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Structural Insights Accelerate the Discovery of Opioid Alternatives

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GPCR, opioid receptor, analgesic, side effects, opioids, nonopioid

Abstract

Opioids such as morphine and oxycodone are analgesics frequently prescribed for the treatment of moderate or severe pain. Unfortunately, these medications are associated with exceptionally high abuse potentials and often cause fatal side effects, mainly through the μ-opioid receptor (MOR). Efforts to discover novel, safer, and more efficacious analgesics targeting MOR have encountered challenges. In this review, we summarize alternative strategies and targets that could be used to develop safer nonopioid analgesics. A molecular understanding of G protein–coupled receptor activation and signaling has illuminated not only the complexities of receptor pharmacology but also the potential for pathway-selective agonists and allosteric modulators as safer medications. The availability of structures of pain-related receptors, in combination with high-throughput computational tools, has accelerated the discovery of multitarget ligands with promising pharmacological profiles. Emerging clinical evidence also supports the notion that drugs targeting peripheral opioid receptors have potential as improved analgesic agents.

Contents

1. INTRODUCTION: CURRENT STATUS OF OPIOIDS

Chronic, or long-lasting, pain is a relatively common condition, with 18 to 20% of the adult population being afflicted at any one time in the United States (1, 2). Chronic pain is one of the most frequent reasons that individuals seek medical care (2), and it is among the most common causes of disability in the United States and other developed countries (1). Both chronic and acute pain (i.e., pain of relatively short duration) can be treated with a variety of medications including nonsteroidal antiinflammatory drugs, opioids, and others. Opioids are medications that exert their actions by interacting with members of a small family of G protein–coupled receptors (GPCRs) known as opioid receptors (3). Although opioids can effectively alleviate pain in many individuals, their use is associated with severe side effects, including a high potential for abuse and addiction, as well as death due to respiratory depression in overdose (3). Indeed, opioid overdose now represents a major cause of death in the United States, with 46,802 deaths reported for 2018 (4)—only a slight decrease from the 47,600 reported in 2017 (5). Opioid abuse, addiction, and overdoses are currently considered to be at epidemic proportions in the United States (6). The magnitude of these problems has led to a search for nonopioid medications for the treatment of pain and related conditions.

As the neural pathways and molecular substrates for pain have been extensively mapped (**Figure 1**) (reviewed in 7), a path for the development of potential nonopioid pain therapeutics can be gleaned from an appreciation of the relationships between the pathways and molecules. As shown in **Figure 1**, nociceptors (i.e., pain receptors) in the periphery sense various types of pain and transmit this painful signal to so-called first-order neurons. These first-order neurons make synaptic connections with nerves in the spinal cord, which then project to the thalamus and, ultimately, to higher brain centers where the pain is perceived (reviewed in 8). As shown schematically, at each location where nerves form functional synapses, molecular targets for regulating the transmission of the sensation of pain are present (**Figure 1**). The μ-opioid receptors (MORs), which are the main targets of prescribed opioid medications, are found in the dorsal horn and are essential for the analgesic actions of these medications (9, 10). MORs are also found in many higher brain areas where they are essential for the transmission and perception of pain and associated affective components (9, 11). In addition to MORs, other opioid receptors including κ-opioid receptors (KORs) (12, 13), δ-opioid receptors (DORs) (14), and nociceptin opioid peptide receptors

Analgesics: A class of drugs that act as painkillers by reducing or slowing down pain signal transmission, e.g., nonsteroidal antiinflammatory drugs and opioids

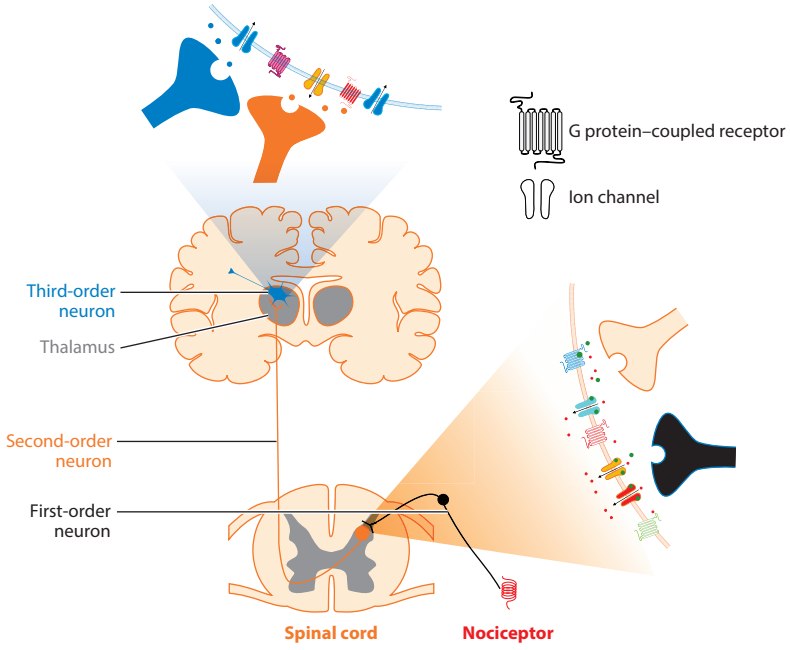


Figure 1

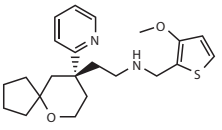
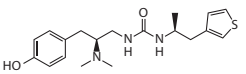
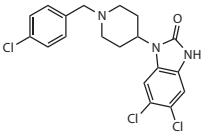
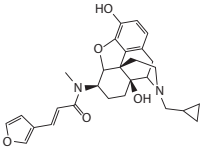
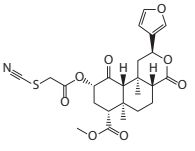
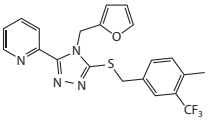
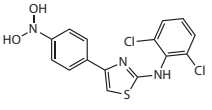
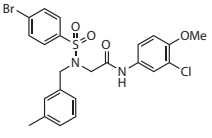
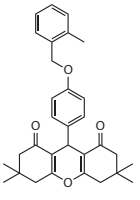
The general architecture of pain signaling sensation. This process involves multilevel regulation and multiple targets. The pain signal is first sensed by nociceptors at the peripheral site and then transmitted to the spinal cord (first-order neurons). In the spinal cord, pain signals are processed by functionally distinct nociceptors, such as ion channels and G protein–coupled receptors (second-order neurons). These signals are finally transmitted to the supraspinal site and produce associated sensations, emotional reactions, and affective states (third-order neurons).

(NOPs) (15, 16) have been identified as potential targets for both nonopioid medications as well as opioids (3). Other molecular targets involved in the pain circuit have been identified, including various ion channels (17) and many other GPCRs (18) (**Table 1**). In this review we focus on GPCRs as potential targets for creating safe and effective nonopioid medications for pain. Given recent breakthroughs in the convergent fields of GPCR structural biology and computational approaches for drug discovery, this review provides a critical perspective in this area.

2. INSIGHTS FROM HIGH-RESOLUTION STRUCTURES OF OPIOID RECEPTORS

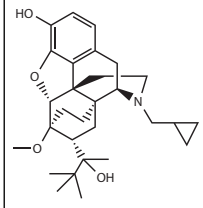
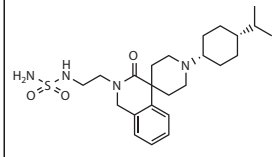
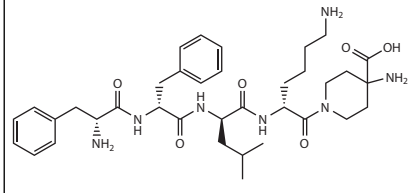
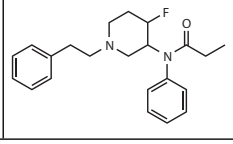
Opioid receptors were first proposed as specific biochemical entities based on the distinct analgesic activity of stereoisomers of synthetic analgesics, from which Beckett & Casy (38, p. 998) concluded in 1954 that, "Active analgesics are shown to have structures which enable them to present similar surfaces to allow of their association with a proposed 'analgesic receptor surface.'" The concept of a specific receptor for opioids was further elaborated in the 1960s by Portoghese (39) and others (reviewed in 40). The direct demonstration of opioid receptors by biochemical techniques was achieved by Pert & Snyder in 1973 (41) via radioligand binding technology. In the early 1990s, 20 years after their biochemical demonstration, four distinct classes of opioid receptors (i.e., MOR, DOR, KOR, and NOP) were cloned, and their distinctive pharmacology elucidated (42–45). Nearly 20 years later, the first X-ray structures of these four opioid receptors

Table 1 Summary of strategies and targets for alternatives to opioids

Category	Receptor	Example(s)	Chemical structure	In vitro	In vivo	Reference(s)
Biased agonists	MOR	Oliceridine (TRV-130)		G protein biased	Analgesia Reduced tolerance Reduced addiction and respiratory depression[b]	19–21
		PZM21		G protein biased	Long-lasting analgesia Decreased respiratory depression and constipation[b]	22, 23
		SR-17018		G protein biased	Analgesia No respiratory suppression	24
	KOR	Nalfurafine		G protein biased	Analgesia Antipruritic activity No dysphoria or aversion	25
		RB-64		G protein biased	Long-lasting analgesia No sedative effect Aversion	26
		Triazole 1.1		G protein biased	Analgesia Antipruritic activity No sedation or dysphoria observed	27
Allosteric modulators	MOR	BMS-986121/2		PAM	NA	28
		MS1		PAM	NA	29
	DOR	BMS-986187		G protein biased PAM	NA	30

(*Continued*)

Table 1 (*Continued*)

Category	Receptor	Example(s)	Chemical structure	In vitro	In vivo	Reference(s)
	MOR/DOR/KOR	Na^+	Na	NAM	NA	31
	MOR/DOR/KOR	Nanobody 6		NAM	NA	32
Multitarget ligands	MOR/KOR/DOR	Buprenorphine		Partial agonist at MOR Antagonist at KOR and DOR	Effective in opioid use disorder No side effects such as addiction and respiratory depression	6
	MOR/NOP	AT-121		Partial agonist at both MOR and NOP	Analgesia No side effects such as addiction and respiratory depression No physical dependence No hyperalgesia	33
	MOR/NK1R	TY027	**H-Tyr-D-Ala-Gly-Phe-Met-**Pro-Leu-Trp-NH-[3′,5′-$(CF_3)_2$Benznidazole]*	Agonist at MOR Antagonist at NK1	Analgesia No side effects such as addiction and constipation	34, 35
Peripherally restricted ligands	KOR	Difelikefalin		Agonist	Analgesia No side effects such as aversion and dysphoria	36
	MOR	NFEPP		Agonist	Analgesia No side effects such as addiction and constipation	37

[a]The bold part of the structure is the opioid pharmacophore; the rest of the structure is the NK1 pharmacophore.
[b]Controversial side effect profiles have been reported by different groups.
Abbreviations: DOR, δ-opioid receptor; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NA, data not available; NAM, negative allosteric modulator; NFEPP, fluorinated fentanyl; NK1R, neurokinin-1 receptor; NOP, nociceptin opioid peptide receptor; PAM, positive allosteric modulator.

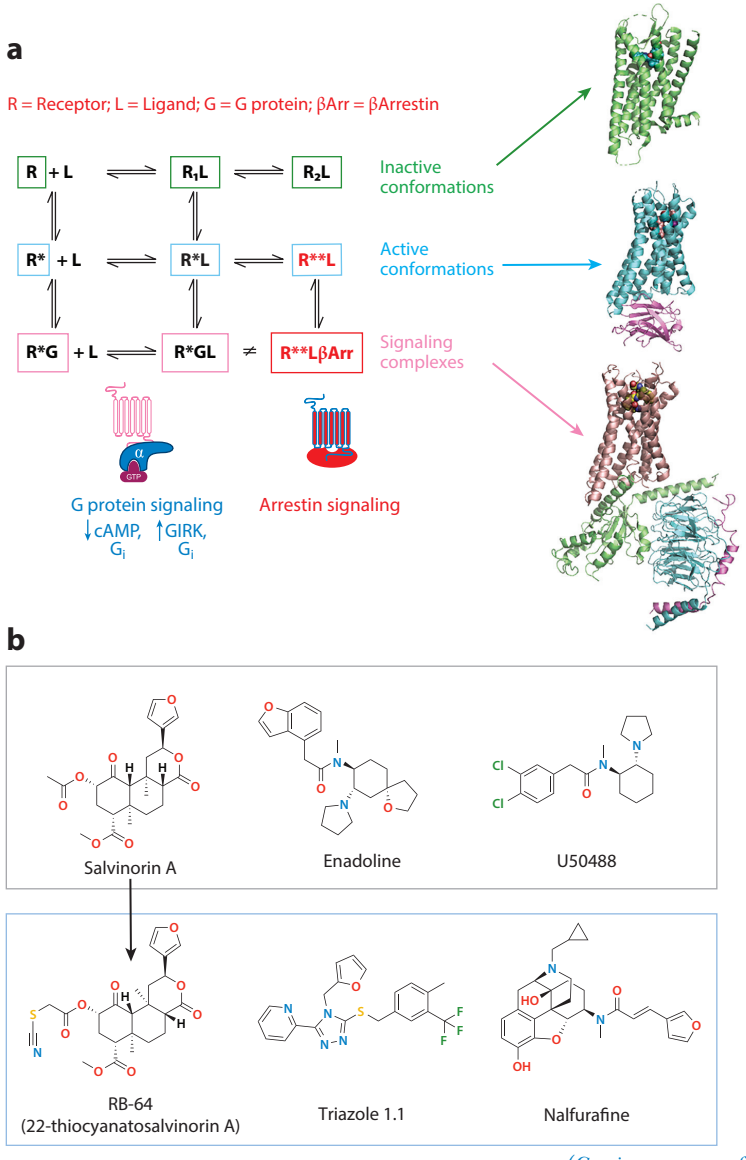
were obtained (46–49) (**Figure 2*a***). Since then, high-resolution cryo–electron microscopy (cryo-EM) and X-ray structures of active (50–52) and inactive (31, 32) states of opioid receptors have been published (**Figure 3**).

Here, we focus on insights gained from studies of KOR, as it represents a fruitful target for novel nonopioid medications. KOR agonists represent potentially safe and effective nonopioid

Agonist: A ligand that binds to a receptor and activates it to produce a biological response

Inverse agonist: A ligand that has the opposite activity to that of an agonist; compared with antagonists, inverse agonists suppress receptor activity below the basal level

medications, as they lack the lethal side effects of conventional opioids (13), albeit with side effects ranging from sedation (26) to hallucinations (53, 54). For many years, it has been suggested that KOR agonists that target the canonical G protein pathways and avoid nonclassical signaling pathways may represent safe and effective nonopioid analgesics (55–57). In support of this hypothesis, G protein–biased KOR agonists as diverse as the salvinorin derivative RB-64 (26, 58), triazole 1.1 (27, 59), and nalfurafine (25) have been demonstrated to have a variety of therapeutic effects in animal models (26, 27, 58, 59) and humans (60) and to lack the side effects associated with unbiased KOR agonists (**Figure 2*b***).



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

The conformational complexity and signaling bias. (*a*) Scheme of an extended ternary complex model of GPCR activation. The conformation of GPCRs is dynamic in that they switch between inactive and active conformations when they bind to antagonists and agonists, respectively. It is noteworthy that, even in the inactive or active conformation, GPCRs still display ligand-dependent intermediate states. These intermediate states can be captured via stabilization by mutations or conformationally specific nanobodies. To achieve a fully active state usually requires the engagement of G proteins or arrestin coupling. These ligand-dependent conformational states may affect the activation of downstream transducers and regulators, resulting in biased signaling. (*b*) Examples of unbiased (SalA, enadoline, U50488) and G protein–biased (RB-64, triazole 1.1, nalfurafine) KOR agonists. Unbiased agonists equally activate downstream G proteins and arrestins, leading to inhibition of cAMP production, activation of GIRK channels, and arrestin-dependent signaling. These unbiased agonists usually produce both analgesia and typical KOR-mediated side effects such as dysphoria and aversion. G protein–biased agonists preferentially activate G protein signaling with reduced arrestin activation. Behavioral studies have shown that G protein–biased KOR agonists maintain analgesic activity but produce less dysphoria or aversion. Abbreviations: cAMP, cyclic AMP; GIRK, G protein–coupled inwardly rectifying potassium channel; GPCR, G protein–coupled receptor; KOR, κ-opioid receptor; SalA, salvinorin A.

Thus far, there are X-ray structures of an inactive state of KOR complexed with the inverse agonist JDTiC (PDB ID: 4DJH) (46), an agonist-bound active state, a nanobody-stabilized structure (PDB ID: 6B73) (52), and a nanobody-stabilized inverse-agonist-bound ground-state structure (PDB ID: 6VI4) (32). These structures, along with that of the G_i-coupled signaling complex for MOR (PDB ID: 6DDF) (51), provide structural insights into unbiased and biased agonist activity (**Figure 3**).

Inactive state: A conformation stabilized by the binding of antagonists or inverse agonists

Active state: The conformation of a receptor stabilized by the binding of agonists; for GPCRs, a fully active state usually requires further stabilization from the receptor's cognate G protein or arrestin

Biased agonist: A ligand that preferentially activates one signaling pathway over another compared with a balanced agonist

Antagonist: A ligand that competes for the binding of agonists and blocks the action of the agonists; neutral antagonists do not change the basal activity

2.1. Structural Determinants of Ligand Selectivity in Opioid Receptors

The availability of these structures affords opportunities to better understand the mechanisms of ligand binding, receptor activation, and transducer binding and ultimately to design safer medications (22). The structural basis and molecular mechanisms underlying receptor activation or transducer binding have been extensively reviewed elsewhere (61–63). Briefly, several molecular switches (e.g., the CWxxP, NPxxY, and DRY motifs) in most GPCRs (64) and all opioid receptors are involved in transferring signaling from the orthosteric binding site to the intracellular portions of the receptor, which interact with various transducers. Ligand-stabilized conformations are encoded by the displacement of engaged residues, leading to a breakdown of energy barriers and stabilization of the shift of the receptor from an inactive to an active state. This review focuses on how unique features of opioid receptors could guide safer drug design. Although they have ∼60–70% sequence identity, the four opioid receptors have some selectivity for different endogenous ligands (MOR, endorphins; DOR, enkephalins; KOR, dynorphins; and NOP, nociceptins) (3). These preferences imply that variations exist in the binding pockets of opioid receptors that could potentially be exploited for the structure-guided discovery of selective ligands for each receptor.

Sequence alignment of the four opioid receptors reveals both conserved and nonconserved residues that may drive ligand selectivity (**Figure 4*a***). Here, we use the Ballesteros-Weinstein numbering convention (65), in which the most conserved residue of each transmembrane (TM) helix is assigned position 50, and other residues within the helix are numbered relative to this position. Each residue is then labeled with a superscript *x.yy*, where *x* is the TM helix number, and *yy* is the position in the helix. Within the opioid receptor subfamily, the highly conserved residue $Asp^{3.32}$ is a universal feature that is critical for anchoring both endogenous peptides and most exogenous agonist and antagonist small molecules (**Figure 4*b*,*c***) (reviewed in 3). The side chain of $Asp^{3.32}$ typically forms a salt bridge with the amino terminus of opioidergic peptides

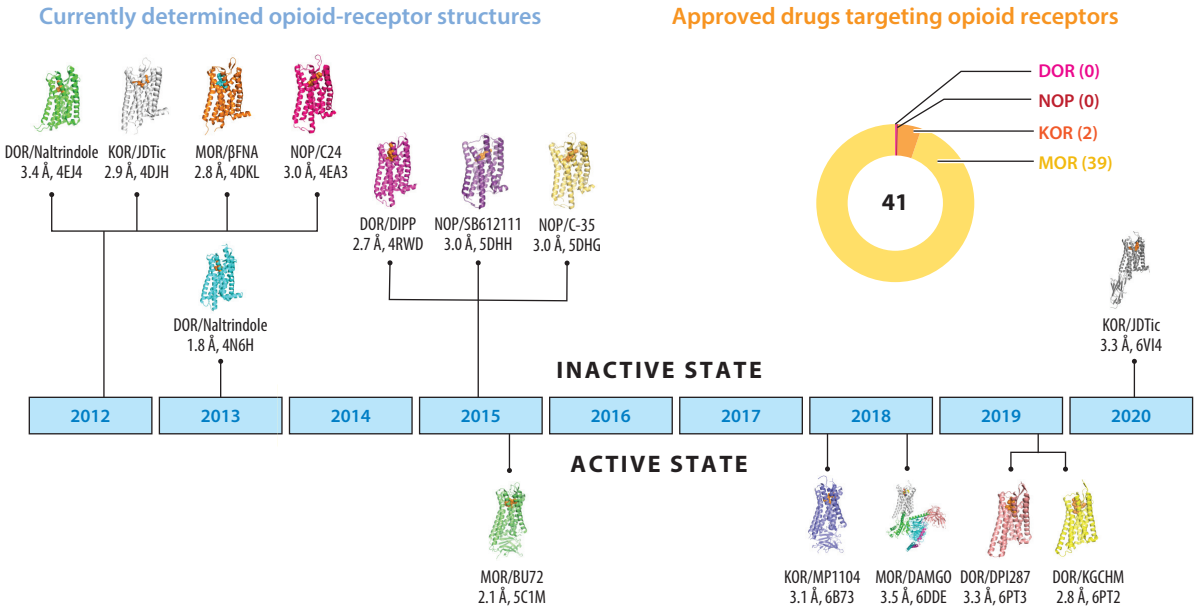


Figure 3

Opioid receptor structures and approved drugs. The structures of all four opioid receptors (DOR, MOR, KOR, and NOP) have been determined with small-molecule or peptide ligands in their inactive or active states. The structures are shown along a timeline with the receptor name and its complexed ligand, PDB ID, and resolution. Notably, the structure of DOR and naltrindole (PDB ID: 4N6H) represents one of the highest-resolution GPCR structures and shows a clear sodium-binding site. Several other structures are stabilized by nanobodies bound to their intracellular binding interface (PDB IDs: 5C1M, 6B73, and 6VI4). The structure of MOR with DAMGO is the only opioid receptor structure determined to date in complex with heterotrimeric G proteins (PDB ID: 6DDE). The approval status of the drugs was taken from the GPCR database (**https://gpcrdb.org/drugs/drugbrowser**). Most approved opioid drugs nonselectively target more than one opioid receptor. The number in the pie chart represents drugs that act primarily or selectively via that receptor. Abbreviations: DOR, δ-opioid receptor; GPCR, G protein–coupled receptor; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NOP, nociceptin opioid peptide receptor; PDB ID, Protein Data Bank identifier.

(51, 66) as well as the basic amine of opioids like morphine and other morphinans (31, 49) and nonmorphinan ligands (46, 67). Consistent with the critical role of $Asp^{3.32}$, opioid receptors with $Asp^{3.32}Ala$ mutations fail to recognize endogenous and most small molecular ligands (67). Exceptions are the KOR agonists salvinorin A (SalA) (67) and, to a lesser extent, MP1104 (52). SalA is a natural product isolated from the hallucinogenic plant *Salvia divinorum* and is a highly selective KOR agonist (54) that produces a dissociative, hallucinatory state in humans (68). SalA is structurally distinct from other opioids and contains no basic amine (**Figure 2*b***), a feature that explains why the binding of SalA does not require $Asp^{3.32}$. Still, molecular modeling studies suggest that SalA occupies a pocket like the other opioids, because it competes with radioligand binding in the orthosteric site (67). A high-resolution structure of KOR bound to SalA would help us to understand both the action of SalA and the physiological role of KOR. The KOR is the only opioid receptor that can produce psychotomimetic side effects (53), which is the main hurdle preventing KOR agonists from entering clinical trials.

Orthosteric site: The site on a receptor where the endogenous ligand binds

2.2. Structural Determinants of Opioid Receptor Activation and Signaling

Although many residues in the orthosteric site are highly conserved among opioid receptors, it is the shape, more than the composition of the binding pocket, that apparently determines ligand

a

Receptor/position	3.31	3.32	3.33	3.34	3.35	3.36	6.47	6.48	6.49	6.50	7.35	7.36	7.37	7.38	7.41	7.42	7.43	7.44
DOR	I	D	Y	Y	N	M	C	W	A	P	L	H	L	C	L	G	Y	A
KOR	I	D	Y	Y	N	M	C	W	T	P	Y	Y	F	C	L	G	Y	T
MOR	I	D	Y	Y	N	M	C	W	T	P	W	H	F	C	L	G	Y	T
NOP	I	D	Y	Y	N	M	C	W	T	P	L	R	F	C	L	G	Y	V

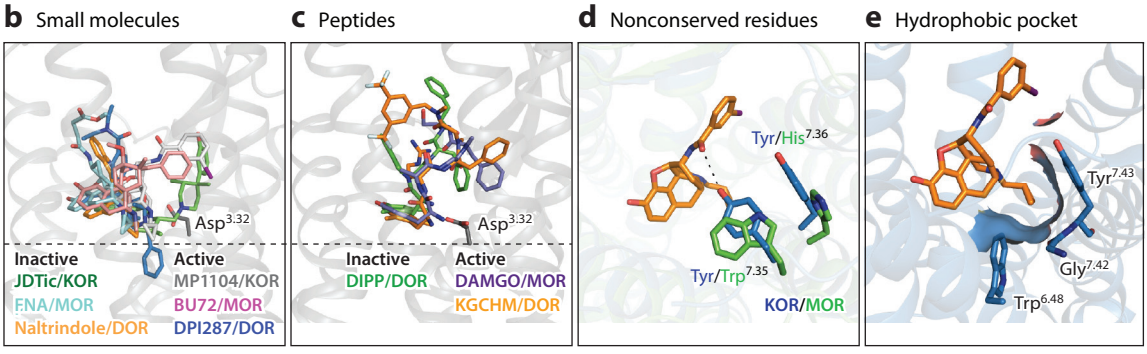


Figure 4

Structural comparison of opioid receptors. (*a*) Sequence alignment of orthosteric-binding-pocket residues in opioid receptors. Highly conserved residues that are critical for ligand recognition and functional activity are shown in red; nonconserved residues that have receptor-specific roles are shown in green. Structural alignment showing similar binding poses for (*b*) small-molecule ligands and (*c*) peptide ligands. The ligand in each structure, either inactive or active, forms one or more H-bonds with the side chain of the $Asp^{3.32}$ residue, anchoring the ligand in the correct position. Specifically, small-molecule ligands adopt a deeper binding mode than peptides. (*d*) Nonconserved residues display a receptor-specific role in a particular ligand's functional activity. Structural alignment of MOR (*green*) and KOR (*blue*) shows that nonconserved residues are present at positions 7.35 and 7.36, respectively. These residues have been shown to contribute to a receptor's biased signaling or functional selectivity. (*e*) A hydrophobic pocket in opioid receptors is critical for KOR activation. This hydrophobic pocket consists of the highly conserved residues $Trp^{6.48}$, $Gly^{7.42}$, and $Tyr^{7.43}$. The mutation of residues in this pocket in KOR has been shown to decrease agonist activity. Abbreviations: DOR, δ-opioid receptor; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NOP, nociceptin opioid peptide receptor.

selectivity. Opioid receptors are peptide GPCRs whose binding sites display a more complex interaction interface than those of receptors for small molecules. Structures of MOR bound to a synthetic peptide, DAMGO (51); KOR bound to an endogenous peptide, dynorphin A (69); and DOR bound to a synthetic peptide (66) have displayed conserved interactions. For DAMGO, for instance, the tyrosine at its N terminus forms the major interactions with the receptor residues $Asp^{3.32}$, $Met^{3.36}$, and $Val^{5.42}$, while the rest of the peptide engages the receptor's N terminus and extracellular loops in addition to the TM helices (51). These buried regions of the receptor are potentially useful for the design of opioid alternatives; because they are nonconserved, they are unique to each receptor, and they may contribute to the selectivity or functional activity of novel ligands. For example, structural comparisons between active-state structures of MOR and KOR revealed that a switch in residue at position 7.35 (Trp in MOR, Tyr in KOR) (**Figure 4*d***) could change the orientation of one ligand (IBNtxA) sufficiently to alter its downstream signaling (52). Indeed, the structure-guided discovery of ligands has had some success in published studies targeting opioid receptors (22, 70). Recent advances in large-scale (71) and ultralarge-scale docking of >100 million compounds (72, 73) have enabled the discovery of novel chemotypes with optimized signaling properties; using a similar approach for opioid receptors could enable the discovery of safer and more effective nonopioid medications.

Partial agonist: A ligand that produces a weaker maximal activity than a full agonist

A third pocket that includes the hydrophobic residues $Trp^{6.48}$, $Gly^{7.42}$, and $Tyr^{7.43}$ has recently been shown to have different roles in different opioid receptors (**Figure 4*e***). Ligand moieties interacting with this pocket at MOR confer antagonist activity (50) while simultaneously conferring agonist activity for KOR ligands (52). This is another example showing how both similarities and differences in opioid receptors contribute to complexity in the agonist and antagonist efficacies of opioids. These findings may partially explain why KOR agonists frequently appear to be MOR antagonists (74). For instance, BU74 and diprenorphine are both antagonists at MOR, while displaying full and partial agonist activity at KOR, respectively (32, 75, 76). Physiologically, MOR agonists like morphine produce side effects including addiction and respiratory depression; KOR agonists like U50488 instead produce dysphoria and respiratory stimulation (3). A deeper understanding of the molecular mechanisms responsible for these opposing observations requires insights into how opioids inhibit or activate individual receptors and how different downstream or environmental signaling partners are engaged. A ligand that nonspecifically binds to MOR or KOR may present new pharmacology, as exemplified by buprenorphine, which is a partial agonist at MOR and an antagonist at KOR that is an approved medication effective for opioid use disorders. Conceivably, such medications would represent useful alternatives to conventional opioid medications.

3. ALTERNATIVES TO OPIOIDS

3.1. Biased Agonists

As stated in Section 2.2, the classical paradigm of GPCR signaling includes activation of heterotrimeric G proteins, with this signaling being terminated by receptor phosphorylation (77) followed by arrestin binding (78). Opioid receptors signal via a subclass of G proteins, G_i and G_o, which leads to inhibition of cyclic AMP production (79), closure of calcium channels (80), and opening of G protein–activated inwardly rectifying potassium channels (81). The termination of opioid signaling is initiated by the binding of GPCR kinases (GRKs) (82, 83), which have high affinity for active-state receptors. GRKs then phosphorylate Ser/Thr residues in the intracellular loops or C termini of opioid receptors and recruit β-arrestins (84, 85), leading to receptor internalization and degradation and termination of G protein signaling (84, 86, 87). However, β-arrestins not only are negative regulators of G protein signaling but can also act as scaffold proteins to mediate G protein–independent pathways such as MAPK or ERK1/2 activation (88) and other kinase cascades (56, 89).

An appreciation of these relatively independent signaling events suggested that the pathways could be separable by using ligands that promote receptor coupling to one type of signal transducer but not to others (e.g., to G proteins but not β-arrestins, or vice versa). This notion that GPCR ligands can differentially promote the activation of distinct intracellular signaling cascades was proposed many years ago (90) and has been subsequently dubbed functional selectivity (91) or biased signaling (92). Agonists that direct signaling toward a specific pathway are conventionally referred to as biased agonists. The concept of biased signaling was supported by experiments evaluating MOR agonists using β-arrestin2 knockout mice (93). These studies supported the hypotheses that (*a*) G protein signaling is key for beneficial effects and (*b*) blocking the β-arrestin pathway could reduce the undesired side effects of opioids (26, 55, 93, 94). Since then, many studies have focused on the search for or design of G protein–biased agonists for opioid receptors that are analgesic without side effects. Several examples of drugs that preferentially activate the G protein pathway versus the β-arrestin pathway have been reported (e.g., TRV130, PZM21, and SR17018 for MORs and triazole 1.1, RB-64 and nalfurafine for KORs) and have demonstrated promising analgesic responses with attenuated side effects in preclinical rodent models (22, 24, 25, 58). In addition, recent findings have shown that G protein–biased KOR agonists are associated

with fewer side effects in nonhuman primates (95). The findings with MOR remain controversial, as several studies have questioned the role of arrestin signaling in MOR-related side effects. Thus, for instance, either phosphorylation-deficient knock-in (96) or β-arrestin2 knockout mice (97), which exhibit reduced or eliminated β-arrestin recruitment, still produce profound respiratory depression, constipation, and hyperlocomotion upon morphine or fentanyl administration. Notwithstanding these apparently contradictory findings, studies from the same group recently reported that modestly G protein–biased drugs like PZM21 and SR17018 are indeed analgesic with a lower propensity to induce respiratory depression and other side effects (23). As none of the currently available tool compounds has substantial G protein bias (23), definitive testing of the biased signaling hypothesis for MOR agonists requires better compounds—an area that could be accelerated via structure-guided discovery.

Allosteric site: A binding site on a receptor that is different from the orthosteric site

Obviously, each of the physiological responses to opioids represents a complex phenomenon encompassing multiple cellular and molecular mechanisms. A plethora of studies has suggested that G protein–mediated signaling via Gβγ subunits is involved in MOR-mediated side effects, such as respiratory depression (98), sedation (99), constipation (100), nausea, and vomiting (101). The cellular mechanisms of DOR-mediated side effects such as convulsions remain unidentified and do not seem to involve β-arrestins (102). KOR-induced aversion has been correlated with the activation of p38 MAPK (103–105) and mTOR (106, 107), both of which could involve arrestinergic signaling (56). Currently, controversy remains regarding the roles that β-arrestins play, if any, in mediating MOR-associated side effects, although there are consistent findings from several groups that KOR-mediated side effects may require arrestin signaling. Given the lack of definitive biochemical findings regarding the mechanism(s) by which arrestins mediate their actions in the pain pathways, it remains unclear whether the in vivo actions of biased agonists result from differential G protein signaling, from complex pharmacological properties, or from as yet unidentified signaling pathways.

The use of biased ligands as both tool compounds and therapeutics will ultimately prove useful when a clear signaling process is determined to be responsible for a certain in vivo action in a defined physiological setting. However, the role of specific signaling pathways in the normal perception of pain and in pathological pain states remains poorly defined. Here, it is instructive to recognize that opioid receptors, for example, can couple to seven Gα subtypes within the $G_{i/o}$ family (G_i1, G_i2, G_i3, G_oA, G_oB, G_z, and Gustducin) and two β-arrestins (β-arrestin1 and β-arrestin2) (108) (**Figure 5*a***). Indeed, our recent profiling of a dozen MOR and KOR agonists against the seven G proteins and two β-arrestins has demonstrated distinct ligand-specific signaling signatures (108). Others have reported a similar degree of Gα subtype-specific signaling by opioids (109). Thus, in addition to G protein versus arrestin signaling, there exists bias among the various Gα subtypes, thereby further complicating the imputation of signaling bias for a desired therapeutic effect.

Another challenge regarding the design of biased ligands as alternative medications is related to assay design and cellular context (for a discussion, see 108). GPCR signaling strongly depends on and substantially varies with the in vitro assays and conditions employed (e.g., cell line, native tissue, mouse or human receptors, signaling pathway subtypes) (110). Thus, relatively unambiguous assays are urgently needed for measuring the capability for an agonist to preferentially signal via one pathway over another, relative to a reference ligand (108).

3.2. Allosteric Modulators

GPCRs, including opioid receptors, can be conceptualized to function as allosteric machines to facilitate the transmission of a signal from extracellular ligand binding to intracellular signaling responses. Many allosteric sites (sites that are different and distant from the orthosteric site where

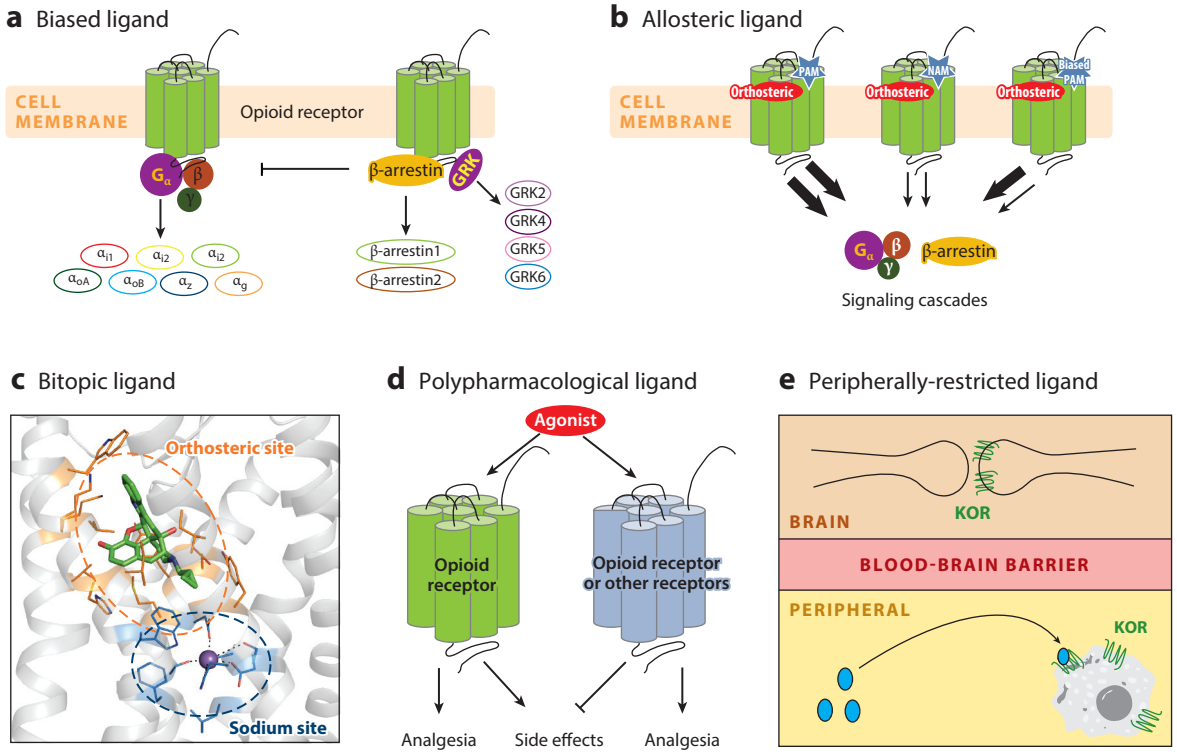


Figure 5

Emerging strategies for alternatives to opioids. (*a*) The complexity of opioid receptor-mediated signaling. Upon activation, opioid receptors predominantly couple to a family of $G_{i/o}$ proteins, which include G_i1, G_i2, G_i3, G_oA, G_oB, G_z, and Gustducin. Independent of G protein signaling, opioid receptors also interact with GPCR kinases (GRK2, 4, 5, 6) and, subsequently, arrestins (β-arrestin1 and 2). Ligands that activate a selective signaling pathway may produce beneficial effects with reduced side effects. Currently, the correlation between an individual signaling pathway and a behavioral response is not yet clear. (*b*) The potential effects of allosteric modulators for opioid receptors. A PAM could enhance the functional activity of an orthosteric ligand, such as G protein or arrestin signaling. Alternatively, a NAM could decrease the functional activity of an orthosteric ligand. An allosteric modulator may also have biased activity. For example, a biased PAM may preferentially enhance a selective downstream signaling pathway, such as G protein signaling, without affecting arrestin signaling. (*c*) The conjugation of orthosteric and sodium sites. The image was made from the structure of DOR (PDB ID: 4N6H). The orthosteric site includes the ligand naltrindole (*green*) and surrounding residues (*yellow*). The sodium site is deeper than the orthosteric site. It consists of a positively charged sodium (*purple sphere*) and nearby negatively charged residues (*blue*). A ligand that occupies both the orthosteric and sodium sites simultaneously is called a bitopic ligand. (*d*) The potential effects of multitarget opioid receptor agonists. A multitarget agonist could bind and activate two or more opioid or nonopioid receptors. Individually, they could produce both analgesia and side effects. However, the combined effects could be potentially beneficial, as the opioid receptors produce different or even opposite adverse effect profiles. (*e*) The application of peripherally restricted opioid receptor agonists. A peripherally restricted KOR agonist, for example, would be unable to penetrate the blood-brain barrier and would target only the KOR in the peripheral system. Such compounds could thus avoid any side effects elicited by the KOR in the central nervous system. Abbreviations: DOR, δ-opioid receptor; GPCR, G protein–coupled receptor; GRK, GPCR kinase; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NAM, negative allosteric modulator; PAM, positive allosteric modulator; PDB ID, Protein Data Bank identifier.

endogenous and standard exogenous ligands bind) have been identified in GPCRs and include the extracellular loops, TM and lipid interfaces, and intracellular regions (64, 111). Allosteric modulators are ligands that bind to the allosteric sites and modulate the effect of the orthosteric ligand; they can be either positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs) (111) (**Figure 5*b***). As early as the 1970s, years before the opioid receptor sequence was cloned,

radioligand binding assays demonstrated that small ions such as sodium (Na^+) or manganese (Mn^{2+}) could allosterically decrease or increase agonist binding, respectively (112, 113). These ions represent the first GPCR NAM (Na^+) and PAM (Mn^{2+}). The high-resolution crystal structure of DOR enabled the first visualization of a well-orchestrated sodium-binding pocket in an opioid receptor, in which the sodium ion is stabilized by nearby negatively charged residues (31) (**Figure 5*c***). This sodium-binding pocket collapses during receptor activation, and sodium is then expelled from this site; this is a hallmark of the activation of most class A GPCRs (50). No binding site for cations such as manganese has been determined yet, although a zinc- and a calcium-binding site have been recently visualized for the 5HT2A serotonin and MC4 melanocortin receptors, respectively (114, 115). Other natural allosteric modulators include heterotrimeric G proteins, which also function as PAMs to enhance agonist binding because they interact with the intracellular part of GPCRs and affect the affinity and/or efficacy of orthosteric ligands (116). The first small-molecule PAM for opioid receptors was discovered via high-throughput screening campaigns using a β-arrestin recruitment assay with MOR or DOR (28). Others were discovered using virtual screening approaches (29). Opioid receptor PAMs have been proposed as potentially safer nonopioid analgesics (117), although definitive data supporting this notion are not yet available.

Allosteric modulator: A ligand that binds to an allosteric site of the receptor and affects receptor responses to orthosteric ligands

To date, there are no selective and potent small-molecule NAMs for opioid receptors. Recently, however, a ground-state structure was determined for KOR bound to an intracellular nanobody (Nb6), in which Nb6 binds to a crevice between TM5 and TM6 rather than the classical core engaged by G proteins and other reported nanobodies (117). A detailed biochemical characterization of its actions revealed that Nb6 is a NAM that negatively affects the affinity and efficacy of KOR agonists (32). Thus, the pocket engaged by the receptor and Nb6 represents an allosteric site in KOR that could be used for the discovery of NAMs at KOR.

Theoretically, allosteric modulators offer an approach for the therapeutic modulation of opioid receptor activity. PAMs could potentiate the effects of endogenous opioids that are released during pain, which would restrict analgesia both temporally and spatially (117). NAMs could potentially be used to lower the analgesic dose of opioid agonists, decreasing the possibility of adverse effects associated with opioid administration. In particular, the propensity of allosteric modulators to provide probe dependence has the advantage that allosteric modulators might be effective only with a specific analgesic such as morphine, without disturbing the activity of endogenous ligands. Previous efforts to search for allosteric modulators have relied upon physical screening using cell- or animal-based assays. Although several small-molecule allosteric modulators for opioid receptors have been identified, optimization of these allosteric modulators for therapeutic candidates remains challenging, in part due to a lack of knowledge about allosteric modulation of opioid receptors. Structures of allosteric modulators bound to different GPCRs (111) provide essential details regarding where and how these modulators bind and interact with GPCRs—information that is essential for the structure-guided optimization of allosteric modulators.

The available structures combined with high-throughput computational screening have accelerated the discovery of novel scaffolded allosteric modulators by targeting identified allosteric sites (118). Although no structures of opioid receptors bound to small-molecule allosteric modulators have been determined so far, allosteric modulators can and have been discovered based on the identification of theoretical binding sites in modeled GPCRs (119). It is still difficult to predict the binding mode of allosteric modulators because, in theory, one could bind anywhere inside or on the surface of the receptors. Molecular docking (119) and dynamics simulations are providing testable predictions regarding the structures and locations of the allosteric sites in relation to the orthosteric site, and this approach is expected to facilitate the development of this class of compounds (120).

Appreciation of allostery as a potential avenue for the development of new medications provides another advantage for designing so-called bitopic agonists, which are compounds that communicate with both the orthosteric and allosteric sites (64). As stated above, sodium acts as a NAM to decrease receptor activity, and the sodium pocket is a highly conserved allosteric site in many class A GPCRs. Potential efficacy switches have been implicated in the sodium pocket, because mutation of these residues can transform classical opioid antagonists into full agonists (31, 52). The relatively short distance between the orthosteric site and the sodium site makes the design of a bitopic ligand feasible (**Figure 5*c***). A bitopic agonist with one side (e.g., the morphinan scaffold) binding to the orthosteric site and the other side (e.g., the positively charged group) extending to the sodium pocket has the potential to achieve subtype selectivity as well as spatiotemporal control of functional activity. Recent success with this strategy has been achieved with the LTB4 leukotriene receptor (BLT1); a bitopic ligand (BIIL260) occupies both ligand- and sodium-binding sites, stabilizing the receptor in the inactive state (121).

3.3. Polypharmacological Ligands

In the molecular era, roughly since 1985, drug discovery has been dominated by single-target approaches—finding molecules with high potency and selectivity for single receptors—although there have been concerns regarding this approach (122). Morphine and other clinically used opioids produce analgesia through the specific activation of single opioid receptors such as MOR (9), along with nearly all of their desired and undesired effects (9). Recently, other targets, such as the orphan receptor MRGPRX2, have been proposed as sites of action for specific side effects such as itch, for example (123). However, pain transmission or sensation is the outcome of many protein networks that include both opioid and nonopioid receptors (7). Therefore, efforts to rationally develop analgesics against multiple pain-related receptors and pathways could provide a possible path for the design of opioid alternatives with additional benefits and fewer side effects.

Two types of multifunctional ligands that can act as novel analgesics have been frequently tested and reported in preclinical studies (124, 125). The first type, also the main focus of this review, is a single ligand with a polypharmacological profile that promiscuously interacts with two or more targets (**Figure 5*d***) (126). A similar concept has been applied clinically by prescribing active analgesics together with antagonists that reduce overdose-related side effects, such as the oxycodone and naloxone combination (127). The advantages of a polypharmacological single compound over the coadministration of multiple ligands include more predictable pharmacokinetics and better control of side effects (126).

The rationale for multitarget analgesics is that pain, like other neuronal activities, rarely involves just a single target. The primary focus of the search for such ligands is on the opioid receptors because of their undisputed roles in pain management. All four opioid receptors mediate analgesia at different levels and have nonoverlapping side effects. This is mostly attributed to their variable regional expression, plasticity, and functional activity in the central nervous system (CNS) and the periphery (128). Ligands that have dual agonist activity at MOR/DOR (129, 130), MOR/KOR (131), or MOR/NOP (33) have demonstrated promising preclinical analgesic profiles with limited side effects, including dependence, hyperlocomotion, and respiratory depression. It must be acknowledged that it is a complicated and challenging task to create multitarget drugs using traditional medicinal chemistry (132). Achieving exquisite selectivity over other drug targets or the desired polypharmacology usually requires the identification of a structural basis for ligand selectivity (126).

Polypharmacology could also occur between opioid and nonopioid receptors. Several nonopioid receptors (e.g., CB1 cannabinoid receptors, MRGPRX2) have been identified by genomic

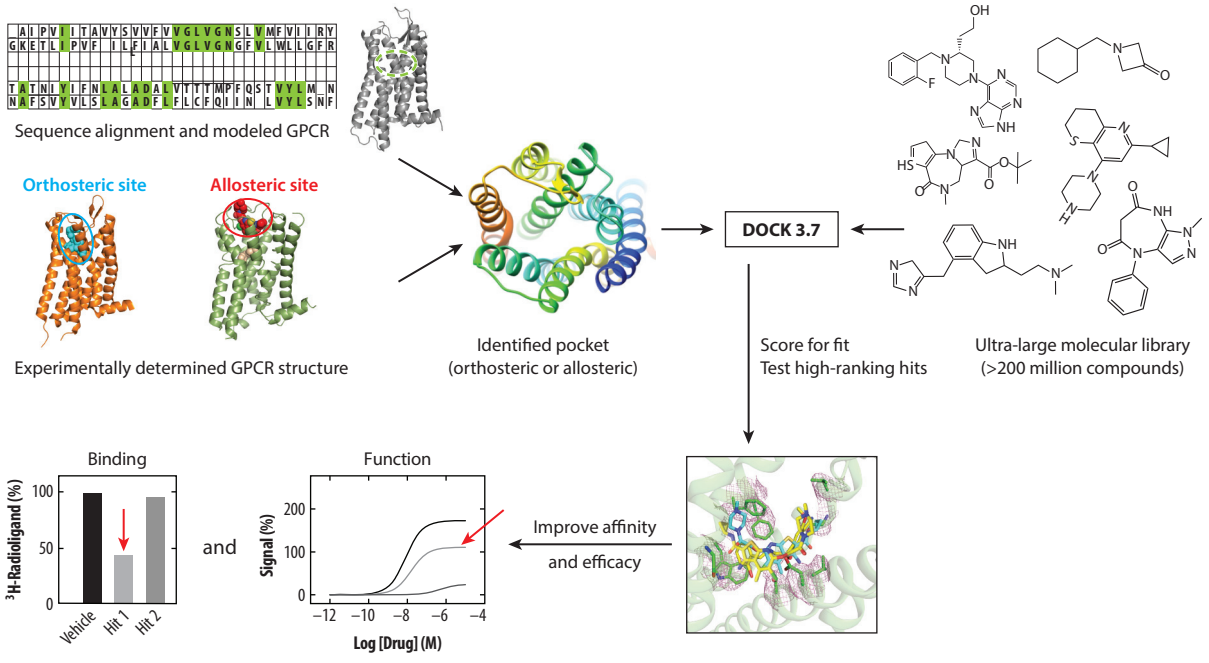


Figure 6

Ultralarge-scale docking for the discovery of new scaffolded ligands. For targets without available structures, sequence alignment and homology models can be used to identify potential binding pockets. Using the modeled or experimentally determined structure, a library of up to 200 million novel small-molecule compounds is then docked to the binding site (orthosteric or allosteric) of the structure to predict hits. The top-scoring hits are tested by physical screening, e.g., using cell- or animal-based assays. Active hits are then further optimized by medicinal chemistry or structure-guided design. To obtain a probe with sufficient activity usually requires several rounds of optimization. Abbreviation: GPCR, G protein–coupled receptor.

and proteomic methods to be involved in the regulation of variable pain models (123, 126). Up- or downregulation of these receptors at the genomic or protein level has provided valuable insights into which subtype of the ligand may have therapeutic potential (e.g., full agonist, partial agonist, antagonist). Opioid receptors also colocalize with other GPCRs [e.g., neurokinin-1 receptor (NK1)] and jointly regulate pain sensation (133). Ligands that activate MOR and inhibit the NK1 receptor have shown both analgesic efficacy for pathological pain and promising adverse effects profiles (35).

Although designing drugs with a specific multitarget profile is challenging, solutions have emerged with the development of computational methods. Using an automated design approach, for instance, a clinically approved drug (e.g., an acetylcholinesterase inhibitor) could be evolved into a ligand that selectively targets designated GPCRs (126). The synergy between available X-ray or cryo-EM receptor structures, high-throughput computational screening, and access to an ultralarge-scale virtual compound library has already taken drug discovery to a new level (72, 134) (**Figure 6**). Although this combination is still in its infancy, it is imaginable that ligands could be docked into the structure or homology model of an individual target, followed by counter-screening of potential hits against unwanted targets to exclude side effects (135).

The second type of multifunctional ligands would be drugs that can bind to two or more targets simultaneously, such as bivalent ligands. In vitro cell-based studies have indicated that heteromers can be formed between opioid receptors or between opioid and nonopioid receptors (136).

However, there is conflicting evidence regarding whether such dimers exist in endogenous systems (137) and even less evidence regarding whether a bivalent ligand could directly communicate from the ligand-binding pocket of one receptor to the binding pocket of a neighboring receptor. Considering that oligomerization is a common phenomenon in class C GPCRs (e.g., glutamate receptors), more direct evidence is needed to identify the physiological roles of homo- or heterodimers of opioid receptors in their functional activity and pain sensation (138). Cryo-EM technology has already provided structures for both homo- and heteromeric $GABA_B$ receptors (a class C GPCR) (138), and this approach could likely be extended to investigate oligomerization in class A GPCRs.

3.4. Peripherally Restricted Analgesics

The rationale behind drug design targeting peripheral opioid receptors is trifold: (*a*) all opioid receptors are expressed in both the CNS and peripheral systems; (*b*) activating opioid receptors in the periphery produces adequate analgesia; and (*c*) the most serious opioid-associated side effects are due to the activation of opioid receptors in the brain, such as respiratory depression from activation of MOR in the brainstem medulla. Constipation is an exception, since it is mostly mediated by MORs in the periphery such as those in the gastrointestinal tract, although spinal and supraspinal MORs may also be involved (127, 139). Thus, peripherally restricted opioids are expected to avoid many of the lethal side effects associated with opioids targeting the CNS (**Figure 5*e***). Clinical studies using intraarticular morphine administration or other locally applied opioids have supported the notion that peripheral opioid receptors mediate a large proportion of the analgesic effects produced by systemically administered opioids (127, 139).

Several strategies have been developed to limit the ability of opioids to cross the blood-brain barrier. The first approach involves introducing chemical modifications to increase the hydrophilicity of current opioids (140, 141). In particular, difelikefalin is a tetrapeptide (Phe-Phe-Leu-Lys) under development by Cara Therapeutics as an intravenous agent for the treatment of both postoperative pain and pruritus. An extra amino carboxylate at the C terminus confers a charge on difelikefalin and thus peripherally restricts its distribution (142). Difelikefalin has demonstrated significant clinical efficacy without any of the side effects associated with KOR agonists in the CNS (142). Based on these beneficial activities, difelikefalin proceeds to clinical trials as a first-in-class drug for pain relief after abdominal surgery (143).

The second approach is to use a specific type of conjugation or modification to allow opioids to be delivered or activated only in one specific location. Liposomes conjugated with anti–intercellular adhesion molecule-1 antibodies have been used to deliver the peripherally restricted MOR agonist loperamide to injured tissues for pain control (144). Fluorinated fentanyl (NFEPP) is a pH-sensitive MOR agonist and has been shown to be active only in peripherally injured tissues due to the acidic pH of those tissues (37, 145). A similar approach involves fusing morphine and hyperbranched polyglycerol through a cleavable pH-sensitive linker (146). This conjugate prevents blood-brain barrier permeation and allows selective release of morphine in injured tissue.

3.5. Other Strategies for Opioid Alternatives

Other approaches targeting endogenous peptides have also been extensively investigated. Endogenous opioid peptides have the advantage of being highly efficacious at pain relief without any toxicity. However, endogenous peptides as drugs have been limited by their poor pharmacokinetics, bioavailability, and permeability. Efforts have been first focused on improving the half-life

of peptides and preventing peptide degradation in the brain or plasma. Dual enkephalinase inhibitors, for instance, have shown promising profiles in preclinical tests (147, 148).

4. CONCLUSIONS AND PERSPECTIVES

Nearly all of the opioid drugs approved for clinical use to date target the MOR. The activation of MOR produces serious and potentially fatal side effects, which underlie the current opioid overdose crisis. Here, we have reviewed potential strategies and targets for developing alternatives to opioids. Biased signaling is a universal phenomenon due to the conformational heterogeneity that occurs upon GPCR activation. Future evaluation of the therapeutic potential of biased agonism should take into consideration the complexity of signal transduction pathways. Additionally, the possible existence of biased agonists should be considered for all potentially biased signaling pathways to better correlate in vitro pharmacology with in vivo activities. Allostery is an intrinsic property of GPCRs, and allosteric modulators provide an alternative means to more precisely modulate the actions of endogenous or exogenous ligands. The combination of high-resolution structures and high-throughput computation has made the discovery of polypharmacological ligands feasible. It is important to note that pain is not regulated by a single molecular target. Targeting multiple opioid and nonopioid receptors simultaneously may yield safer analgesics. The broad expression of opioid receptors in the peripheral system suggests that the application of peripherally restricted ligands could avoid the side effects elicited from such receptors in the CNS. Still, attention should be paid to the side effects mediated by peripheral receptors and the deliverability of ligands to the targeted receptors. The regulation of chronic pain involves the multifactorial engagement of various cellular regions (**Figure 1**). Therefore, for the ultimate development of safer analgesics, an integrated biochemical, pharmacological, and physiological approach is needed.

DISCLOSURE STATEMENT

B.L.R. is a founder of Epiodyne, Inc. and has received royalties from the licensing of patents to this company. T.C. is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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