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From Models to Molecules: Opioid Receptor Dimers, Bivalent Ligands, and Selective Opioid Receptor Probes[†]

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Opiates have been the most widely investigated class of natural products.[1] The two most prominent opiates, morphine and codeine, have had a long history of use and abuse. Numerous semisynthetic opiates have been synthesized in an effort to obtain analgesics that are free of tolerance, physical dependence, and addiction liability. The development of totally synthetic analgesics subsequently led to the development of diverse structural classes of ligands that mimic the actions of the opiates. Compounds with mixed agonist−antagonist activity during that period represented a new approach to reducing the abuse potential and some of the side effects associated with the classical opiates, and several of the analgesics in this group are presently employed clinically.

In this presentation I will draw on selected examples from my research to illustrate how key conceptual models have led to the design of selective ligands, some of which are widely employed as pharmacologic tools for the investigation of opioid receptors. I will also illustrate how site-directed mutagenesis, when combined with the classical structure−activity relationship (SAR) approach, has led to the identification of amino acid residues on opioid receptors and groups on ligands that participate in molecular recognition.

The Opioid Receptor Family

The discovery of the endogenous opioid peptides, Leu- and Met-enkephalin, and their interaction with specific receptors was a turning point in opioid research.[2] These revelations confirmed the 1954 concept[3] of a specific "analgesic receptor" that recognizes morphine and highlighted the importance of an endogenous opioid system in the central nervous system. The endogenous opioid peptide family is characterized by a common tetrapeptide sequence (Tyr-Gly-Gly-Phe), and it now comprises a group of over a dozen ligands.[4] More recently, two additional endogenous ligands, endomorphin-1 and -2 (Tyr-Pro-X-Phe-NH_2 X = Trp or Phe), whose tetrapeptide sequence differs somewhat from that of the classical opioid peptides have been reported.[5]

Multiple opioid receptors and multiple modes of binding to opioid receptors were first proposed over 35 years ago based on SAR analysis.[6] Subsequent in vivo and in vitro pharmacological studies led to the identification of three major types of opioid receptors, named δ, κ, and μ.[7,8] With the cloning of these receptors in 1992−3, a new chapter in opioid research was opened because their known amino acid sequences made it possible, for the first time, to investigate ligand−receptor interactions from the perspective of *both* the ligand and the receptor.[9]

The opioid receptor family is a member of the rhodopsin subfamily in the superfamily of over 1000 G protein-coupled receptors. Members of the opioid receptor family are highly homologous (∼60% amino acid identity) and recognize structurally diverse ligands that include peptides, opiates, and a variety of synthetic non-peptides.[9] The diversity of ligands, both endogenous and exogenous, and the multiplicity of opioid receptors provides a distinct advantage in exploring the basis for molecular recognition among G protein-coupled receptors in gen-

eral and opioid receptors in particular. Site-directed mutagenesis and molecular modeling in conjunction with classical SAR studies is proving to be a powerful combination for identifying the basis for molecular recognition of ligands at opioid receptors.

Revisiting Bivalent Ligands: Early Evidence for Opioid Receptor Dimers

Over 20 years ago, a small but growing number of membrane-bound receptors were known to exist as dimers. Also, ligand-induced clustering had been reported for opioid receptors on the surface of neuroblastoma cells.[10] These reports stimulated us to consider the design of double pharmacophore ligands as probes for bridging hypothetical dimeric opioid receptors. We hoped that such an approach would lead to selective ligands as tools for distinguishing between different types of opioid receptors. The term, bivalent ligand, was defined as a molecule that contains two pharmacophores linked through a spacer. I am revisiting bivalent ligands because recent reports have provided convincing biochemical evidence for homo- and heterodimers among opioid receptors.[11]

We envisaged that a bivalent ligand with a spacer of optimal length would exhibit a potency that is greater than that derived from the sum of its two monovalent pharmacophores.[12,13] Such synergy was based on the assumption that a bivalent ligand should first undergo univalent binding, followed by binding of the second pharmacophore to a recognition site on a neighboring receptor (Figure 1). When the bivalent ligand is in the univalently bound state, the pathway to bivalent binding should be favored over univalent binding of a second ligand because of the small containment volume of the tethered, unbound pharmacophore that is in the region of the unoccupied neighboring receptor site. However, if the neighboring receptors are allosterically coupled, any affinity change due to occupation of a neighboring site by a single ligand (bridging) may also be a function of the type of cooperativity that is involved. For example, if the neighboring sites are negatively allosterically coupled, it is possible that the binding enhancement may be mitigated.

Spacer length is obviously a critical factor with respect to the ability of the bivalent ligand to bridge neighboring receptors. A spacer of insufficient length would not permit bridging, and an excessively long spacer would tend to reduce bridging by increasing the confinement
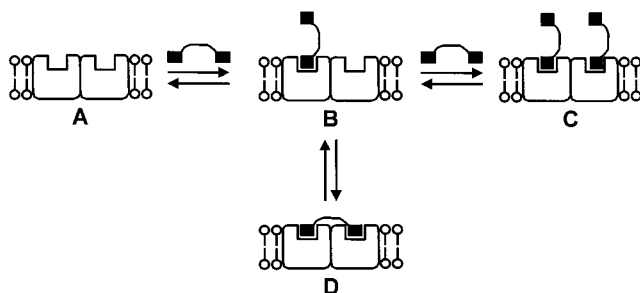
Figure 1. An original schematic illustration[12,13] of the bivalent ligand concept for bridging opioid receptor dimers. The unoccupied dimer (A) undergoes univalent binding that leads to state B. The unoccupied site in B can be "bridged" to give D, or be bound by a second ligand to give the dimer with both sites occupied (C).
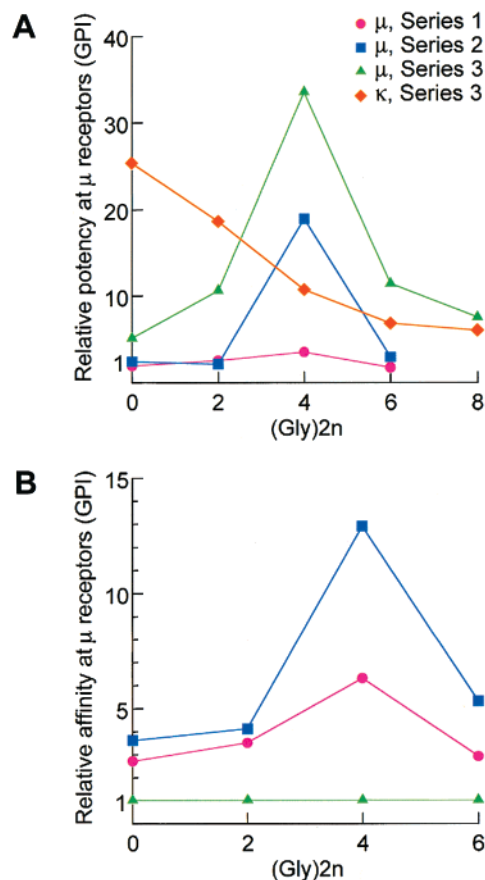
Figure 2. Relationship between spacer length and potency (panel A) or affinity (panel B) of bivalent ligands. The potencies and affinities are relative to control monovalent ligands.

volume of the free pharmacophore so that it would spend less time in the vicinity of the unoccupied, neighboring recognition site.

Our first studies on bivalent opioid ligands employed spacers that were derived from oligoethylene glycols.[13] However, subsequent series (**1**–**3**) contained spacers with glycine oligomers because of their ease of synthesis.[12,14,15] The use of glycyl units avoided cumulative

1 (6β-series), R = CH₃
2 (6α-series), R = CH₃
3 (6β-series), R = CH₂CH(CH₂)₂
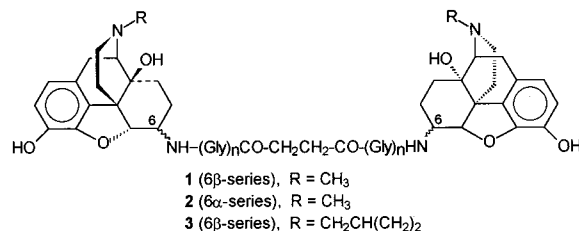
incremental increases in hydrophobicity that would occur upon homologation if an alkyl chain were employed. Symmetry was introduced into the oligoglycine spacers by the inclusion of a central succinyl or fumaryl moiety. Both moieties were employed to compare the relationship between conformational flexibility of the spacer and activity. Monovalent ligand analogues containing capped spacers were synthesized as controls in order to factor out possible contributions of the spacer to activity.

The SAR profiles obtained from testing agonists (**1**, **2**) and antagonists (**3**) in these series on the guinea pig ileum preparation are summarized in Figure 2. The data
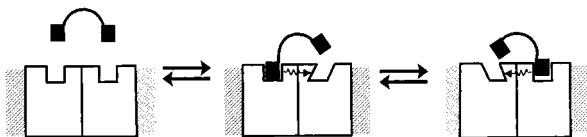
Figure 3. Model for negative cooperativity in the interaction of neighboring receptor sites in a homodimer with a bivalent antagonist. The binding of ligand to one site induces a change in the conformation of its neighboring site of the homodimer. The rapid switching of univalently bound sites would lead to the blockage of both sites.

Figure 4. Model of interlocking transmembrane (TM) helices in a homodimeric $\mu-\mu$ opioid receptor, as viewed from the extracellular side. The interface between each hybrid 7TM domain (I−V,6,7 and 1−5,VI,VII) is V,VI:6,5. The distance between binding sites is ~27 Å.

illustrate the relative agonist and antagonist potencies at μ receptors as a function of the number of glycyl units in the spacer. The most noteworthy feature is that maximum potency was observed when the spacer contained a total of four glycyl units. Paradoxically, while μ receptor affinity paralleled agonist potency, there was no significant difference in the binding of bivalent antagonists with different length spacers. The possible significance for this lack of correspondence will be discussed subsequently in light of recent reports on opioid receptor dimers.

There have been a number of reports of enhanced binding or potency at δ opioid receptors with bivalent enkephalins.[16−18] However, it is not known whether such enhancements reflected bridging of opioid recognition sites on a receptor dimer or binding to an accessory site. In order to evaluate the involvement of two opioid recognition sites in the bridging, we synthesized isomeric (meso) bivalent ligands containing both (−)- and (+)-enantiomeric opiate elements.[19] The (+)-enantiomeric units in these ligands were derived from either (+)-oxymorphone or (+)-naltrexone which are inactive at opioid receptors. These meso isomers (**4** and **5**, respectively) contained the same length spacer ($n = 2$) as the (−)(−)-bivalent ligands that afforded peak activity at μ receptors. The agonist ligand **4** was ~$1/5$ as potent as its (−)(−)-isomer (series **1**, $n = 2$), and **5** was ~$1/30$ that of its corresponding antagonist bivalent ligand (series **3**, $n = 2$). This study suggests that agonist **1** and antagonist **3** ($n = 2$) bivalent ligands each bridge discrete opioid receptor recognition sites.

4 R = CH₃
5 R = CH₂CH(CH₂)₂
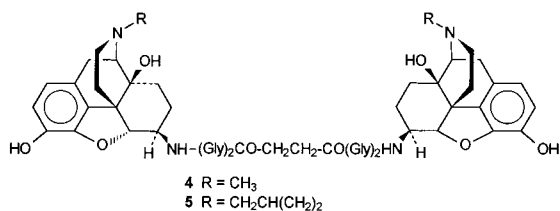
The lack of enhanced antagonist potency of the meso isomer **5** suggested that the corresponding bivalent ligand **3** ($n = 2$) bridges two stereochemically similar or identical neighboring opioid recognition sites. While the binding of the agonist series paralleled the potency, the robust enhancement of antagonism in series **3** ($n = 2$) was not accompanied by a concomitant increase in binding (Figure 2). One interpretation of these data is that strong negative coupling between neighboring recognition sites may be involved in the antagonist conformational state of the receptor (Figure 3). If these the neighboring sites are unequally reciprocally coupled, this could be a complex process.
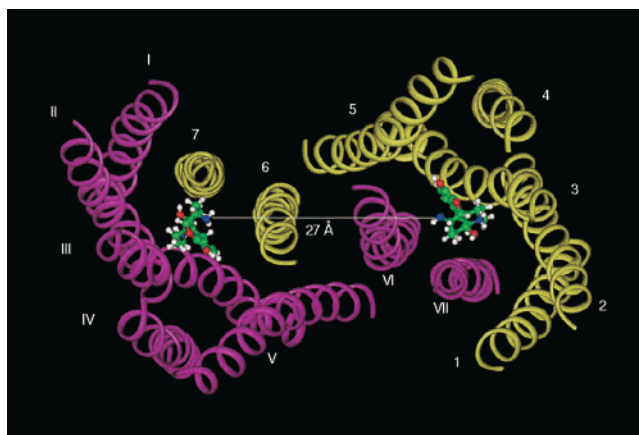
The recent reports[11] of dimers among opioid receptors and a variety of other G protein-coupled receptors (GPCRs) suggests that dimerization/oligomerization is a feature that is common to the superfamily of receptors.[20] The structural organization of such dimers has not yet been established, but it is reported that the monomeric subunits may be joined through disulfide linkages or associated through noncovalent interactions. "Rescue" experiments involving the restoration of function and/or binding upon coexpression of deficient mutant or chimeric receptors have provided some support for interlocking receptor dimers. Based on molecular modeling, it has been proposed that interlocking dimers with a TM5,6-interface between the 7TM domains may be the dominant form of dimers (Figure 4), although other types of interlocking dimers (e.g., TM4,5-interface) as well as contact dimers also were considered among the possibilities.[21] At this time, no definitive studies on the structural organization of opioid receptor dimers have been reported.

Given that our data are consistent with a receptor model that accommodates a single bivalent ligand through a bridging mechanism, the spacer length of the most potent member of the bivalent ligand series (e.g., **1**, $n = 2$) may provide some insight into the organizational arrangement of monomeric subunits within the dimer. Although the distance (~22 Å) between the agonist pharmacophores in the most potent member of series **1** ($n = 2$) probably does not correspond to the distance between the two opioid recognition sites of the dimer because of the large incremental difference (six atoms or ~8 Å) in spacer lengths between members of the series, it may allow us to distinguish between dimers with a TM5,6- and TM4,5-interface. Molecular modeling has suggested that the distance between the recognition sites of either the interlocking or contact dimers with a TM5,6-interface is ~27 Å, while it is substantially greater (~32 Å) in dimers with a TM4,5-interface.[22] Thus, our data are more consistent with the presence of dimers with a TM5,6-interface. We are currently revisiting the bivalent ligand approach in an effort to more reliably assess the intersite distance in opioid receptor dimers.
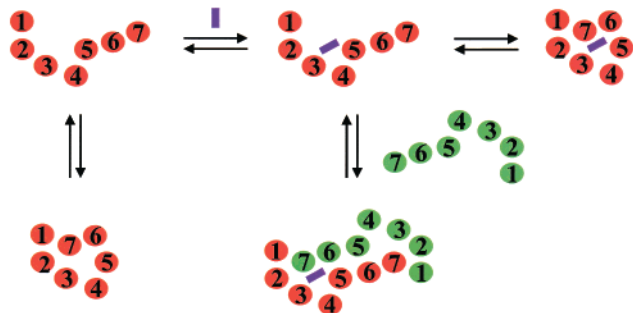
Figure 5. Cartoon illustrating the binding of a membrane-localized ligand with opioid receptors, as viewed from the extracellular side. A lipophilic ligand may gain access to the binding pocket in the center of the 7TM bundle if there is an equilibrium between monomer and dimer. The ligand binds to the recognition site during the transition between monomer and dimer.
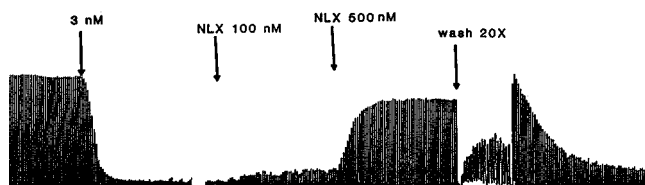

Figure 6. Inhibition of the electrically stimulated guinea pig ileum preparation (GPI) by N-benzoyl-β-naltrexamine (3 nM) and its reversal by naloxone (NLX). Note that inhibition of the twitch returns after washing.

Can Ligands Access Opioid Receptors via the Lipid Bilayer?

There have been reports of access of receptors by membrane-localized ligands.[23] The possibility of interlocking dimers offers some intriguing possibilities with respect to access of ligands to the recognition locus of opioid receptors. If opioid receptor monomers are in equilibrium with interlocking dimers, the monomer–dimer transition may involve an open form of the 7TM domain (Figure 5). One scenario is that an open form of the receptor may capture a lipophilic opioid ligand that is localized in the lipid bilayer. This would be consistent with wash-resistant activity in smooth muscle preparations that we have observed over the years.[24] For example, N-benzoyl-β-naltrexamine was observed to be a κ agonist whose activity in the guinea pig ileum persists after extensive washing. Naltrexone antagonized the agonist effect, but upon washing the naltrexone-pretreated preparation, the agonism returned (Figure 6). One interpretation of these results is that the lipophilic ligand partitions into the lipid bilayer and is captured by an open form of the κ receptor which then closes to monomer or dimer. Addition of naltrexone would antagonize the agonist effect by displacing it from the recognition site. Upon washing, the agonism returns because the less lipophilic naltrexone is selectively removed from the recognition site and replaced by the agonist from the membrane depot.

The localization of a lipophilic opioid ligand in the form of a depot in a lipid bilayer highlights the likelihood that the partition coefficient may determine whether access to the recognition site occurs directly from the aqueous biophase or from the lipid bilayer. Thus, different access pathways to the recognition site of the
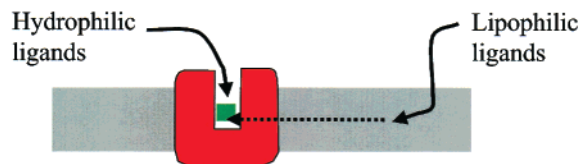

Figure 7. Cartoon illustrating different access pathways of hydrophilic and lipophilic ligands to an opioid receptor site. The hydrophilic ligands gain direct access from the aqueous biophase, whereas lipophilic ligands first partition into the lipid bilayer followed by lateral diffusion and entry via an open form of the receptor (see Figure 5).

receptor may be relevant in determining the duration of action. In this regard, hydrophilic ligands would be generally expected to access the recognition site directly from the biophase, while lipophilic ligands would gain access via the lipid bilayer from a depot (Figure 7).

Another aspect of a depot within a lipid bilayer is concerned with the significance of the affinity or potency of ligands. If a lipophilic ligand is present as a depot, its concentration in the lipid bilayer may be orders of magnitude higher than in the aqueous phase. As such, affinity constants and potency values would be difficult to equate with molecular recognition when they are expressed in terms of the aqueous concentration of ligand.

Separate Recognition Sites for Opioid Agonists and Antagonists on Dimeric Receptors?

In view of early evidence for opioid receptor dimers through our bivalent ligand studies, we considered the possibility that agonists and antagonists bind to separate, coupled opioid recognition sites. Our interest in this possibility was prompted by the observation that the μ-selective affinity label, β-funaltrexamine (β-FNA, **6**), selectively and irreversibly antagonizes μ agonists in the guinea pig ileum preparation (GPI), while its N-methyl analogue **7** is a reversible μ agonist.[25] It has been reported that β-FNA **6** irreversibly blocks μ opioid receptors by alkylating the ϵ-amino group of Lys233 located at the top of the fifth transmembrane helix (TM5).[26]

6 R = CH$_2$CH(CH$_2$)$_2$
7 R = CH$_3$
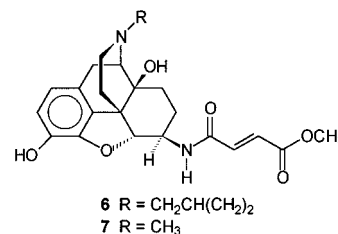
We therefore conducted μ receptor protection experiments to test this hypothesis using μ-selective agonists (**7** and morphine) and antagonists (naloxone and naltrexone) to determine their effectiveness in blocking irreversible μ antagonism by **6** in the GPI. We found that only relatively high concentrations (0.5–1 μM) of the μ-selective agonists weakly protected μ receptors against inactivation, whereas antagonists in the low nanomolar range were highly effective in blocking the irreversible effect of **6**.

Based on these experiments and our bivalent ligand studies that suggested opioid receptor dimers, we
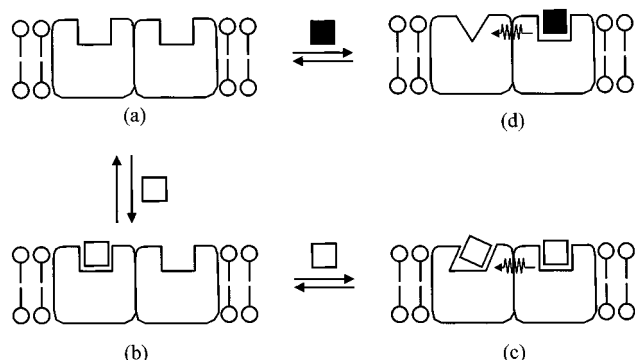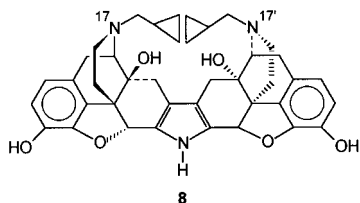
Figure 8. Model for the interaction of a μ agonist (□) and antagonist (■) with an opioid receptor dimer.[25] An agonist at low concentration binds selectively to the μ receptor (a → b), and at higher concentrations to the second site (b → c). The latter interaction triggers a decrease in affinity for the first site. Exogenous antagonist ligands bind to the second site (a → b) to cause a conformational change in the first site.

7TM-A   7TM-B

Figure 9. Schematic model of an extracellular view of an interlocking $\kappa-\delta$ opioid receptor heterodimer having a TM5,6 interface. The full circles represent TM helices of the κ opioid receptor, whereas the open circles indicate TM helices of the δ receptor. The 7TM hybrid bundle (7TM-A) contains Glu297 which is the key determinant for κ antagonist (norBNI **8**) binding and selectivity. The 7TM-B bundle contains the δ selectivity determinant which is located in extracellular loop 3 (EL3). Binding of **8** to 7TM-A would trigger a conformational change of 7TM-B that would result in the antagonism of its bound δ agonist.

proposed that μ opioid receptor activation and antagonism are mediated through separate negative allosterically coupled recognition sites in a dimer.[25] A two-site receptor dimer model was proposed, and we suggested that such a system may serve to modulate the activity of endogenous opioid peptides (Figure 8). Accordingly, an opioid peptide could modulate its own activity as a function of concentration. Single occupancy of the dimer would occur at low levels of the peptide, and at higher levels, occupation of the second site would dampen the overall binding and activation of the opioid receptor through negative cooperativity. The bell-shaped dose–response curve that is frequently observed in the guinea pig ileum preparation is consistent with this proposal. In contrast to endogenous agonists, exogenous antagonists were envisaged to have greater affinity for this second site.

Given the recent definitive evidence for opioid receptor dimers,[11] the proposed model is now on a more firm structural basis. However, if heterodimeric opioid receptors[27] are involved, there is the issue of pharmacologic selectivity. For example, in the porcine ileum, the neural co-localization of δ and κ receptors, together with the results of studies using selective opioid ligands, has suggested an allosterically coupled heterodimer model.[28] The finding that the selective κ opioid receptor antagonist, norbinaltorphimine **8** (norBNI), potently antago-

8

nized the effect of δ-selective agonists is consistent with conformationally coupled recognition sites in the $\kappa-\delta$ heterodimer. If κ and δ receptors are associated as interlocking subunits (Figure 9), it is possible that the 7TM-A recognition site that contains the κ "address" subsite (Glu297) would bind norBNI and antagonize δ agonists by inducing a conformational change in 7TM-B which contains the δ "address" (OL3). A key point here is that the use of norBNI **8** as a tool to identify the
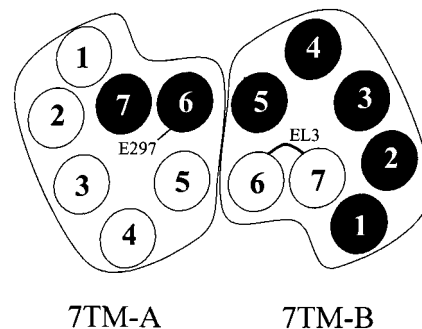
receptor type involved could be open to misinterpretation when $\kappa-\delta$ heterodimers are involved.

Similarly, the apparent transition from μ to δ agonism on chronic exposure of mice to μ agonists (methadone and heroin) may reflect possible changes in the distribution of heterodimers.[29] For example, if there is an increase in the density of $\mu-\delta$ heterodimers, it is possible that μ agonists that bind to the μ-selective recognition site of the dimer could be antagonized by the interaction of a δ antagonist at the δ-selective recognition site in the dimer.

The presence of heterodimeric receptors has profound ramifications not only with regard to interpretation of biological data but also in screening procedures that use a homogeneous population of cloned receptors. Moreover, it is possible that dimeric receptors may activate transduction pathways that are different from those activated by monomers.

Norbinaltorphimine: A Bivalent Ligand with a "Message" and "Address"

Although the antagonist SAR profile of the bivalent ligand series **3** at μ opioid receptors exhibited peak potency at a spacer length of $n = 2$, the greatest antagonist potency in this series at κ opioid receptors was associated with the shortest spacer ($n = 0$) (Figure 2A). The relatively short interpharmacophore distance suggested that potency enhancement occurred through the bridging of neighboring recognition sites located on a single receptor, rather than on a dimer. We therefore continued our investigation by synthesizing naltrexone-derived bivalent ligands with spacers shorter than a succinyl group. The shortest possible spacer in the form of a pyrrole afforded norbinaltorphimine **8** (norBNI) which proved to be a highly potent and selective κ opioid receptor antagonist that is presently widely employed as a research tool.[30]

Synthesis of the meso isomer **9** was undertaken to determine whether the neighboring recognition sites are stereochemically similar.[31] Since **9** contains one enantiomeric element derived from the inactive, (+)-naltrexone, it should not be recognized by an identical
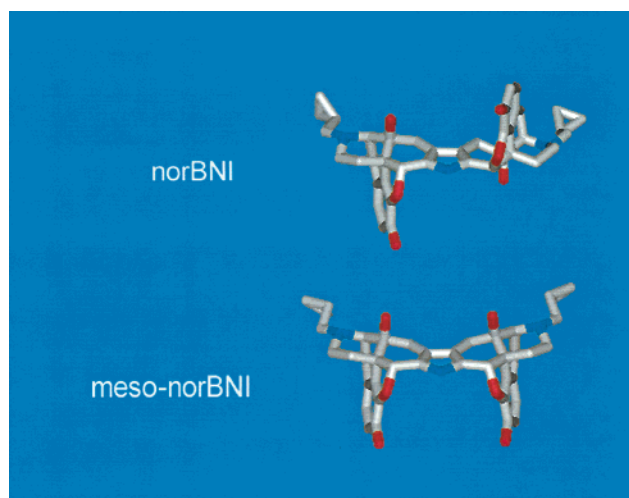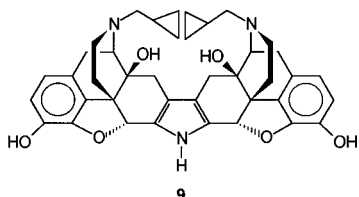
Figure 10. Comparison of the three-dimensional structure of norBNI **8** with its meso isomer **9**. Note the similar position of the basic nitrogens in the right half of the molecule.

neighboring opioid receptor recognition site. Testing revealed **9** to be κ-selective and somewhat more potent


9

than norBNI **8**. Therefore, we concluded that a specific moiety in the second pharmacophore, and not the second pharmacophore itself, is responsible for the κ selectivity of norBNI **8**. Superposition of **8** upon **9** revealed overlap of the N17′ basic nitrogen, suggesting that this group is responsible for the enhanced affinity and κ selectivity of norBNI (Figure 10).

Structure−activity relationship studies in smooth muscle preparations have supported the important role of the N17′ basic nitrogen. Most noteworthy was the finding that neutralization of N17′ through amidation greatly reduced the antagonist potency and selectivity at κ receptors.[32] Also, an analogue **10** without the critical

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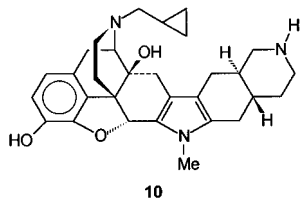
groups required for an antagonist pharmacophore in the second half of the molecule retained activity.[33] This suggested that the decahydroisoquinoline moiety within the second pharmacophore of norBNI **8** acts as a scaffold to rigidly hold and direct its N17′ basic nitrogen to a subsite that is unique to the κ receptor. Given that the analogue **11** with an isosteric thiophene[34] in place of the pyrrole moiety possesses binding selectivity similar to that of **8**, and the finding that a bivalent ligand whose scaffold geometry differs substantially from that of **8** is not κ-selective,[35] the important directive role of the scaffold is supported. Consequently, the binding of

norBNI **8** to the κ receptor was postulated to involve two major subsites: the first subsite recognizes one of the antagonist pharmacophores, while the second subsite containing an anionic group associates with the cationic protonated N-17′ moiety in the second half of the molecule.

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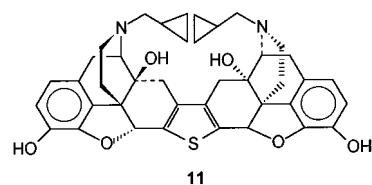
This model bears a formal resemblance to the message−address concept proposed by Schwyzer,[36] who employed it to analyze the structure−activity relationship of ACTH and related peptide hormones. Accordingly, peptide hormones contain a "message" sequence and "address" sequence. The message component is a common molecular feature of the series that is recognized by a family of receptors. The address recognizes a unique subsite on one of the receptors in the family and provides additional binding affinity to the ligand.

Site-directed mutagenesis of the κ receptor revealed the "address" subsite to be the anionic residue, Glu297, located at the top of the transmembrane spanning helix 6 (TM6) (Figure 11).[37] In this regard, the Glu297Lys and Glu297Ala mutants possessed greatly reduced affinity for norBNI **8**, whereas the affinity of naltrexone was not affected. Homology modeling suggested that the residue corresponding to Glu297 is a tryptophan (Trp284) in the δ opioid receptor and a lysine (Lys303) in the μ opioid receptor. Given that the nonconserved Glu297 is primarily responsible for conferring high affinity to norBNI, residues at the equivalent position in the μ (Lys303) and δ (Trp284) receptors would tend to hinder binding through electrostatic repulsion in the former or steric hindrance in the latter. In the μ receptor, additional steric hindrance appears to arise from Trp318 located the top of TM7, as we have determined that the Trp318Ala mutant has greatly increased affinity for norBNI.[38] The increased affinity most likely is due to the smaller size of Ala relative to Trp, thereby making the binding pocket more accessible to norBNI.

The role of Glu297 as an anionic residue that interacts with the cationic "address" of κ antagonists has received added support from studies with the μ receptor mutant whose Lys303 was replaced by glutamate (Lys303Glu).[39] The δ receptor was also mutated in an equivalent position (Trp284Glu).[38] In these mutants, the glutamate residue is in a position equivalent to that of Glu297 in the κ receptor. The mutant μ and δ receptors displayed enhanced affinity for norBNI and its active congeners when compared to the wild-type receptors, again highlighting the critical role of Glu297 in the κ receptor. These data support the proposal[40] that the "message" recognition locus for the antagonist pharmacophore of norBNI and other naltrexone-derived ligands is the central cavity created by the 7TM helices, as this domain has the highest homology of the potential binding sites. A key conserved residue that interacts with the antagonist pharmacophore within this cavity is believed to be the conserved TM3 aspartate (Figure 12).[41]
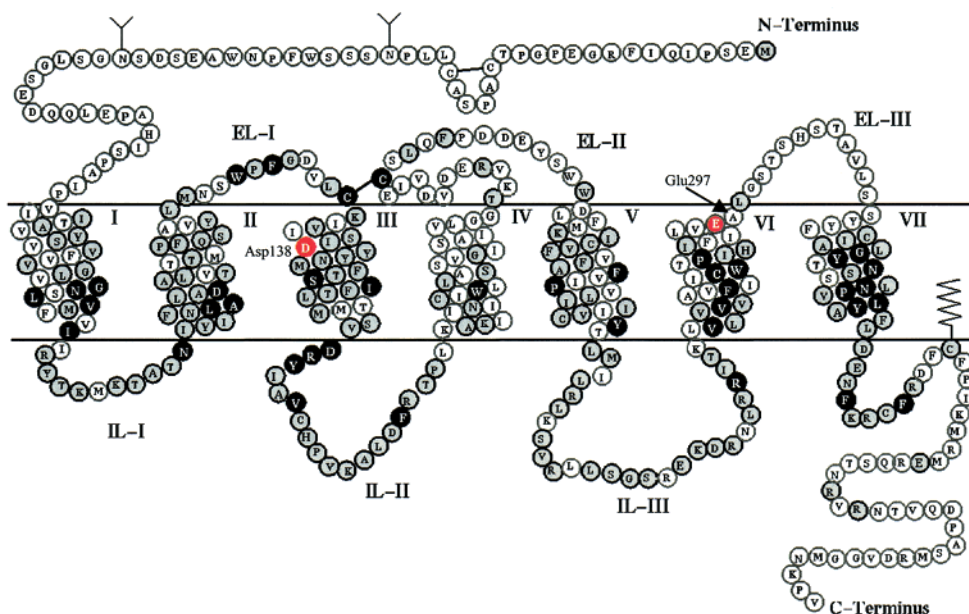
Figure 11. Two-dimensional serpentine model of the κ opioid receptor. The gray circles represent residues that are conserved in the opioid receptor family. Filled circles represent residues that are conserved among receptors in the rhodopsin subfamily of G protein-coupled receptors. The nonconserved Glu297 residue is implicated in the κ selectivity of **8** and other κ antagonists.
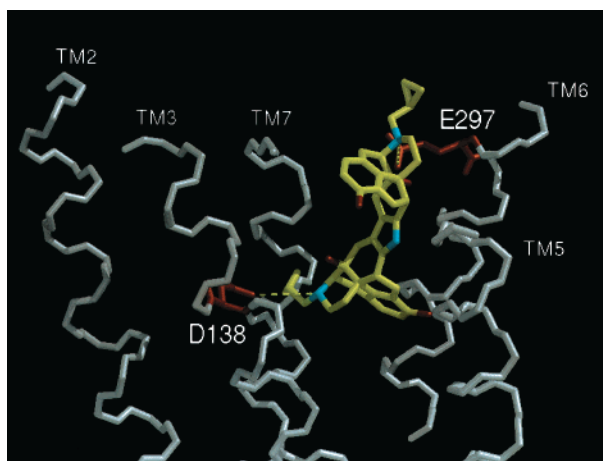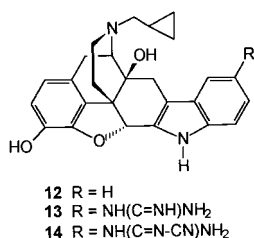

Figure 12. Model of norBNI **8** bound to the K opioid receptor. Note the fit between the conserved Asp138 and the nonconserved Glu297.

Figure 13. Three-dimensional relationship between norBNI **8**, naltrindole **12**, and GNTI **13**.

New "Address" Moieties for the Design of κ Opioid Antagonists

In view of the structural requirements for the κ antagonist activity of norBNI **8** and the importance of a rigid scaffold for the orientation of its N17′ basic nitrogen, we have combined an indole scaffold with new "address" moieties in order to design novel κ antagonists.[42] Superposition of the conserved structural motif of the δ opioid receptor antagonist, naltrindole **12**

12 R = H
13 R = NH(C=NH)NH₂
14 R = NH(C=N-CN)NH₂

(NTI),[30] upon that of norBNI led to the use of the indole

moiety as a rigid scaffold for projecting a variety of protonated amine or cationic groups within ion-pairing distance of the Glu297 residue in the κ receptor (Figure 13).

Ligands with high κ antagonist potency contained 5′-substituted guanidinium, amidinium, amine, or quaternary ammonium substituents.[42] The high basicity of guanidines and amidines, and the fact that the quaternary ammonium group is totally ionized, strongly support the notion that a cationic group at the 5′-position is required for potent κ antagonist activity. The importance of a cationic 5′-substituent for κ antagonist activity was shown by comparing the potency of the 5′-guanidine compound **13** (GNTI) with its closely related *N*-cyano derivative **14**. GNTI **13** was approximately 30-fold more potent than the nonbasic cyanoguanidine, highlighting the contribution of a positively charged group at the 5′-position of the indole scaffold.[42] Binding studies of GNTI on wild-type and Glu297Lys mutant κ receptors have implicated the involvement of counterionic association

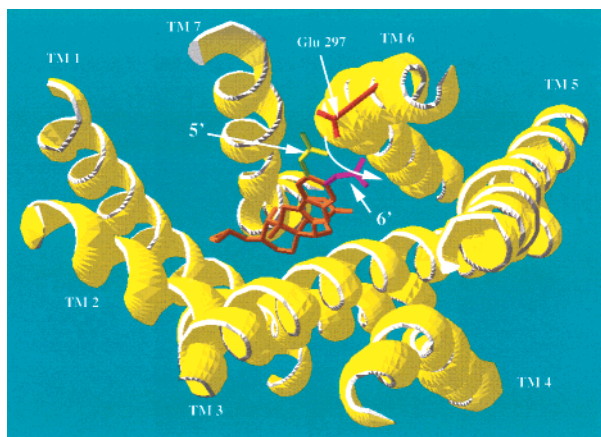**Figure 14.** Model of the interaction of the guanidinium group of GNTI **13** (green) with Glu297 (red) of the κ receptor and the counterclockwise rotation of helix 6 (curved arrow) upon interaction with the guanidinium group (magenta) of the 6′-regioisomer **15b**. Transmembrane helices are shown as ribbons in yellow.

between the cationic 5′-guanidinium group and the carboxylate group of Glu297.[40]

The importance of the 5′-position for directing the basic group to the glutamate-containing locus on the receptor was demonstrated by the finding that the 4′-, 6′-, and 7′-regioisomers (**15a**−**c**) either were inactive as

	Position
15a	4′
15b	6′
15c	7′
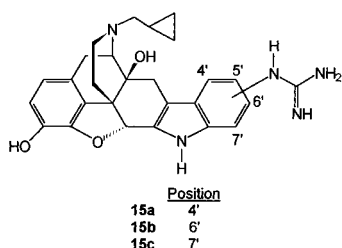
antagonists or had greatly reduced κ antagonist potency.[43] Significantly, the 6′-regioisomer **15b** showed potent κ agonist activity (∼50-fold greater than morphine in the GPI) and a significant decrease in binding to the Glu297Ala mutant κ receptor. These data have suggested that an ionic interaction between the 6′-guanidinium group and the Glu297 residue at the top of TM6 may be associated with the κ agonist activity of **15b**. This unprecedented transition from potent antagonist (**13**) to potent agonist (**15b**) can be rationalized by a ligand-induced conformational change of TM6 in the κ receptor, as illustrated by the model (Figure 14). A change of the guanidinium group from the 5′- to 6′-position may result in a counterclockwise axial rotation of TM6 (as viewed extracellularly) in order for the counterions to maintain favorable association. Such axial motion would lead to a conformational change of inner-loop 3 which is the key domain involved in G protein coupling and activation. There are a number of reports that implicate the rotation of TM6 in this process.[44−47]

Where is the κ Opioid Receptor "Address" Recognition Locus for Dynorphin?

Given that extracellular loop 2 (EL2) of the κ opioid receptor has multiple negative charges (Figure 13), together with reports that dynorphin A binds with high

affinity to a κ−μ chimera containing a κ EL2 but not to chimera containing a μ EL2, it is generally accepted that EL2 is the recognition locus for the "address" of this endogenous opioid peptide.[48,49] The implication was that the cation-rich "address" of dynorphin A is involved in ionic bonding with anionic residues in EL2. For this reason, we had originally assumed that the N17′ basic group of norBNI **8** was a mimic of the guanidinium group of Arg[7], a key residue in the "address" of dynorphin A, and that both ligands might be involved in ionic interaction with the same "address" recognition locus in EL2. The finding that the "address" recognition locus for norBNI is located at the top of TM6 (Glu297) rather than EL2[37] prompted us to investigate the role of EL2 in the binding of dynorphin A to the κ receptor.

The role of EL2-based acidic residues was investigated with a series of mutant κ opioid receptors in which Asp and Glu were neutralized by converting them to Asn and Gln, respectively.[50] Though it was not possible to totally replace all of the acidic residues, the mutant receptors afforded K_i values for dynorphin A(1−13) that were comparable to those of the wild-type κ receptor, and function was not significantly different from wild-type activity. These results are consistent with modeling studies of dynorphin A(1−10) docked to the κ receptor which have suggested that hydrophobic interactions arising from EL2 rather than ionic attraction may be the dominant factor in recognition.[51] Therefore, it appears that the address recognition locus for dynorphin differs from that for norBNI **8**.

Aromatic "Address" Moieties in the Design of δ Opioid Receptor Antagonists

The design of the prototypical δ opioid receptor antagonist, naltrindole **12**, was based on the "message−address" concept[36] that was discussed earlier in connection with the selectivity of norBNI **8**. A study[52] that showed that the Phe[4]Leu[5] C-terminus of Leu-enkephalin confers δ selective binding when attached to the 6-position of an opiate agonist pharmacophore led to the idea that Phe[4] may be a key contributor to its δ selectivity. We therefore synthesized opiates containing a benzene "address" moiety in an effort to impart δ selectivity to non-peptide ligands. This led to the synthesis of NTI **12** and a variety of substituted indolomorphinans that are potent δ opioid receptor antagonists.[53] The high δ antagonist potency and selectivity of a benzofuran isostere **16** (NTB)[54] implicated the benzene component common to NTI **12** and NTB **16** as the δ "address". The finding that the pyrrolomorphinan **17** was not a selective δ antagonist supported this conclusion.[55]

16

17
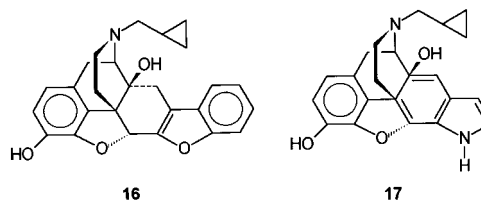
The aromatic address of NTI **12** confers δ selectivity by enhancing the affinity for its target receptor and reducing affinity for non δ opioid receptors. Thus, antagonist potency is decreased when the benzene

moiety of the indole is substituted with a cyclohexane **18** or with alkyl groups (series **19**).[54,56] Although both


18

19 R and R' = alkyl
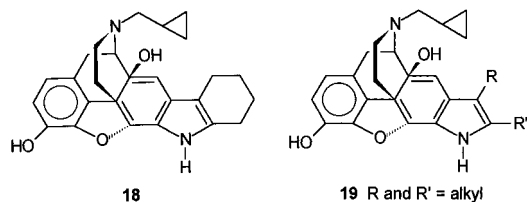
18 and members of series **19** are less potent at δ receptors, they retain δ selectivity, presumably because the alkyl groups attached to the pyrrole moiety sterically interfere with binding to μ and κ receptors. An analogous exclusion mechanism also may be the basis for the δ selectivity of other pyrrolomorphinans.[57]

Site-directed mutagenesis studies of residues located at the top of TM7 in the μ and κ receptors afforded results that are in harmony with the idea that steric hindrance contributes to the lower potency of δ antagonists at wild-type μ and κ receptors. The increased affinity of NTI **13** for the mutant μ (Trp318Ala) and κ (Tyr312Ala) receptors suggests that reduction in the steric bulk of these residues increases access of NTI into the central cavity that recognizes the antagonist pharmacophore. On the other hand, the presence of Trp284 in the δ receptor may play a role in the selective binding of NTI by increasing its affinity.[38]

The conformational role of the "address" in conferring δ opioid receptor antagonist potency and selectivity was investigated with ligands having orthogonally restricted aromatic groups with respect to ring C of the morphinan structure. These ligands included benzylidenenaltrexone **20** (BNTX) and spiroindans **21a** and **22**.[58–60] We have found that ligands with a coplanar-oriented aromatic group (e.g., **13** and **16**) are more potent δ antagonists than those with an orthogonally oriented "address" (**20**–**22**).

20

21a R = CH₂CH(CH₂)₂
21b R = CH₃

22
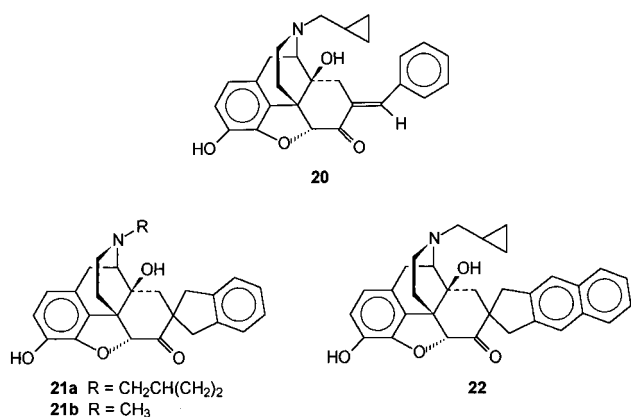
In vivo pharmacological studies have suggested the possible existence of putative δ-1 and δ-2 receptor "subtypes".[61,62] Presently, it is not known whether these "subtypes" represent δ receptors whose amino acid sequences differ or if they are a single receptor that exists both as monomer and dimer. Two non-peptide ligands that are widely employed as δ-1 and δ-2 antagonists are BNTX[59] **20** and NTB[61] **16**, respectively. BNTX selectively antagonizes the agonist effect of

[D-Pen²,D-Pen⁵]enkephalin,[63] while NTB selectively blocks [D-Ser²,D-Leu⁵,Thr⁶]enkephalin[64] and deltorphin II.[65]

Given that the "address" in NTB **16** is coplanar relative to ring C of the morphinan structure, while in BNTX **17** it is restricted to an orthogonal-like conformation, we investigated whether the conformation of the "address" contributes to the "subtype" δ selectivity. This was the basis for the design of antagonist and agonist 7-spiroindanylmorphinans whose aromatic "address" moiety is fixed in an orthogonal orientation. All of the *N*-cyclopropylmethyl members of this series exhibited selective δ opioid antagonist activity in smooth muscle preparations, and the benzospiroindan analogue **22** (BSINTX) was found to be a selective δ-1 antagonist in vivo.[60] The δ-1 agonist, 7-spiroindanyloxymorphone **21a** (SIOM),[66] was designed based on a molecular dynamics study of the δ-1 selective peptide, [D-Ala²,Leu⁵]-enkephalin.[67] In this case its Phe⁴ phenyl group was found to be oriented in a fashion that approximated that of the spiroindanyl aromatic moiety.

Summary and Conclusions

The idea that opioid receptors may be organized as dimers, together with the concept of "bridging" such neighboring receptors by ligands containing two pharmacophores linked through a spacer of appropriate length, led to the design of bivalent ligands with greatly enhanced potency. The finding that both opioid agonist pharmacophores in the bivalent ligand bound to neighboring opioid recognition sites provided circumstantial evidence for dimeric receptors. The recent biochemical characterization of opioid receptor dimers has provided more definitive evidence for their existence.

Our model for dimeric receptors has provided a structural basis for rationalizing data that was not easily interpretable using classical receptor models. Based upon our evidence for dimers and the large difference in the ability of μ agonists and antagonists to protect μ receptors against irreversible blockage by β-FNA **6**, we have proposed that opioid agonists and antagonists bind to separate allosterically coupled recognition sites on a receptor dimer. Such cooperativity is consistent with the results of recent studies in the porcine ileum which contains co-localized κ and δ receptors. The ability of the κ antagonist, norBNI **8**, to potently antagonize δ-selective agonists has suggested a κ–δ heterodimer model in which norBNI antagonizes δ agonists via a separate recognition site that is κ-selective. Also, the apparent transition from μ to δ agonism upon chronic exposure of mice to μ agonists may reflect a shift in the distribution of μ and δ receptors to μ–δ heterodimers. In such a case, it is conceivable that a μ agonist may be antagonized by a δ antagonist via the allosteric mechanism that has been discussed.

The existence of receptor dimers may also explain how lipophilic ligands that become localized in the cell membrane lipid bilayer can gain access to the central cavity of opioid receptors when the receptors are organized as interlocking dimers. Access via the lipid bilayer calls into question the significance of "affinity" when a high concentration of ligand is localized in the membrane. On the other hand, hydrophilic peptidic ligands would be expected to access the recognition site directly

from the aqueous phase. Differential access would account for the generally higher apparent "affinity" of lipophilic opioid ligands relative to peptides.

Exploration of the relationship between spacer length and antagonist potency of bivalent ligands led to the development of the first highly selective and potent κ opioid antagonist, norBNI **8**. κ selectivity was found to be due to a cationic "address" moiety. The concept of a "message" and an "address", as originally proposed for rationalizing the SAR of peptide hormones, was subsequently employed to design several other selective nonpeptide opiates that include NTI **12**, GNTI **14a**, NTB **16**, BNTX **20**, BSINTX **22**, and SIOM **21b**. These ligands are presently employed as pharmacologic tools.

Our approach for the design of the κ opioid antagonist, GNTI **14a**, required a detailed analysis of the SAR of the series, a knowledge of the amino acid sequences for all three opioid receptors, and the construction of credible opioid receptor models. Taken together, our studies have revealed that the high potency and selectivity of a ligand is not merely a consequence of enhanced affinity for the target receptor. The origin of such selectivity changes have been found to be due to a combination of molecular exclusion of the ligand at lower affinity receptors and increased affinity at the high affinity receptor.

Finally, structure—activity relationships are rarely straightforward and often more complicated than they appear. For this reason, the use of site-directed mutagenesis as a complementary tool to analyze SAR has been invaluable. Indeed, site-directed mutagenesis is a useful technology that is adaptable to a medicinal chemistry environment. Given the paucity of high-resolution crystal structures for membrane-bound receptors, the use of a coordinated 'two-dimensional' paradigm that involves molecular modification of both the ligand and the receptor affords a useful approach to the study of molecular recognition. Through such a paradigm, greater insight into the molecular recognition process may be obtained, particularly when combined with molecular modeling. It therefore behooves the medicinal chemist to employ such technology to analyze molecular recognition from the perspective of both the ligand and the receptor, in an effort to obtain a more penetrating analysis of the SAR. The power of this approach has been illustrated in the analysis of molecular recognition of selective opioid antagonists at opioid receptors.

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