 2010 as a constrained version of **TAN-67** with the addition of a hydroxyl group. **KNT-127** displayed a high affinity for DOR (K_i = 0.16 nM) over MOR and KOR (μ/δ = 134-fold, κ/δ = 961-fold).[187] Subsequent studies proved a marked decrease in the side-effects seen with its use; compared to **SNC-80**, **KNT-127** produced no convulsions up to doses of 100 mg/kg in mice upon s.c. administration, in addition to antidepressant-like effects, as determined via a forced swim test at mice. Furthermore, antinociceptive effects were observed in both a writhing and formalin test.[188] Also, Nozaki *et al.* described the reduced side effects of **KNT-127**. Inflammatory hyperalgesia was reversed by **KNT-127** upon acute treatment, together with the production of antidepressant-like effects. However, upon chronic administration of **KNT-127**, analgesic tolerance and cross-tolerance with **SNC-80** was detected. Nevertheless, **KNT-127** did not induce DOR internalization *in vivo*, in contrast to **SNC-80**.[189]

9. BMS-986187

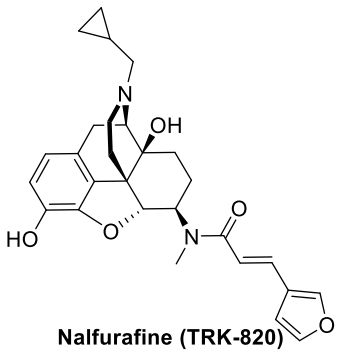
BMS-986187 (Figure 6) is a biased allosteric DOR agonist discovered *via* high-throughput screening (HTS), showing no direct agonist activity, but did produce positive allosteric modulator (PAM) activity. It demonstrated an increase in potency to orthosteric agonists.[190] Later studies based on free-energy interfaces identified specific binding sites and conformational states for **BMS-986187**.[191] Subsequently, **BMS-986187** was identified as a G protein-biased allosteric agonist, albeit less potent, but showing no significant level of β-arrestin-2 recruitment. This is a result of reduced phosphorylation, internalization and desensitization of the receptor, which consequently generates a bias factor of 1.53 towards G protein-signaling using the operational model (Equation 2) with **SNC-80** as a reference ligand. Additionally, through the use of orthosteric antagonists such as naltrindole and naxolone, it was shown that **BMS-986187** could mediate agonism on other sites than just the orthosteric site.[192]

C. Biased κ-opioid receptor ligands

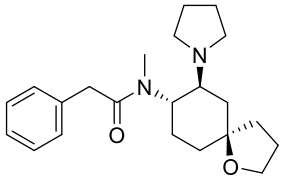
The third opioid receptor that we consider here is the κ-opioid receptor (KOR). Since KOR is widely described in, not only the CNS, but also the PNS, potent analgesic effects can be produced without CNS-based side effects; as a result KOR is often considered as the 'safest' of the three classical receptors.[13]. Alongside their antinociceptive effects, KOR agonists have antiaddictive and antipruritic properties, in addition to effects on anhedonia, dysphoria, sedation,[19, 32, 193] anxiety.[27, 33, 193] Importantly, in β-arrestin KO mice both antinociceptive and antipruritic efficacies at KOR are retained.[33] G protein-biased KOR agonists are capable of inducing analgesic effects, without producing dysphoria,[194] sedation, abuse potential,[195] anxiety, stress, and depression.[34] To determine the biased activity of KOR ligands, salvinorin A, **U50,488**, and **U69,593** are employed as reference ligands. Different biased κ-opioid receptor ligands are listed and discussed here. These ligands can vary from morphine-like compounds, to peptides, to small molecules derived from KOR agonists.



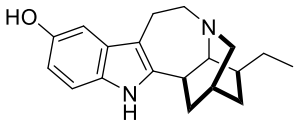
Naltrindole: R = H
6'-GNTI: R = guanidyl



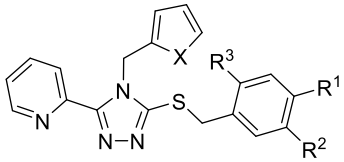
Nalfurafine (TRK-820)



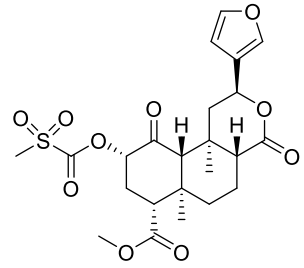
U69,593



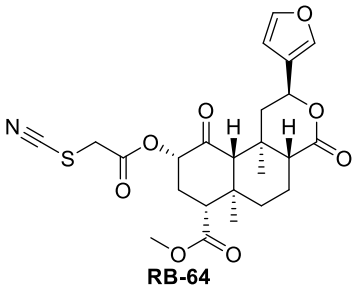
Noribogaine



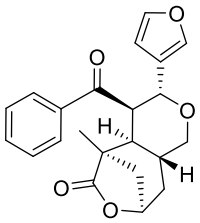
Triazole probe: X = O, R^1 = Cl, R^2 = Cl, R^3 = H
Triazole 1.1: X = O, R^1 = Me, R^2 = CF_3, R^3 = H
Triazole 1.5: X = S, R^1 = Cl, R^2 = CF_3, R^3 = H
1.2: X = S, R^1 = H, R^2 = CF_3, R^3 = Cl



Mesyl Sal B



RB-64



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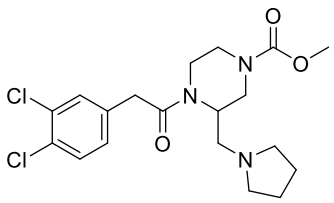

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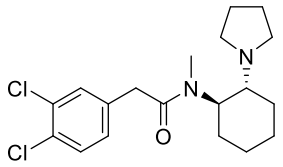
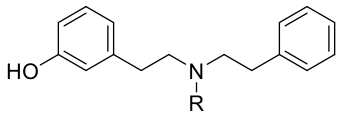
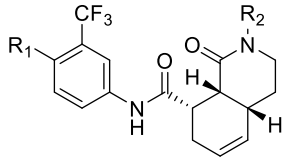


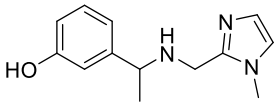
***(+)*-U50,488**



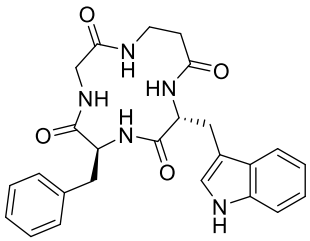
HS665: R = cyclobutylmethyl
HS666 (MCBPHA): R = cyclopropylmethyl
BPHA: R = butyl
MCPPHA: R = cyclopentylmethyl



Lead compound: R^1 = Cl, R^2 = benzyl
2.1: R^1 = Me, R^2 = 2-fluorobenzyl
2.2: R^1 = Br, R^2 = phenyl



compound 81



LOR17

Dyn A: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
Dyn B: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-OH
Dyn 1-13: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH
Dyn 1-11: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-OH
Dyn 1-9: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH
Dyn 1-8: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-OH

Figure 7: Structures of biased KOR ligands and reference ligands

1. Morphine-like compounds

6'-guanidinonaltrindole (**6'-GNTI**; Figure 7) is a morphine-like compound developed by Sharma *et al.* in 2001. A shift of the guanidium group from the 5'- to 6'-position transformed the antagonist naltrindole into the potent KOR-agonist, **6'-GNTI**.[196] This last one is able to selectively activate G proteins, without recruiting β-arrestins. It thus serves as an antagonist to the undesired pathway by blocking internalization and β-arrestin recruitment in general. Generally, this is called biased agonism. **6'-GNTI** produced antinociceptive effects in rodent models of thermal allodynia.[197] In previous studies, **6'-GNTI** had been described as a DOR/KOR heterodimer-selective ligand,[198] but when assessed using the radiant heat tail-flick assay in DOR KO mice, only a small decrease in nociception was observed, pointing to the maintained activation of KOR without the presence of DOR.[197] In striatal neurons, **6'-GNTI** did not activate ERK1/2 (linked to β-arrestin recruitment) but was able to activate Akt (linked to G protein-signaling) whereas **U69,593**, a KOR agonist (Figure 7) activates both kinases.[199] A bias factor for **6'-GNTI** was determined using the operational model (Equation 2), with **Sal A** (*vide supra)* as a reference ligand, resulting in a value of 0.76 towards G protein-signaling.[200]

Nalfurafine (**TRK-820**; Figure 7) is a morphinan-like compound derived from 4,5-epoxymorphinan, developed by Nagase *et al.,* that is a highly potent and selective KOR agonist.[201] Later studies demonstrated the antipruritic activity of nalfurafine.[202] After successful results from clinical trials, nalfurafine hydrochloride subsequently entered the market (trade name Remitch®) in Japan as an antipruritic agent.[203] More recently, Lui *et al.* provided data on nalfurafine where it displayed analgesic and antipruritic effects without causing sedation, anhedonia, reduced motor coordination or conditioned place aversion (CPA), with a potency of 0.11 nM in a GTPγS assay in mouse neuro2A cells.[204] In earlier studies, nalfurafine had been reported to produce only sedation (with ED_{50} = 27 μg/kg) when dosing the drug at levels much higher than required for producing antinociception (ED_{50} = 3.3 μg/kg).[205] Nalfurafine also ensured a potent attenuation of i.t. morphine-induced itch/scratching responses in primates.[206] The nalfurafine bias factor was calculated by Schattauer *et al.* for both rKOR and hKOR. Comparing ERK1/2 phosphorylation, linked to G protein-signaling, with p38 phosphorylation, linked to β-arrestin-signaling, resulted in biased factors of 1.15 for rKOR and 3.2 for hKOR using the equiactive model relative to **U50,488** (Equation 1). Hence, nalfurafine is a G protein-biased KOR agonist both in rat and human receptor types.[207]

2. Noribogaine

Noribogaine (Figure 7) is the principal active metabolite from the drug ibogaine; a psychoactive alkaloid extracted from the African shrub *Tabernanthe iboga*.[208-210] Noribogaine is, alongside ibogaine, both a KOR agonist and a NMDA receptor antagonist[211] and was later found to be a G protein-biased KOR agonist, as well as a moderately potent MOR antagonist. This latter fact makes noribogaine a dual κ-μ agonist/antagonist. This is in contrast with ibogaine, which is a more potent MOR antagonist and a weaker KOR agonist than noribogaine. Relative to **U69,593**, noribogaine showed partial KOR agonism (E_{max} = 72%) in a GTPγS assay, but displayed much lower levels of β-arrestin-2 recruitment (E_{max} = 13%). Noribogaine can also be considered to be a G protein-biased KOR agonist, since it is more effective at inhibiting β-arrestin agonist signaling as compared to the G protein pathway.[209]

Noribogaine is structurally similar to mitragynine (*vide supra,* Figure 3); both contain an indole attached to an azepine or a piperidine ring. In addition, they both act as G protein-biased ligands,

but each at different receptors. More specifically, noribogaine is a MOR antagonist, suggesting the big impact on agonism when modifying the structure of the ligand. Noribogaine is more constrained compared to mitragynine and lacks the ester and enol ether function.

3. Triazole 1.1 and derived compounds

The triazole probe (Figure 7) was discovered in 2012 from HTS screening studies of the Molecular Libraries Probe Production Centers Network.[212] This triazole probe displayed a high selectivity for KOR over MOR and DOR (κ/μ = 792-fold and δ/κ = 2230-fold). Optimization of the triazole probe by substitution of the chlorine atoms on the aromatic ring afforded a series of potent G protein-biased agonists relative to **U69,593** called triazole **1.1** to **1.5** (Figure 7). The bias factor was the highest for triazole **1.1** and **1.5** (1.79 and 2.05 respectively using the operational model; Equation 2). Triazole **1.1** and **1.5** were obtained by substitution of the two chlorine atoms by a methyl and a trifluoromethyl group for **triazole 1.1** and the substitution of a chlorine atom by a trifluoromethyl group and replacing the pendant furan ring with a thiophene for triazole **1.5**. After further investigation, triazole **1.1** was shown to be the most suitable analog since it displayed much less ERK1/2 phosphorylation than triazole **1.5** and *in vivo* tests proved the brain-penetrability of triazole **1.1**.[213] Subsequently, antinociception in murine tail flick tests was observed after systemic administration, showing triazole **1.1** to be a potent G protein-biased KOR agonist, since it displayed less ERK1/2 phosphorylation linked to β-arrestin recruitment, but still able to induce antinociception.[213] In later studies, triazole **1.1** demonstrated neither aversion nor sedation upon doses resulting in analgesia and antinociception as compared to **U50,488** (*vide infra*).[195] Furthermore, triazole **1.1** did not change the ambulatory behavior in mice, whereas **U50,488** led to dramatically lowered movement.[214] In another study, other analogs of the triazole probe were developed, showing a bias towards G protein-signaling. The compound with the highest biased factor of 1.9 (comparing G protein with β-arrestin-2 and relative to **U69,593**) was compound **1.2** (Figure 7). Here, the 2-furanyl-ring was substituted with a 2-thiophenyl-ring and the 3,4-dichlorophenyl was substituted with a 1'-Cl, 5'-trifluoro-phenyl ring. Compound **1.2** showed a greater potency towards G protein-signaling, but a much lower potency towards β-arrestin when compared to **U69,593**.[215]

4. Salvinorin A derivatives

Mesyl Sal B (Figure 7) is a neoclerodane diterpene analog, derived from Salvinorin A; a non-nitrogenous diterpene isolated from a hallucinogenic plant that acts as a potent selective KOR agonist.[125] Mesyl Sal B was synthesized by Harding *et al.* by substitution of the acetate of **Sal A** by a methanesulfonyl group. Furthermore, Mesyl Sal B had similar potency and affinity as **Sal A** (K_i = 2.3 nM and 1.9 nM, EC_{50} of 30 nM and 40 nM respectively), but Mesyl Sal B showed a higher selectively towards KOR as compared to **Sal A**.[124] Later, Simonson *et al.* described the antiaddictive properties of Mesyl Sal B and it was longer lasting than **Sal A** tested in the antinociception hot water tail-withdrawal assay in mice. Additionally, without altering cell-surface expression of dopamine transporters, Mesyl Sal B increased dopamine uptake in rat nucleus.[216] The bias factor of Mesyl Sal B was later calculated by Kivell *et al.*, as compared to U50,488 (*vide infra)*, resulting is a bias factor of 0.61 meaning that Mesyl Sal B is biased towards the G protein pathway, since they compared cAMP inhibition vs β-arrestin recruitment. For these reasons, Mesyl Sal B is considered as a G protein-biased ligand for KOR. Besides its antinociceptive effect, Mesyl Sal B did cause neither aversion, sedation, anxiety, nor learning and memory impairment in rats.[217]

RB-64 (Figure 7) is a semi-synthetic structural derivative of **Sal A**. **RB-64** was developed by Yan *et al.*, is more potent than **Sal A**,[218] and was found to be a G protein-biased agonist for KOR.[200] In 2015, **RB-64** was described as a biased agonist for G protein-signaling without sedative and anhedonia-like effects. Additionally, **RB-64** was the only KOR agonist that did not reduce motor coordination. Its bias factor, calculated in mice, was 1.98 towards G protein as compared to **Sal A**, using the operational model.[219] The bias factor was later quantified in hKOR leading to a value of 1.55, again using the operational model (Equation 2) relative to **Sal A**.[200]

Collybolide (Figure 7) is a non-nitrogenous sesquiterpene, first extracted in 1974 from the fungus *Collybia maculata*.[220] Collybolide shares a furyl-δ-lactone core with **Sal A** and was shown to be a G protein-biased KOR agonist. It produced antinociception in a tail-flick assay in male mice together with a reduction of pruritus and was aversive. Additionally, upon doses where antinociception was observed, no sedation was detected. Moreover, it induced higher levels of anxiety than **Sal A**. Interestingly, at higher concentrations, collybolide can bind to a second site in hKOR behaving as a allosteric modulator.[221]

5. GR89696

GR89696 (Figure 7) is a highly potent and selective KOR agonist, developed by Naylor *et al.* in 1993, showing well-defined antinociceptive effects (ED_{50} = 0.52 ng/kg upon s.c. administration).[222] **GR89696** ensured a potent attenuation of i.t. morphine-induced itch/scratching responses in primates.[206] It had also been suggested that **GR89696** could interact with KOR/DOR heterodimers to mediate antinociception.[223] A bias factor for **GR89696** of 0.67 towards β-arrestin-2 was calculated by White *et al.* (**Sal A** as a reference ligand, using the operational model for quantification; Equation 2) which produces an unfavorable bias for **GR89696**,[200] which was also reported by Kenakin *et al.*[224]

6. U50,488

U50,488 is a compound developed by Van Voigtlander *et al*. in the search for opioid analgesics.[225] It is a highly selective KOR agonist and exhibited antitussive effects in rats.[226] The authors made a distinction between ***(+)*-U50,488** and ***(-)*-U50,488**, since a shift in biased signaling occurred between the enantiomers. Taking **Sal A** as a reference ligand, ***(+)*-U50,488** (Figure 7) was a slightly G protein-biased KOR agonist with a bias factor of 0.91, whereas ***(-)*-U50,488** proved to be a modestly β-arrestin-biased KOR agonist with a bias factor of 0.31 towards β-arrestin recruitment. These bias factors were both calculated using the operational model (Equation 2),[200] which makes ***(+)*-U50,488** the better biased KOR ligand. Compared to **U69,593**, ***(+)*-U50,488** was also a KOR agonist slightly biased towards G protein-signaling (bias factor of 0.60, calculated with the operational model).[227]

7. Diphenethylamines

The design and synthesis of different compounds with a diphenethylamine structure backbone has previously been described by Spetea *et al.*[228] The most favorable *N*-substitution of the diphenethylamines were cyclopropylmethyl (CPM) and cyclobutylmethyl (CBM) over *N*-alkyl groups for an increase in affinity and selectivity towards the KOR. The *N*-CBM analog, **HS665**, demonstrated a remarkable selectivity for KOR over MOR (>1100-fold) and DOR (>20000-fold) and displayed potent antinociceptive effects after s.c. administration in mice. The *N*-CPM analog, **HS666**, showed lower but still significant selectivity for KOR over MOR (140-fold) and DOR (>1700-fold), and revealed itself to be a partial KOR agonist.[228] Later investigations into those two compounds showed antinociceptive responses in murine models of acute thermal nociception. **HS665** (Figure 7) was

reported to be more potent than **HS666** in the generation of antinociception upon intracerebroventricular (i.c.v.) (ED_{50} of 3.74 nmol and 6.02 nmol, respectively). When comparing to **U50,488** (*vide supra*), **HS665** was also more potent and **HS666** showed a similar level of potency. However, **HS666** showed reduced liability for aversive effects after i.c.v. administration in mice.[229] More recently, Dunn *et al.* have described three of these diphenethylamine analogs as biased agonists: **BPHA**, **MCBPHA** (viz. **HS665**) and **MCPPHA**. **MCBPHA** was validated as equally potent as **U50,488** in peripheral analgesia. Upon quantification of the bias, **BPHA**, **MCBPHA**, and **MCPPHA** (Figure 7) were all found to be biased towards G protein-signaling (bias factors of 1.8, 1.6 and 1.3 respectively all compared to **U69,593**, using the operational model of Black and Leff; Equation 2). Hence, **BPHA** was proven to be a full agonist with full efficacy in GTPγS assay without β-arrestin-2 recruitment, which makes it a highly G protein-biased KOR agonist. **MCBPHA** and **MCPPHA** have a lower bias factor than **BPHA**, since they showed partial efficacy towards β-arrestin-2 recruitment.[227]

8. Isoquinolinone analogs

Isoquinolinone lead compounds were discovered by Frankowski *et al.* using a 72-member library synthesized by Diels-Alder acylations and followed by screening of these compounds for binding at potential GPCR targets. The isoquinolinone lead compound (Figure 7) was found to be highly selective for KOR over both MOR and DOR.[230-231] Later, this isoquinolinone lead compound was optimized by substituting the chlorine on the aromatic ring and the benzyl-group on the nitrogen of the isoquinolinone moiety by a methyl and 2-fluorobenzyl respectively, affording **2.1**, and by a bromide and phenyl, giving **2.2** (Figure 7). The bias factors of these analogs were calculated using the operational model (Equation 2) with **U69,593** as a comparison, resulting in factors of 1.50 and 1.67 respectively for **2.1** and **2.2** towards G protein-signaling. Analog **2.1** demonstrated the best potency (EC_{50} of 84.7 nM vs 264.5 nM for **2.2**) *in vitro* and was for this reason more extensively investigated *in vivo*, showing antinociceptive responses in the mouse tail flick test. Additionally, **2.1** proved to be brain-penetrating *in vivo* by taking brain samples after 30 and 60 min from C57Bl-6 mice.[213]

9. Compound **81**

In 2017, Zheng *et al.* reported the discovery of compound **81** (Figure 7), a potent G protein-biased KOR agonist with little β-arrestin recruitment. It was discovered by a multi-template screening using the KOR crystal structure with the corresponding ligand-optimized atomistic models to discover new KOR chemotypes with distinct functional features and submicromolar activities, followed by SAR, resulting in 11 hits. Compound **81** showed a high affinity towards KOR (K_i = 0.16 nM) and had a bias factor of 0.78 towards G protein-signaling over β-arrestin recruitment relative to **Sal A**. Subsequent docking of compound **81** demonstrated H-bonding between the amine moiety of the ligand with Asp^{138} of KOR.[64]

10. Dynorphins

Dynorphin A and B are endogenous opioid peptides with a high selectivity for KOR over MOR and DOR.[5] White *et al.* screened different dynorphin sequences for their propensity for biased signaling. The sequences tested were **Dyn A**, **Dyn 1-8**, **Dyn 1-9**, **Dyn 1-11** and **Dyn 1-13** (Figure 7). **Dyn A** is a 17-mer from which the other sequences are truncated derivatives; **Dyn 1-8**, **Dyn 1-9**, **Dyn 1-11** and **Dyn 1-13** represents the first eight, nine, eleven and thirteen amino acids from **Dyn A** respectively (starting from the *N*-terminus). From their studies, the authors arrived at bias factors of 1.56, 0.68, 1.22, 1.67 and 1.56 (operational model; Equation 2) respectively, pointing clearly towards the G protein pathway with **Sal A** as a reference ligand. This suggests that **Dyn 1-8** and **Dyn 1-9** are

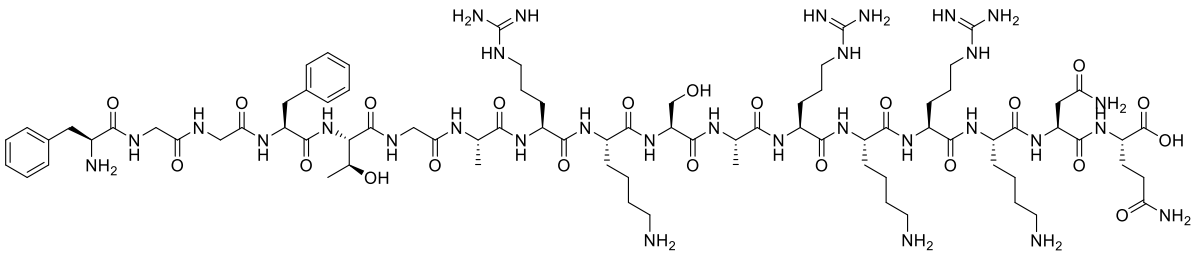
moderately biased towards G protein-signaling with **Dyn A**, **Dyn 1-11** and **Dyn 1-13** showing a higher level of bias towards G protein-signaling.[200]

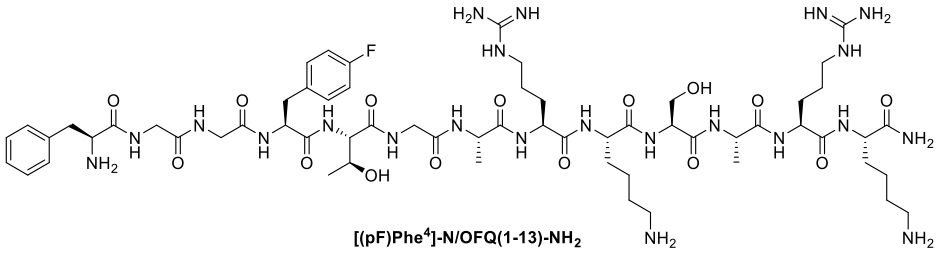
11. **LOR17**

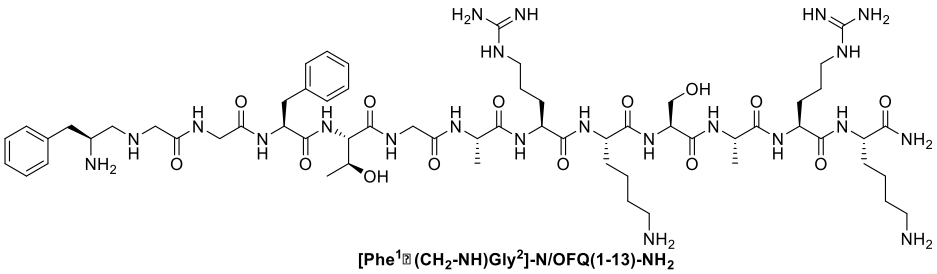
A recently discovered KOR agonist, **LOR17**, was shown to exhibit biased signaling. **LOR17** (Figure 7) is a cyclized form of the tetrapeptide H-Gly-β-Ala-D-Trp-Phe-OH, inhibiting adenylyl cyclase in a similar way to **U50,488**, but without significant β-arrestin-2 recruitment at KOR. This was quantified by the calculation of the bias factor (operational model; Equation 2) using **U50,488** as a reference ligand, revealing a bias factor of 2.93 towards G protein-signaling. Additional *in vivo* experiments showed **LOR17** to be effective for acute nociception, together with a reduced thermal hypersensitivity of induced neuropathic pain, as determined in murine models.[232]

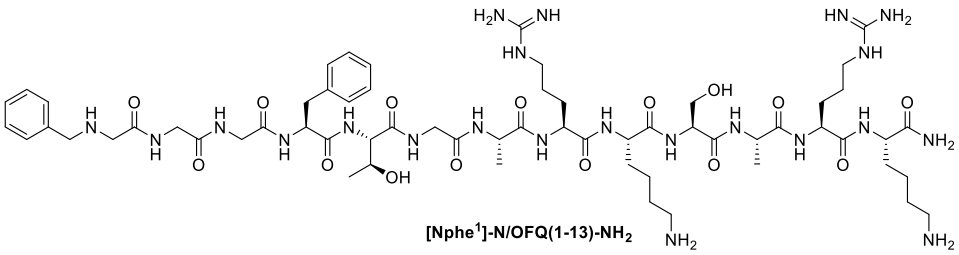
D. Biased nociceptin-opioid receptor ligands

Alongside the three classical opioid receptors, we will also consider the nociceptin-opioid receptor (NOP receptor) for discussion. NOP receptor was discovered many years after the classical opioid receptors MOR, DOR and KOR and was first characterized by Mollereau *et al.* as a result of cloning experiments. It was found to be structurally and functionally related to the classical opioid receptors, with a 49-50% sequence identity to the murine MOR, DOR and KOR.[69] NOP receptor can either induce or block analgesic effects depending on the route of administration of its agonists.[13, 35] More specifically, nociceptin (**N/OFQ**) (Figure 9), the endogenous peptide at NOP receptor, can induce either hyperalgesia, by blocking the MOR-induced analgesia, or analgesia by reducing hyperalgesia during opioid withdrawal.[35, 72] In addition, NOP receptor blockade can have antidepressant effects.[36] To determine the biased activity of NOP receptor ligands, reference ligands are used to compare its activity. These reference ligands are **Ro65-6570** (Figure 9), but mainly nociceptin. Different biased nociceptin-opioid receptor ligands are listed and discussed briefly below.









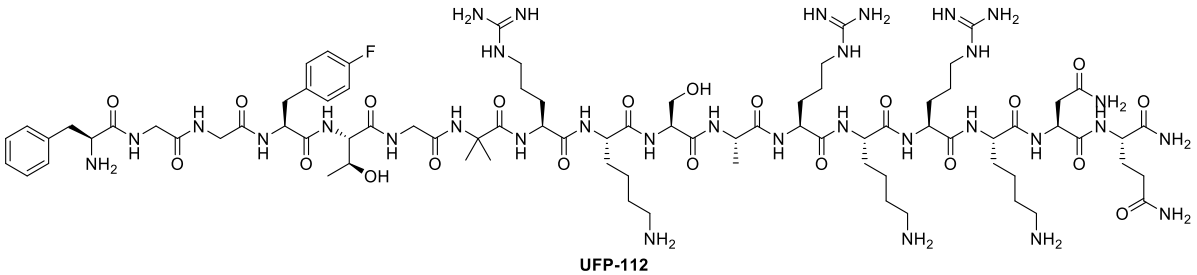


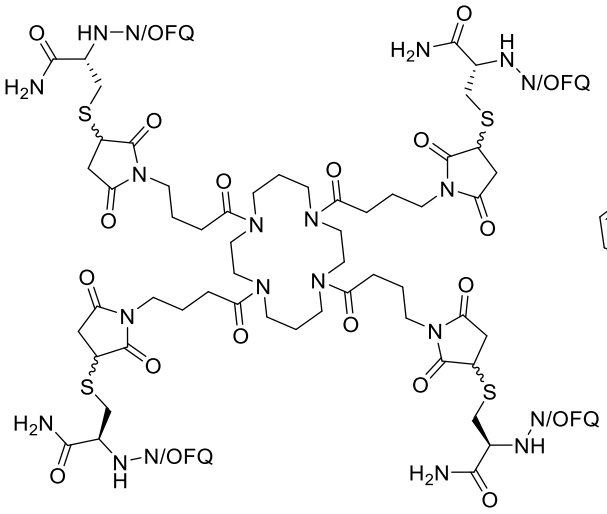
*Figure 8: Structures of **UFP-112** derivatives*

1. UFP-112

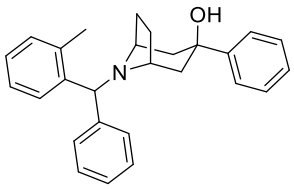
UFP-112 (Figure 8) is a modified peptide analog of nociceptin that was developed by Arduin *et al.* in 2007.[233] Prior to the discovery of **UFP-112**, a number of other modifications on the nociceptin peptide were experimentally validated: increased potency was seen in [Arg^{14}-Lys^{15}]-**N/OFQ**[234] and [(pF)Phe^{4}]-**N/OFQ**(1-13)-NH_2,[235] reduced efficacy on [Phe^{1}ψ(CH_2-NH)Gly^{2}]-**N/OFQ**(1-13)-NH_2[236] or antagonism in the case of [Nphe^{1}]-**N/OFQ**(1-13)-NH_2 (Figure 8).[237] When $C_{\alpha,\alpha}$ dialkylated amino acids were used in place of Ala^{7}, Ala^{11} and Ala^{15} promising results were obtained. As such, the substitution

of Ala^7 by Aib (2-aminoisobutyric acid) on **N/OFQ** led to a 7-fold more potent peptide than **N/OFQ** itself. Taking the previously described potency enhancing modifications into account, **UFP-112** was obtained with the sequence [(pF)$Phe^4$$Aib^7$$Arg^{14}$$Lys^{15}$]-**N/OFQ**-$NH_2$ (Figure 8) developing full agonism on NOP receptor.[233] In the same year, Rizzi *et al.* reported **UFP-112** as a selective and potent full agonist for NOP receptor with long-lasting effects *in vivo*.[238] On top of this, the long lasting effects of **UFP-112**, which are comparable to those of morphine, were shown in hyperalgesia and acute pain. Upon i.t. administration in primates, **UFP-112** did not produce itch/scratching responses, with an exclusive NOP receptor activation.[239] Additionally, the bias for **UFP-112** was quantified in 2015 by Malfacini *et al.* using the operational model (Equation 2). The obtained bias factor for **UFP-112** was 0.71 relative to nociceptin towards the G protein pathway versus β-arrestin-2 recruitment.[240]

Nociceptin (N/OFQ): H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH



PWT2-N/OFQ



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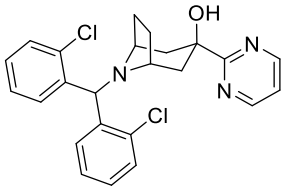

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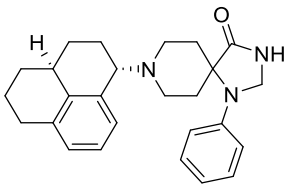
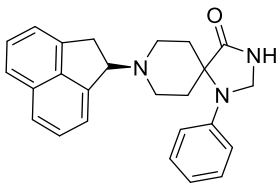
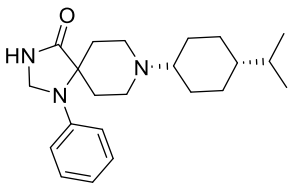


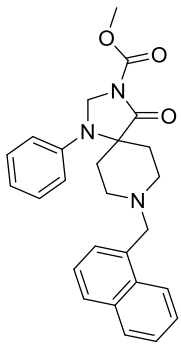
Ro64-6198



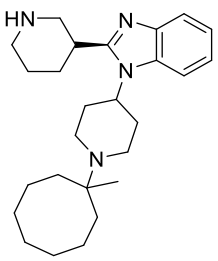
Ro65-6570



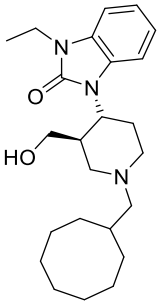
Ro2q



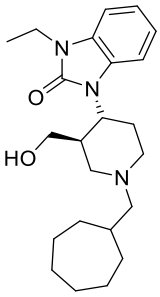
NNC 63-0532



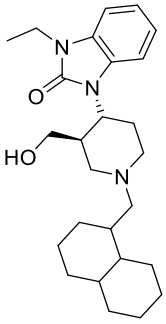
MCOPPB



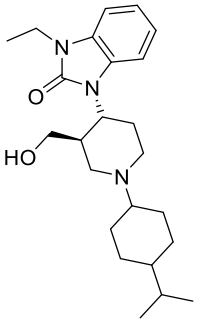
J-113397



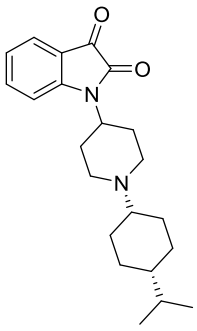
RTI-816



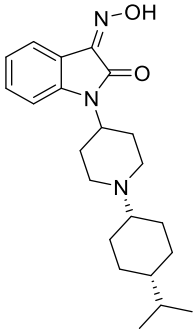
RTI-819



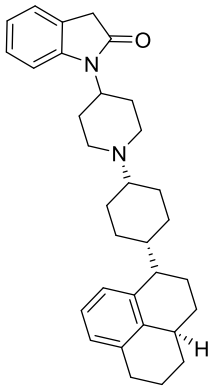
RTI-856



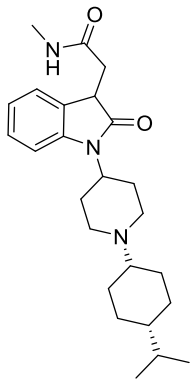
AT-090



AT-127



AT-202



AT-403

Figure 9: Structures of biased NOR ligands and reference ligands

2. PWT2-N/OFQ

PWT2-N/OFQ (Figure 9) is a branched derivative of **N/OFQ** with four-fold symmetry, where PWT stands for *peptide-welding technology*. The first examples of homotetravalent **PWT-N/OFQ** were described by Guerrini *et al.* in 2014. The PWT core (a cyclam in the case of **PWT2**) is linked to maleimido moieties, and then linked to [Cys^{18}]-**N/OFQ**-NH_2 *via* a thiol-Michael reaction.[241] **PWT2-N/OFQ** was found to be 40-times more potent than the native **N/OFQ** peptide with longer lasting effects.[241] This PWT technique was later applied on other opioid ligands (e.g. dermorphin, **N/OFQ** analogs, **UPF-101**).[242] In 2015, Rizzi *et al.* demonstrated the spinal antinociceptive effects of **PWT2-N/OFQ** for both neuropathic and nociceptive pain in mice and primates, exhibiting a duration of action of more than 24 hours in primates and 40-fold more potency than **N/OFQ**.[243] Moreover, **PWT2-N/OFQ** displayed a biased action towards G protein-signaling, since the calculated bias factor was 1.09 quantified with the operational model relative to **N/OFQ** (Equation 2).[240]

3. SCH 221510

SCH 221510 (Figure 9) is an orally-available NOP receptor agonist with anxiolytic-like effects and a high affinity (K_i = 0.3 nM) towards NOP receptor and selectivity for the NOP receptor over MOR, KOR, and DOR (217-, 437- and > 9500-fold, respectively). The anxiolytic-like effects were established through preclinical animal models, showing similar effects than CDP (chlordiazepoxide), but no disruption of overt behavior, such as locomotor activity, was observed, as is the case for CDP. **SCH 221510** is also capable of attenuating vocalizations in guinea pig pups and, even upon chronic administration, the effects did not decrease. Whereas benzodiazepines are associated with sedation, muscle relaxation, amnesia, tolerance and dependence, **SCH 221510** is not.[244] More recent studies on **SCH 221510** have shown that **SCH 221510** can attenuate the reinforcing effects of MOR agonists and does not function as a reinforcer in rats. Whenever an organism's future behavior is preceded by a specific antecedent stimulus, reinforcement is a consequence applied that will strengthen that behavior. For this reason, **SCH 221510** is now considered a potential drug candidate against addiction.[245] Additionally, Sobczak *et al.* reported the anti-inflammatory and antinociceptive effects of **SCH 221510** in mice with acute inflammation, thereby suggesting a potential therapeutic strategy for the treatment of inflammatory bowel diseases.[246] The same research group also stated anti-transit and antinociceptive effects. In mice, **SCH 221510** inhibited the gastrointestinal tract contractibility both *in vitro* and *in vivo*.[247] Regarding biased signaling, Malfacini *et al.* determined a bias factor of 0.77 relative to **N/OFQ** with a preference for the G protein pathway.[240] **N/OFQ** is the endogenous peptide ligand for NOP receptor. A few years later, Ferrari *et al.* reported a bias factor for **SCH 221510** using the operational model for the calculation (Equation 2). A bias factor of 1.10 was obtained towards G protein-signaling over β-arrestin-2 recruitment, in comparison with **N/OFQ**.[248]

4. SCH 486757

SCH 486757 (Figure 9) is a non-peptidic, orally bioavailable agonist for NOP receptor with a selectivity of 211-, 128- and 3206-fold over the 'classical' opioid receptors (MOR, KOR, and DOR, respectively). **SCH 486757** was first shown to be a potent and efficacious antitussive agent in coughing models;[249] it inhibited capsaicin-induced coughing in both acute and chronic dosing regimens by 46 ± 9% and 40 ± 11% respectively (as compared to codeine). In guinea pig, rat, dog and

cat models, **SCH 486757** is well tolerated without overt behavioral effects and it was also demonstrated that **SCH 486757** had a high affinity for human, guinea pig, dog, cat and rat NOP receptor.[249] **SCH 486757** was tested in phase I clinical trials for subacute cough in 2010, which was the first randomized placebo-controlled study of a NOP receptor agonist. The studies showed that virtually no difference in cough was observed for both **SCH 486757** and codeine when compared to placebo. Additionally, patients treated with **SCH 486757** reported sedation, however less gastrointestinal effects compared to codeine were reported.[250] Due to lack of efficacy, the somnolence of patients and sedation, the continued clinical development of **SCH 486757** was abandoned.[250-251] Nevertheless, years later the biased character of **SCH 486757** was determined by calculating the factor, compared to **N/OFQ**, of 0.81, calculated with the operational model (Equation 2), resulting in a G protein-biased ligand.[248]

5. Ro compounds

Ro64-6198 (Figure 9) is a small molecule spirocycle synthesized by Wichmann and colleagues.[252] **Ro64-6198** is a full agonist, displaying high affinity for NOP receptor and a more than 100-fold selectivity over MOR, DOR, and KOR, eliciting anxiolytic-like effects upon i.p. injection in an elevated plus-maze test.[252-253] Furthermore, **Ro64-6198** produced anxiolytic effects similar to benzodiazepines in rats.[254] Even though **Ro64-6198** showed limited bioavailability (around 4%), it exhibited excellent brain penetration following parenteral administration. Upon high dosage of **Ro64-6198** (10 mg/kg i.p.), the forced motor behaviors and panic escape latencies in rats were disrupted.[253] These effects were entirely absent in NOP receptor KO mice.[255] After daily administration of **Ro64-6198** for 15 days in rats, no tolerance to the anxiolytic-like effects was observed upon chronic administration and did not interfere with sensorimotor functions. Additionally, **Ro64-6198** desensitized cAMP responses and downregulated the number of cell-surface NOP receptors in NOP receptor-expressing cells pre-exposed to **Ro64-6198**.[256] In primates, it did not produce respiratory depression, pruritic or reinforcing effects in the same way as alfentanil.[257]

Ro65-6570 (Figure 9) is a small molecule with close structural similarities to **Ro64-6198**. **Ro65-6570** was synthesized by Wichmann *et al.* in 1999 and was identified as a non-peptide agonist with high affinity for NOP receptor and modest selectivity over the 'classical' opioid receptors.[258] In CHO h NOP receptor cells, **Ro65-6570** acted as a full agonist with a 7-fold higher potency than **N/OFQ**.[259] In 2002, Kotlinska *et al.* demonstrated that **Ro65-6570** did not change the effect of cocaine upon i.c.v. administration, in contrast to **N/OFQ** which suppressed the cocaine effects. However, acute administration of **Ro65-6570** increased the time spent in the drug-associated compartment of the conditioned place preference (CPP) apparatus in control rats.[260] On the other hand, when **Ro65-6570** was co-administered with opioid drugs – such as heroin, morphine, oxycodone etc. – it reduced the acquisition of place preference induced by opioid drugs.[261] The bias for **Ro65-6570** was quantified by the calculation of the bias factor using the Black & Leff operational model (Equation 2), resulting in a bias factor of 1.07 towards the G protein pathway versus the β-arrestin-2 recruitment, and relative to **N/OFQ**.[240] The bias factor for **Ro65-6570** was also calculated by Ferrari *et al.* and appeared to be 1.00 towards the G protein pathway (with **N/OFQ** as reference ligand).[262] But in a later study, a different bias factor was obtained, resulting in a value of 1.64.[248] Surprisingly, significant different bias factors were obtained, even though the same method was used in both studies.

Ro2q (Figure 9) is a small molecule, structurally similar to **Ro64-6198** and **Ro65-6570**, discovered by Röver *et al.* coming out of their SAR studies around the central core 8-cycloalkyl-1-phenyl-1,3,8-

triaza-spiro[4.5]decan-4-one. The cis-isomer of **Ro2q** is an agonist which is 40-fold more potent at NOP receptor than its *trans*-counterpart. Additionally, **Ro2q** is 40-fold and more than 200-fold more selective towards NOP receptor over MOR and KOR, respectively.[263] The bias factor for **Ro2q** was later calculated using the operational model (Equation 2), resulting in a value of 0.93 towards the G protein cascade in comparison with **N/OFQ**.[248]

6. NNC 63-0532

NNC 63-0532 (Figure 9) was first synthesized by Thomson and Hohlweg in 2000[264] and is structurally similar to the Ro-compounds (*vide supra*). **NNC 63-0532** showed a high affinity towards NOR (both human and rat receptor, K_i = 7.3 nM and 11 nM, respectively). Additionally, it demonstrated a selectivity of 20-fold over MOR and KOR and 14-fold over dopamine D_{2S}, D_3 and $D_{4.4}$ receptors upon radioligand binding displacement.[264] On top of its NOP receptor selectivity, **NNC 63-0532** showed an efficacy of 72%, making it a partial agonist relative to **N/OFQ** in a cAMP inhibition assay, together with insufficiently promoting β-arrestin-1 and 2, also relative to **N/OFQ**. This latter fact made it impossible to calculate a bias factor.[265] Another study was able to prove the lack of induced phosphorylation by **NNC 63-0532** at NOP receptor, which is linked to the β-arrestin pathway.[266] Since **NNC 63-0532** was not able to produce sufficient β-arrestin-2, and lacked induced phosphorylation at NOP receptor, it could be considered as a G protein-biased NOP receptor ligand.

7. MCOPPB

MCOPPB (Figure 9) is a small molecule agonist of NOP receptor. It was developed by Hayashi *et al.*[267] and has a high affinity for hNOP receptor as well as exhibiting high selectivity over the other opioid receptors (12-, 270- and >1000-fold over MOR, KOR, and DOR respectively).[268] **MCOPPB** is a full agonist of NOP receptor with an EC_{50} of 0.39 nM, contrary to MOR, DOR, and KOR, where weak or partial agonism was observed.[267] In the one-trial passive avoidance test, **MCOPPB**, unlike diazepam, did not produce amnesia. In addition, **MCOPPB** is effective upon oral administration and acceptable penetration of the blood-brain barrier penetration were observed.[268] In later studies, **MCOPPB** was described as a biased NOP receptor agonist towards the G protein pathway relative to **N/OFQ** and 10-fold more potent than the latter one. The bias factor was calculated using the operational model of Black and Leff (Equation 2), resulting in a value of 1.52 and 1.55 for G protein over β-arrestin-1 and β-arrestin-2 respectively. This makes **MCOPPB** significantly biased towards the G protein cascade over both β-arrestins.[265] In another study, the bias factor was calculated for **MCOPPB**, also relative to **N/OFQ** using the same equation, with the obtained value of 0.97, a bias towards G protein over β-arrestin-2, but using the GTPγS assay instead of the cAMP assay used by Chang *et al.*[248]

8. RTI-compounds

RTI-819 and **RTI-856** (Figure 9) are both small molecules, derived from **J-113397** (Figure 9), a potent and selective NOP receptor antagonist. The synthesis of **J-113397** was first described in 1999 by Kawamoto *et al.*[269] Later, **RTI-816** (Figure 9), **RTI-819** and **RTI-856** were synthesized starting from the un-*N*-substituted piperidine ring, by performing a reductive alkylation. Upon performing a cAMP assay, **RTI-816** showed weak inverse agonist activity at NOP receptor. **RTI-819** and **RTI-856** on the other hand demonstrated both partial agonism towards the G protein pathway with an efficacy of 75% and 77% respectively. Besides their partial agonism at G protein, they only induced very weak β-arrestin-1 and 2 recruitment. Consequently, no bias factor was calculated and **RTI-819** and **RTI-856** were both considered as G protein-biased ligand at NOP receptor.[265]

9. AT compounds

Different AT compounds, e.g. **AT-001**, **AT-004**, **AT-035**, **AT-090**, **AT-127**, **AT-202** and **AT-403**, were synthesized at Astraea Therapeutics, all consisting of a piperidine ring and a 2-indolone moiety.

AT-090 and **AT-127** (Figure 9) behaved as potent partial agonists for NOP receptor in the functional assays performed, being the GTPγS binding, calcium mobilization and BRET assays. Additionally, both compounds showed a higher selectivity over NOP receptor than **Ro65-6570**. The whole set of AT compounds displayed a moderate selectivity for NOP receptor over the other opioid receptors MOR, KOR, and DOR, whereas **AT-090** and **AT-127** showed the highest selectivity (17-fold and 61-fold over MOR, and 42-fold and 126-fold over KOR, respectively), similar to **Ro65-6570**. Upon calculation of the bias factor, **AT-090** was demonstrated to be bias towards β-arrestin-2 recruitment with a bias factor of -0.78, though **AT-127** had a bias factor of 0.27 showing a bias slightly towards the G protein pathway.[262]

An additional two AT compounds were explored for their bias were **AT-202** and **AT-403** (Figure 9), both of which contain a piperidine and dihydroindole core. The synthesis of **AT-202** was described in 2004.[270] **AT-202**, also described as **SR16835**, is a full agonist for NOP receptor and showed a binding affinity similar to that of **SCH 221510** and **SCH 486757**.[74] In a mouse spinal nerve ligation model for neuropathic pain, **AT-202** displayed anti-allodynic activity with Von Frey monofilaments upon systemic administration. No thermal antinociceptive activity in tail-flick test was observed in mice for acute pain.[74-75] Even though **AT-202** proved to be 50-fold less potent than **N/OFQ** in a GTPγS binding assay and 100-fold less potent in a calcium mobilization assay, it showed to be a G protein-biased agonist for the NOP receptor with a bias factor of 0.46 in comparison with **N/OFQ**, and quantified using the operational model (Equation 2). Furthermore, **AT-202** exhibited moderate selectivity over the other opioid receptors. In contrast to **AT-202**, **AT-403** displayed a similar degree of potency as **N/OFQ** in both a GTPγS binding and calcium mobilization assays, but is also a potent full agonist. Additionally, **AT-403** demonstrated excellent selectivity towards NOP receptor over MOR, KOR, and DOR, but its bias factor did not exceed 0.16 relative to **N/OFQ**. This value is not statistically different from 0 and so **AT-403** cannot be considered a biased NOP receptor ligand.[248] Later studies on **AT-403** demonstrated the induction of anti-Parkinsonian and anti-dyskinetic effects. At low doses *in vivo*, **AT-403** improved Parkinsonian akinesia and disrupted motor activity as well as significantly reducing abnormal involuntary movements (AIMs).[271] Based on these findings, it remains unclear whether **AT-403** functions as a biased agonist at NOP receptor or not, since the reduction of side effects were clearly observed, but no significant bias factor has so far been be calculated.

IV. Bifunctional biased opioid receptor ligands

Bifunctional ligands can have the prospect of improved potency, whilst at the same time producing fewer harmful side effects compared to ligands only binding to a single target. Major advantages for bifunctional ligands over drug cocktails are the fact that their PK and PD properties are more predictable and there is less chance of drug-drug interactions.[272] While the advantages of agonism or antagonism at one receptor are maintained, targeting a second receptor with a single molecule can bring along benefits such as attenuated side effects (e.g. MOR/DOR ligands, *vide infra*) or significantly lowered dosages for efficient analgesic responses due to synergistic effects induced through a simultaneous activation of distinct receptors involved in pain signaling (e.g., MOR/NOP

ligands).[Guillemyn JMC and Starnowska Eur J Pharm Sci 2017] Different bifunctional biased opioid receptor ligands are listed and discussed briefly below.

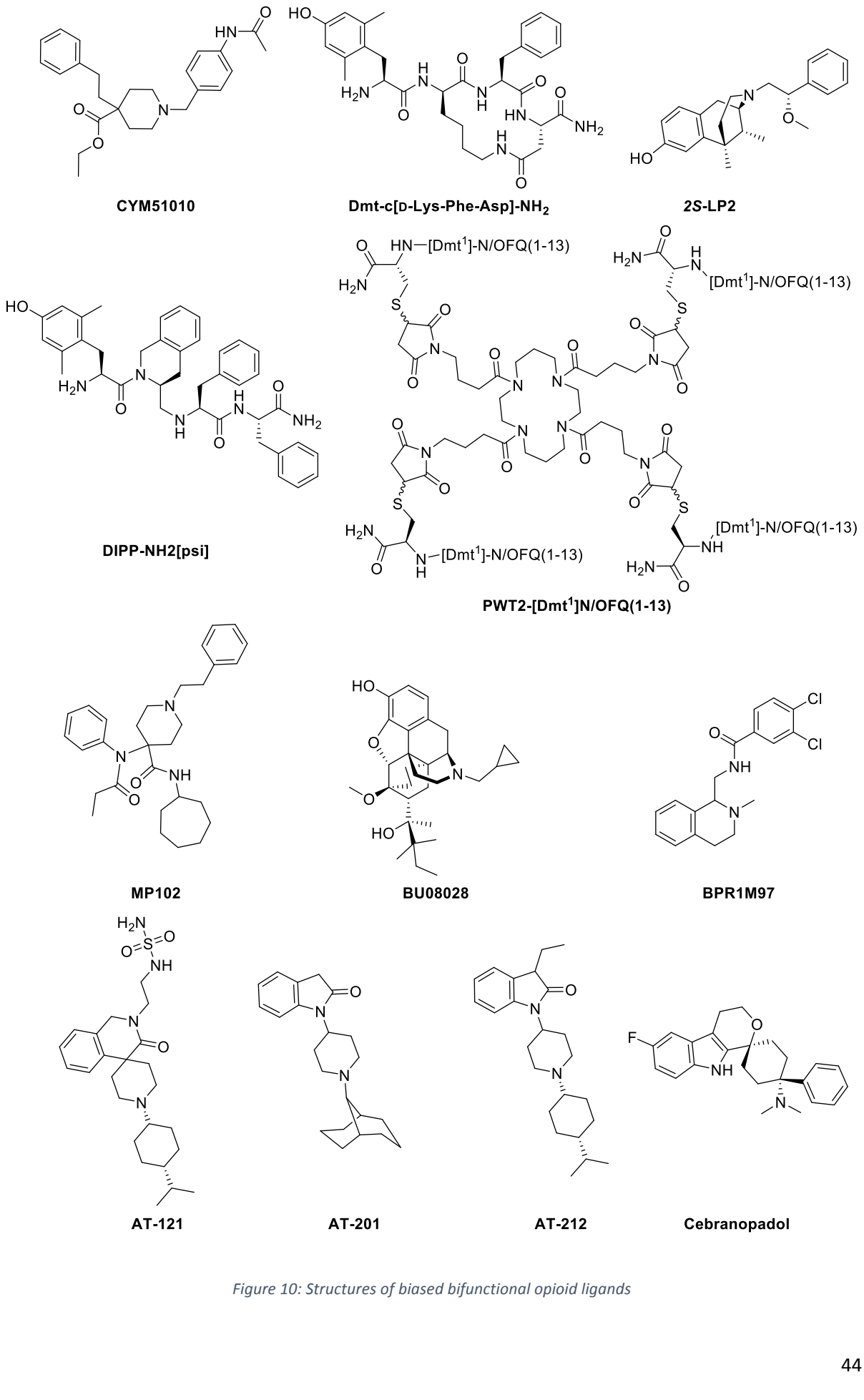


Figure 10: Structures of biased bifunctional opioid ligands

A. MOR/DOR bifunctional ligands

1. **CYM51010**

CYM51010 (Figure 10) is a small molecule biased agonist for the μ-δ opioid receptor heterodimer discovered by Gomes *et al*.[273] Its biased activity was discovered using the MOR-DOR heterodimer-selective monoclonal antibody, since its activity was blocked by this antibody. Upon systemic administration, **CYM51010** exerted antinociceptive effects comparable to morphine, but less antinociceptive tolerance. **CYM51010** displayed a higher potency for G protein activation towards the heterodimer compared with MOR and DOR separately (EC_{50} of 54 nM vs 210 and 300 nM for MOR and DOR respectively) and a lower potency for β-arrestin recruitment (EC_{50} of 8.3 μM vs 1.8 and 2.7 μM for MOR and DOR respectively), suggestive of biased activity towards the G protein pathway.[273-274-275] Additionally, **CYM51010** was found to remain active even in morphine-tolerant systems and is capable of reversing thermal hyperalgesia in rats, thereby acting as an inhibitor of neuropathic pain in rodents. Furthermore, **CYM51010** produced significantly less internalization at MOR relative to **DAMGO** and at DOR relative to deltorphin I.[275]

2. Dmt-*c*[D-Lys-Phe-Asp]-NH_2

Dmt-*c*[D-Lys-Phe-Asp]-NH_2 (Figure 10) is a cyclic peptide, derived from the selective MOR ligand Tyr-*c*[D-Lys-Phe-Asp]-NH_2 by the replacement of Tyr with 2',6'-dimethyltyrosine (Dmt). The sequence was derived from **EM-2**. Dmt-*c*[D-Lys-Phe-Asp]-NH_2 displayed a high efficacy MOR/DOR agonist profile with a high affinity for both receptors. Furthermore, the ligand demonstrated improved antinociception in the hot-plate test when compared to its Tyr counterpart. In addition, it also showed a 5-fold higher antinociceptive effect in murine models than its Tyr-bearing analog. Nevertheless, Dmt-*c*[D-Lys-Phe-Asp]-NH_2 was found to promote the G protein pathway at MOR similarly to **EM-2**, but it recruited β-arrestin in much higher extent, which resulted in in a bias factor of -1.16 relative to **EM-2** (operational model; Equation 2).[276]

3. DIPP-NH_2[ψ]

TIPP-NH_2 is a tetrapeptide Tyr-Tic-Phe-Phe-NH_2 containing the constrained phenylalanine analog Tic and was discovered by Schiller *et al.* as a ligand with a higher selectivity to DOR over MOR (26-fold).[277] TIPP-NH_2 showed a moderate degree of potency at MOR in guinea pig ileum (GPI) assays and extensive antagonism towards the DOR in mouse vas deferens (MVD) assays. This resulted in the first μ-agonist/δ-antagonist bifunctional ligand with mixed MOR-agonist and DOR-antagonist properties.[277] Subsequently, TIPP-[ψ] was described as a highly potent and stable DOR antagonist, while showing no MOR or KOR antagonism, with high selectivity towards the DOR over the MOR, being μ/δ = 10 500-fold). TIPP-[ψ] is a pseudopeptide that is highly stable against enzymatic degradation.[278] Through the substitution of Tyr with Dmt, Schiller was able to demonstrate enhanced potency towards DOR antagonism, but decreased DOR selectivity.[279] Subsequently, the modifications going from DIPP-NH_2 to DIPP-NH_2[ψ] (Figure 10) led to a better opioid profile. After i.c.v. administration, DIPP-NH_2[ψ] produced three-fold more potent analgesic effects in a rat tail flick test, though at the same time producing less acute tolerance, relative to morphine. Chronic tolerance was produced by DIPP-NH_2[ψ], but still less pronounced than morphine. Additionally, no physical dependence was observed upon administration of much higher doses needed to induce analgesic responses.[280]

4. ***2S*-LP2**

***2S*-LP2** (Figure 10) is a *N*-substituted 6,7-benzomorphan compound, synthesized by Pasquinucci *et al.* that acts as a G protein-biased agonist for both MOR and DOR using DADLE as a reference ligand. ***2S*-LP2** displayed a high affinity for MOR and DOR (K_i = 0.5 nM and 2.59 nM respectively), with a 53-fold selectivity of MOR over KOR. In comparison to the *racemic* **LP2** compound, which is also biased for the G protein pathway, ***2S*-LP2** has a higher biased factor (0.82 vs 0.57 for MOR and 2.31 vs 2.03 for DOR) calculated using the operational model (Equation 2). ***2S*-LP2** is hence a compound with more preference to G protein than its *racemic* mixture, and consequently it could provide a safer treatment oppurtunity.[281]

5. **MP102**

MP102 (Figure 10) is a fentanyl-like compound synthesized by Váradi *et al.* using an Ugi multicomponent reaction. It serves as a mixed MOR agonist/DOR partial agonist with respect to **DAMGO** and **DPDPE** respectively, in addition with *in vivo* analgesic potency upon s.c. administration. Importantly, no physical dependence or constipation was observed in mice, together with a lower production of respiratory depression compared to morphine.[282] Consequently, **MP102** is a G protein-biased ligand with preference towards DOR and showed a reduced alcohol intake.[283]

B. MOR/ NOP receptor bifunctional ligands

1. **PWT2**-[Dmt^1]**N/OFQ**(1-13)

PWT2-[Dmt^1]**N/OFQ**(1-13) (Figure 10) is an analog of the previously described compound **PWT2-N/OFQ**(1-13), reported as a G protein-biased NOP receptor agonist.[284] [Dmt^1]**N/OFQ**(1-13)-NH_2 was first described in 2013 by Molinari *et al.* as a potent full agonist behaving as a universal agonist, since it showed high affinity towards all four opioid receptors (K_i = 10.48 nM, 9.43 nM, 9.83 nM and 10.59 nM for MOR, DOR, KOR and NOP receptor respectively). Additionally, [Dmt^1]**N/OFQ**(1-13) displayed a selectivity for NOP receptor over MOR (26-fold).[285] Upon linking [Dmt^1]**N/OFQ**(1-13)-NH_2 to PWT2, the tetrabranched molecule (*vide supra)*, the ligand became a G protein-biased agonist compared to both [Dmt^1]**N/OFQ**(1-13)-NH_2 and **N/OFQ** on NOP receptor, and as compared to both [Dmt^1]**N/OFQ**(1-13)-NH_2 and dermorphin on MOR. Yet, it was still a potent agonist with long-lasting action.[284] Since **PWT2**-[Dmt^1]**N/OFQ**(1-13) activated the G protein pathway over the β-arrestin-2 recruitment for both NOP receptor and MOR, **PWT2**-[Dmt^1]**N/OFQ**(1-13) can be classified as a G protein-biased bifunctional ligand.

2. **BU08028**

BU08028 (Figure 10) is an orvinol compound with structural similarity to buprenorphine, discovered as the first "universal opioid ligand" with a high affinity at all four opioid receptors (MOR, KOR, DOR and NOP receptor affinities of 2.14, 5.63, 1.59 and 8.46 nM respectively). **BU08028** was shown to have long-lasting effects on tail-flick latency, but is liable to the development tolerance in thermal antinociception at a faster rate than for morphine. In addition, **BU08028** was shown to have high-to-moderate activity at both MOR and NOP receptor, low activity at DOR, and no activity at KOR. Furthermore, no doses caused respiratory depression or dependence.[35, 274, 286] Later studies by Ding *et al.* showed that **BU08028** to be safe in primates with an improved side effect profile. Following s.c. administration, it elicited longer-lasting antinociceptive and anti-allodynic effects than buprenorphine. In addition, **BU08028** lacked reinforcing effects together with no production of acute physical dependence.[35, 274, 287]

3. **BPR1M97**

BPR1M97 (Figure 10) is a small molecule containing a tetrahydro-isoquinoline core, discovered by Chen *et al.* on the basis of SAR studies. The authors showed that **BPR1M97** is a potent, high affinity MOR agonist whilst also being a medium-strength KOR agonist, which can be explained by the formation of hydrogen bonds between His^{54}, Asp^{147} and Tyr^{148} observed in molecular docking simulations. In tail-flick tests, **BPR1M97** exhibited strong antinociceptive effects.[288] More recently, **BPR1M97** was shown to be a bifunctional full agonist at both MOR and NOP receptor. Its high potency and efficacy were qualified, in addition to causing less cardiovascular, respiratory and gastrointestinal dysfunction (as compared to morphine as a reference ligand). On top of this, **BPR1M97** induced cAMP inhibition on NOP receptor, while not recruiting β-arrestin-2, making it a G protein-biased NOP receptor agonist. For MOR, it behaved as a full agonist for the G protein pathway and a partial agonist for β-arrestin-2 recruitment.[289] As a result, **BPR1M97** can be classified as a bifunctional G protein-biased ligand at MOR and NOP receptor.

4. AT-compounds

AT-121 (Figure 10) is a sulfamide-containing derivative of the other AT-compounds previously discussed (*vide supra*). It was recently developed by Ding *et al.* in 2018 as a bifunctional NOP receptor /MOR agonist using a combination of receptor structure-guided drug design and SAR analysis. **AT-121** served as a potent analgesic partial agonist, without inducing hyperalgesia or physical dependence. Additionally, it evoked 100-fold more potent antinociceptive effects relative to morphine, together with antiallodynic activity and the lack of reinforcing effects in monkeys (as would be seen with oxycodone and cocaine), while buprenorphine produced mild reinforcing effects.[290] Currently, however, only limited pharmacologic data about **AT-121** is available.

AT-201 (Figure 10), previously named **SR 16435**, was first described by Zaveri *et al.* It consists of a 2-indolone and piperidine moiety,[270] and was one of the first non-peptidic NOP receptor /MOR bifunctional agonists to be characterized. It has a high affinity for both NOP receptor and MOR, viz. 7.49 and 2.70 nM respectively.[270, 291] In a GTPγS functional assay, **AT-201** was demonstrated to be a partial agonist for both MOR[291] and NOP receptor.[270] Following acute administration of **AT-201**, increased latency of the tail-flick in the mouse tail-flick assay was observed, resulting in an antinociceptive effect. Since naloxone was able to block this effect, it was suggested that the antinociceptive effect was mediated by the MOR.[291] Additionally, a significant decrease in the onset tolerance was confirmed relative to morphine.[71, 291]

AT-212 (Figure 10), previously named **SR 16507**, was also described by Zaveri *et al.* and is structurally very similar to **AT-201**.[270] It has been described as a NOP receptor /MOR bifunctional agonist with a high affinity for both receptors, viz. 5.22 and 1.07 nM. Additionally, **AT-212** was experimentally proven to be a potent full agonist for the NOP receptor and a very potent partial agonist for the MOR in a GTPγS functional assay.[292] Even though **AT-212** was able to produce modest conditioned place preference (CPP), it was able to attenuate morphine CPP.[71, 293]

Since only limited studies on the bias of both **AT-201** and **AT-212** are available, their biased activity is still to be fully determined.

5. Cebranopadol

Cebranopadol (Figure 10) is a spiro[cyclohexane-dihydropyrano[3.4-*b*]indole]-amine derivative first reported by Schunk *et al.* in 2014. Cebranopadol acts as an effective analgesic at all four opioid

receptors. Different functional *in vitro* tests are displayed in Table 4. Upon *in vivo* PK experiments in mice, cebranopadol displayed good oral bioavailability (F = 44%), low clearance (Cl = 0.96 L/h·kg), medium distribution volume (V_c = 2.96 L/kg) and moderate half-life ($t_{1/2}$ = 2.57 h). Cebranopadol is equipotent to fentanyl and is highly effective in acute nociceptive animal pain models.[294] In several rat models of both acute and chronic pain – including tail-flick, spinal nerve ligation, bone cancer, rheumatoid arthritis and diabetic neuropathy – cebranopadol showed highly potent, efficacious antinociceptive and antihypersensitive activity. Additionally, the duration of action was long for both i.v. and oral administration (7 and more than 9 hours, respectively). The first indications of bias were observed when cebranopadol was not able to disrupt respiration and motor coordination, which is the case for morphine.[295] For both G protein and β-arrestin activity, cebranopadol remained a full agonist for the MOR in these studies, unlike NOP receptor for which cebranopadol completely lost efficacy towards β-arrestin recruitment. This makes cebranopadol a G protein-biased ligand for NOP receptor, but with a 10-fold higher potency for MOR over NOP receptor towards G protein-signaling.[296] Currently, cebranopadol is in phase II clinical trials, since it showed lower abuse potential than hydromorphine.[297-298]

Table 4: In vitro data of cebranopadol at all four opioid receptors

Receptor	Radioligand binding	[^{35}S]GTPγS binding[a]	
	K_i (nM)	EC_{50} (nM)	Relative efficacy (%)
MOR	0.7	1.2	103.5
DOR	18.0	110.0	105.0
KOR	2.6	17.0	67.2
NOR	0.9	13.0	88.9

[a] *The reference ligands used:* ***DAMGO*** *(MOR),* ***U69,593*** *(DOR),* ***SNC-80*** *(KOR),* ***N/OFQ*** *(NOR)*

V. Conclusion: Past challenges, future perspectives

The interest in the field of biased agonism at the opioid receptors has exponentially increased over the last decade. This review shows the results of tremendous research efforts in the search for biased ligands, particularly on all four opioid receptors. They each have their own unique properties which makes them a singularly and collectively interesting basis for the development of new and improved biased agonists, preferentially towards the G protein pathway. The calculation of bias is already well established *in vitro* by the use of the equiactive or the operational model equations, whereas *in vivo* testing deals rather with the comparison of certain effects between ligands, making the latter more difficult to determine.

The most significant challenge faced by researchers in this field, though, is the selection, and subsequent development of the most appropriate systems in which to study the effects of functional selectivity, since the observed effects are heavily context dependent: the qualitative measurement and quantification of the biased signaling can be influenced by nuanced experimental kinetics, read-out bias, variations related to the cell or tissue cultures employed in the studies, as well as by the dependency of the system on the observed pharmacological effects.[98] The first goal for the development of biased ligands is the identification of agonists exerting their effects through functionally selective mechanisms, which consequently provides opportunities to understand ligand

bias and the means for its quantification, in addition to high-throughput screening to distinguish G protein and β-arrestin efficacy. Despite the range of available possibilities for identification, there is still a limited understanding between *in vitro* and *in vivo* profiles of biased ligands. Furthermore, the cost and complexity of ligand screening as a part of the ligand development process is another substantial challenge to be overcome, as is determining which measure of bias is needed to obtain a change in physiological responses.[91] It is not straightforward to identify the signaling pathways responsible for therapeutic effects and the pathways responsible for the detrimental side effects, and only limited cases were confidently determined.[44, 112-113, 299-300] On the other hand, the means to quantify and illuminate biased agonism are already elaborated to a significant extent.[301] As a consequence, there appears to be a worrying lack of correlation between different studies of calculated bias factors (*vide supra*): the bias of a given agonist has been shown to depend upon the signaling output used for the calculation.[81] This lack of consistency can perhaps be ascribed to insufficiently rigorous data collection or the fact that current methods are not always able to distinguish between system and ligand bias.[302] Caution should be therefore exerted in any transposition from *in vitro* efficacy to *in vivo* biological responses.[91, 303]

Earlier this year, Machelska and Chelik described five potential strategies that are being actively utilized in the design and development of new opioid analgesics.[304] These include the biased activation of opioid receptors, the pH-dependent activation of receptors in peripheral tissue and the targeting of opioid heterodimers. The multifunctional (biased) ligands remain the subject of considerable scientific attention, especially if we consider that a number of studies have shown that DOR is able to heterodimerize with both MOR and KOR. The resulting MOR/DOR and DOR/KOR heterodimers represent novel pharmacological targets with distinct receptor-binding properties.[305-307] Although the (patho)physiological function of these heterodimeric opioid receptors remains to be fully elucidated, initial studies indicate that targeting the MOR/DOR heterodimer specifically may lead to improved analgesics with reduced side effects.[308] A critical goal of research in this area, therefore, is to fully understand these complex, higher-order receptor interactions, particularly in terms of biased agonism, and to harness this knowledge towards the development of novel opioid analgesics devoid of side-effects.

Numerous GPCR crystal structures have been resolved over the past decade in a variety of active conformations and now serve as the most common source of data for structure-based drug design.[309] Some groups have already been able to demonstrate the utility of the approach. On the basis of the crystal structure of MOR, Manglik and coworkers were able to generate a potent small molecule G_i activator by screening more than three million lead-like compounds in the ZINC database.[109] The authors' optimization work yielded the first-in-class molecule **PZM21** (*vide supra*) that, thanks to its MOR selectivity and significant bias, showed long-lasting analgesia devoid of both respiratory depression and morphine-like reinforcing activity. The authors also note that their general approach is able to find scaffolds that stabilize as yet unprobed receptor conformations. Others have also enjoyed success with structure-based design approaches (including [64, 310] and featuring in [311]), and it is expected that more will follow in the future. As our structural understanding of biased signaling and its manipulation improves – for example following the first active crystal structure of the δ opioid receptor by Claff and coworkers in 2019 [312] – there will be increased opportunities available for structure-based design approaches including (virtual) fragment screening and NMR-based methods.[313] Furthermore, Che and coworkers state molecular insights on mechanistic properties of biased signaling at the κ opioid receptor by using the active-state structure

of the receptor stabilized with nanobodies.[314] When considering multi-functional selectivity towards the opioid receptors, structure-guided approaches can also be used. Very recently, Uprety *et al.* demonstrated this for MOR and KOR, but further investigation in this field is needed.[315]

Finally, the issues faced before the approval of oliceridine (**TRV-130**, OLINVYK™) suggests that a better understanding of the signaling pathways, as well as a more rigorous analysis of the signaling data, may prevent potentially costly drug attrition rates in the future. The approval of OLINVYK™, the first functionally selective opioid analgesic, has opened up new avenues for future molecules in the development towards better, superior analgesics. Since **TRV-130** has only limited applications, there is an extensive window for potential improvement, especially in terms of its administration. This provides ample opportunity to develop orally bioavailable molecules, as well as new medicines that can overcome the commonly experienced side effects seen with **TRV-130**: nausea, vomiting, dizziness, headache, constipation, pruritus, and hypoxia.[316] **TRV-130** is now heralded as a paragon that has shown the possibilities of biased GPCR agonism. Even with the FDA approval, however, the purported clinical benefits of these agents remain to be demonstrated.[317] That being said, **TRV-130** can, in any case, serve as a means of comparison for the next generation of biased ligands at MOR and even the other opioid receptors, and shows the *development* potential that can be reached within this area of research, especially when one considers how much remains undiscovered within the field.

In this review, many biased ligands were described both *in vitro* and *in vivo*, but only a few have been or are ready to be tested in clinical trials, e.g. **TRV734**, **CYT-1010**, **PN6047**, **ADL5747**, **ADL5859**, **TRV250**, **SCH 486757**, and cebranopadol. There is still a big need for G protein-biased agonists on the opioid receptors to be tested on humans and a better understanding on how the bias exactly works on the signaling pathways, as evidenced by the extent of efforts undertaken to bring **TRV130** to the market. It is important to mention that even though many biased ligands have been described in this review, none of them represent a profound bias. To fully address the term 'biased ligand', extremely biased ligands, e.g. an infinite bias factor (*in vitro* bias) or zero side effects (*in vivo* bias) will ultimately be needed.

Further down the line, of course, this leads, in turn, to the ongoing need for better and safer analgesics, since the major opioid still used in clinical use is morphine, which even today is associated with a number of severe and dangerous side effects. To overcome this problem, and after discovering the role of the signaling pathways of the opioid receptors, the discovery of G protein-biased ligands will have great impact on both drug design and medicine. This will have long lasting effects on the way new therapeutics are designed, the way they work and the way they are prescribed.

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