Impact of Recombinant DNA Technology and Molecular Modeling on the Practice of Medicinal Chemistry: Structure-Activity Analysis of Opioid Ligands[1]

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INTRODUCTION

These are exciting times. Research in the pharmaceutical sciences, and medicinal chemistry in particular, has undergone considerable change over the past 40 years. As a graduate student in medicinal chemistry, it was inconceivable that we would know as much about drug receptors as we do today. It is mind-boggling to think that in the relatively short span of two decades, the amino acid sequences of well over a thousand G protein-coupled receptors have been deposited in electronic data bases. Twenty-five years ago, I would have considered structure-activity relationship studies of receptors to be science fiction, but through recombinant DNA technology this is rapidly becoming part of the paradigm for investigating the interaction of ligands with membrane-bound receptors. In view of the impact of recombinant DNA technology and molecular modeling on the practice of medicinal chemistry, I will attempt to illustrate in this presentation how such technology has influenced my research on the molecular recognition of ligands for opioid receptors.

BACKGROUND

Exogenous and Endogenous Opioid Ligands

Opiates are the most widely known and notorious natural products. The two most prominent opiates, morphine and codeine, have a long history of use and abuse. After the elucidation in 1925 of the chemical constitution of morphine(1), hundreds of semisynthetic opiates were prepared in an unsuccessful effort to obtain analgesics free of addiction liability and other undesirable side effects.

The discovery of meperidine and methadone in the 1940s led to the realization that totally synthetic and structurally unrelated ligands (opioids) are capable of mimicking the pharmacological actions of morphine (Figure 1)(2). Over the next four decades many diverse classes of synthetic and semisynthetic analgesics were reported. The design of 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. This group of analgesics presently enjoys wide clinical usage(3).

The discovery of the enkephalins (Tyr-Gly-Gly-Phe-X, X=Leu or Met) in 1976 was a major advance in that it represented the first step in establishing the opioid peptides as a major class of endogenous neuromodulators(4). Also, it confirmed that exogenous opioid agonists mimic the effects of the opioid peptides by interacting with common receptors. The opioid peptide family presently consists of over 20 peptides that have a tetrapeptide sequence in common with that of the

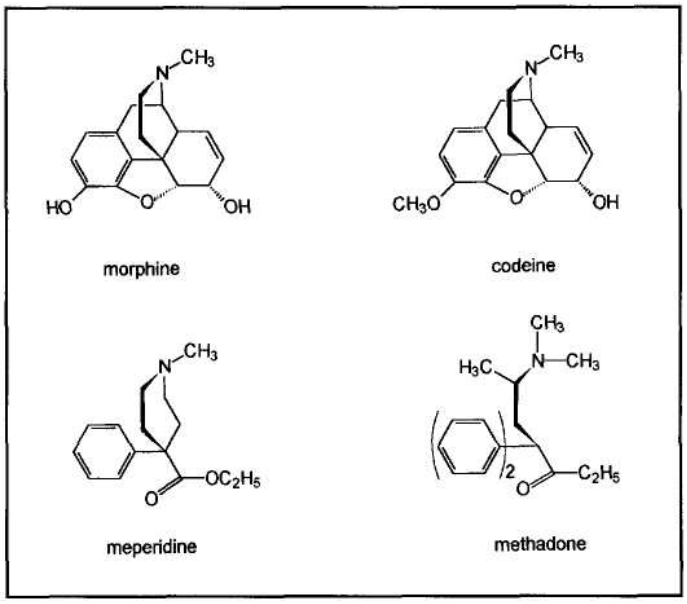

Fig. 1. Morphine, codeine and synthetic opiates of the 1940s.

Peptide	Selectivity	Amino Acid Sequence
Enkephalin	δ	Y-G-G-F-X, X=L or M
Dynorphin A	κ	Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q
β-Endorphin	-	Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F-K-N-A-I-I-K-N-A-Y-K-K-G-E
Endomorphin	μ	Y-P-X-F-NH2, X=W or F

Figure 2. Opioid peptide sequences. A=Ala, R-Arg, N=Asn, D=Asp, Q=Gln, E=Glu, G=Gly, I=Ile, L=Leu, K=Lys, M=Met, F=Phe, P=Pro, S=Ser, T=Thr, W=Trp, Y=Tyr, V=Val.

Fig. 2. Opioid peptide sequences. A=Ala, R=Arg, N=Asn, D= Asp, Q=Gln, E=Glu, G=Gly, I=Ile, L=Leu, K=Lys, M=Met, F=Phe, P=Pro, S=Ser, T=Thr, W=Trp, Y=Tyr, V=Val.

enkephalins (Figure 2)(5). The family is divided into three groups; the enkephalins, dynorphins, and endorphins. Recently, a new class of endogenous opioid peptides, the endomorphins (Tyr-Pro-X-Phe-NH2, X=Trp or Phe), has been reported(6). At first glance there may not be any resemblance between morphine and the opioid peptides. However, some similarity is evident in that a tyramine moiety is present in both morphine and the peptides (Figure 3). Despite the flurry of activity by pharmaceutical companies in developing analogues of the opioid peptides as analgesics, this approach failed because of bioavailability problems and the finding that the opioid peptides produce physical dependence in animal models(7).

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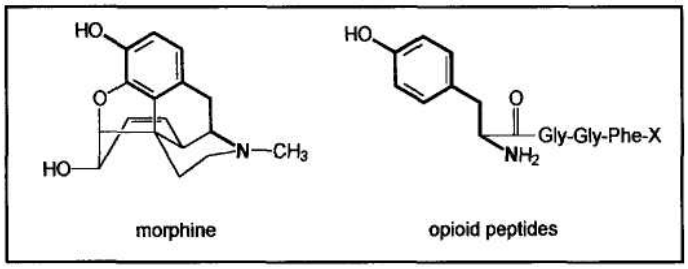

Fig. 3. The tyramine moiety (bold lines) in morphine and in the opioid peptides

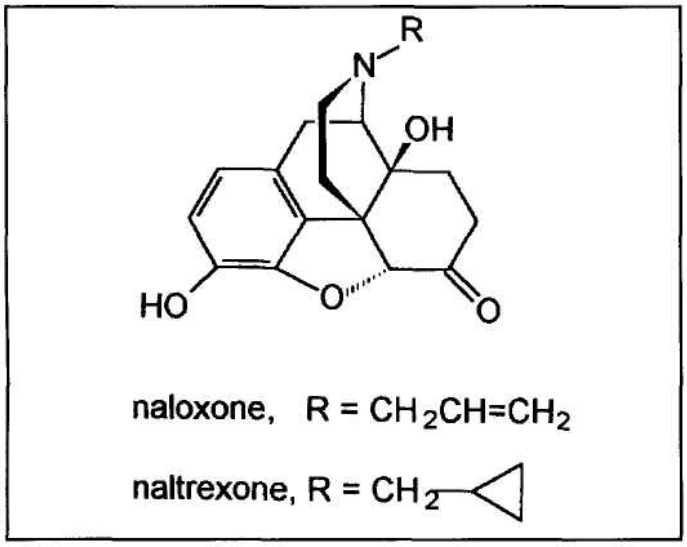

Fig. 4. Prototypical universal opioid antagonists.

Multiple Opioid Receptors

The first serious attempt to model an opioid receptor site was reported by Beckett and Casy in 1954 using the rigid morphine molecule as a molecular template(8). This was based on a three-point contact model which highlighted the importance of stereochemical factors in the interaction of ligands with an opioid receptor site. Based on the analysis of the relationship between molecular structure and analgesic activity, it was proposed in 1965 that different structural classes of opioid analgesics interact with multiple types of opioid receptors(9). Also, the analysis was consistent with multiple modes of interaction of different ligands with the same receptor. A decade later, pharmacological studies in dogs provided support for two types of opioid receptors (mu and kappa)(10) and the following year a third type (delta) was proposed from studies on smooth muscle preparations(11). More recently, site-directed mutagenesis studies of opioid receptors have supported the idea of multiple modes of interaction between different ligands and opioid receptors(12).

Design of Selective Opioid Antagonists

Progress in opioid research has relied heavily on the use of the opioid antagonists, naloxone and naltrexone (Figure 4)(13). Their usefulness stemmed from the fact that they interact with all known types of opioid receptors. Because of this property, naloxone- or naltrexone-induced antagonism has been frequently used as a key criterion to verify the involvement of an opioid receptor mechanism.

The recognition that there are multiple types of opioid receptors has spurred the development of selective antagonists

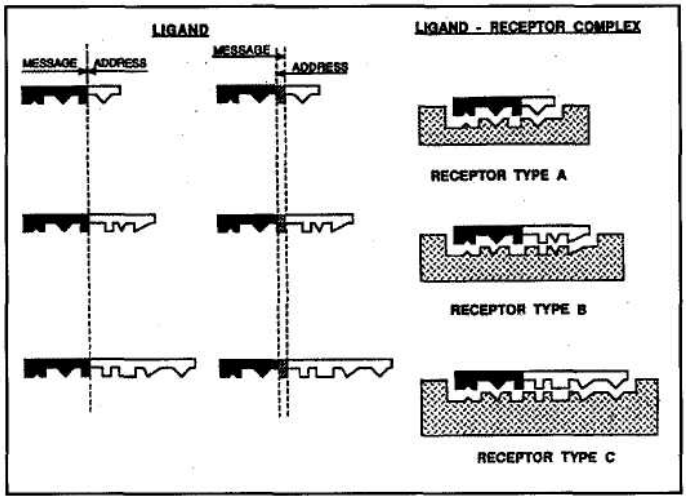

Fig. 5. A schematic illustration of the message-address concept. Note that the message and address components may overlap (center).

as tools to determine the selectivity of opioid agonists and to identify the receptor type that is bound by a ligand(13). The rationale for the design of naltrexone-derived kappa- and delta-selective antagonists share the concept of the occupation of two neighboring recognition subsites by a bifunctional ligand(14). The model was based on the assumption that each type of opioid receptor possesses a highly homologous domain that recognizes the opioid pharmacophore, and a neighboring unique subsite that confers selectivity when it is bound by the ligand. This model bears a formal resemblance to the "message-address" concept proposed by Schwyzer(15) who devised it to analyze the strucure-activity relationship of ACTH and related peptides. Accordingly, the "message" component of the peptide ligand contributes to signal transduction, while the "address" confers selectivity by providing additional affinity for the target receptor and/or reduced affinity for other receptors (Figure 5). This model was originally applied to the analysis of dynorphin and its analogues, in that the N-terminal tetrapeptide sequence, Tyr-Gly-Gly-Phe, of the opioid peptide family was considered to function as the "message" while a segment of the remainder of the molecule acts as an "address"(16).

The application of the message-address concept to the design of selective antagonists was tested initially on hybrid ligands comprised of a nonpeptide "message" component derived from an opiate, and a peptide "address" component from the delta-selective Leu-enkephalin or from the kappa-selective dynorphin(17). In this context, these conjugates were viewed as possessing three key features: a message component consisting of the tyramine pharmacophore; a scaffold or spacer which mimics the connecting linkage, Gly^2-Gly^3, between the "message" and "address" in the opioid peptides; and a segment of the "address" sequence from the opioid peptide that is essential for its selectivity (Figure 6). The selectivity of the conjugates was found to be determined by the "address" sequence, in that enkephalin-derived Phe^4-Leu^5 enhanced delta selectivity while a dynorphin-derived sequence (Phe^4-Leu^5-Arg^6-Arg^7-Ile^8-OMe) afforded kappa selectivity. These results provided the basis for a design strategy for highly selective nonpeptide antagonists.

Examples of nonpeptide opioid antagonists that contain "message" and "address" components are the delta-selective, naltrindole (NTI), and the kappa-selective, norbinaltorphimine

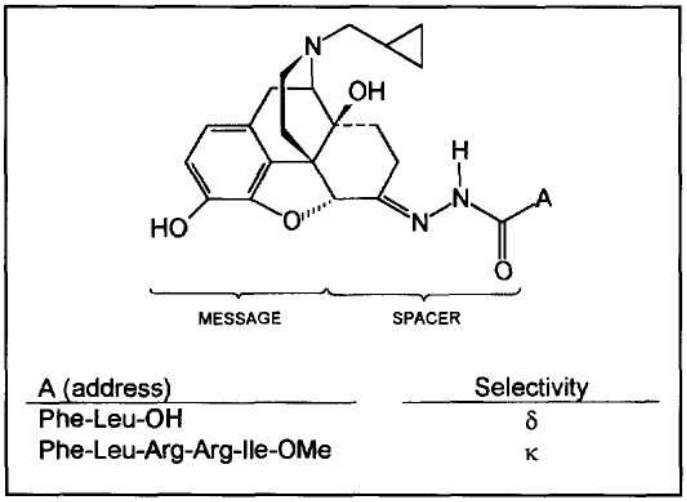

Fig. 6. Segment of "address" sequence essential for opioid peptide activity.

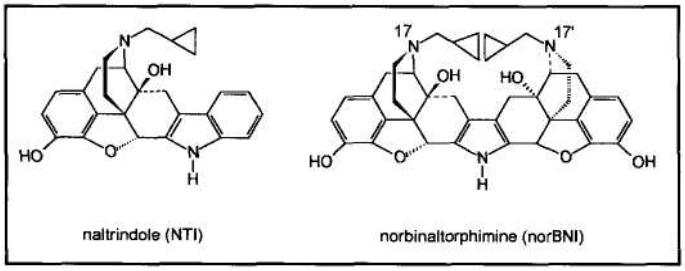

Fig. 7. Delta-selective naltrindole and kappa-selective norbinaltorphimine.

(norBNI) (Figure 7)(13). These antagonists are widely employed as tools in opioid research. Although the idea of discrete "message" and "address" elements has led to the design of selective ligands, it is noteworthy this strategy has been useful only in a conceptual sense because of the possible overlap in the functional roles of these elements and the possibility that agonists and antagonists possess different modes of binding to opioid receptors. In the case of NTI, the naltrexone-derived antagonist pharmacophore (N-cyclopropylmethyltyramine) is attached through a rigid scaffold to a benzene moiety which serves as a mimic of a key residue (Phe^4) of the presumed delta "address" of enkephalin (Figure 8). With regard to norBNI, the "address" was determined to be the N-17' basic group. Thus, norBNI consists of two (-)-naltrexone-derived antagonist pharmacophores, only one of which is required for antagonist activity. The second pharmacophore functions mainly as a scaffold to hold and orient its N-17' basic group. The original assumption was that N-17' is a mimic of Arg^7 which is an important residue of the kappa "address" sequence of dynorphin. Also, it was postulated that both the protonated N-17' and the guanidinium group of Arg7 are involved in ion-pairing with an acidic residue in a location that is unique to the kappa receptor. As is discussed below, this conclusion was not entirely correct, as it was found through recombinant DNA technology that the "address" subsite for norBNI is different from that for dynorphin.

Cloning of Opioid Receptors

While pharmacological and binding studies have strongly suggested the existence of at least three major types of opioid receptors, unequivocal proof for the three types was obtained

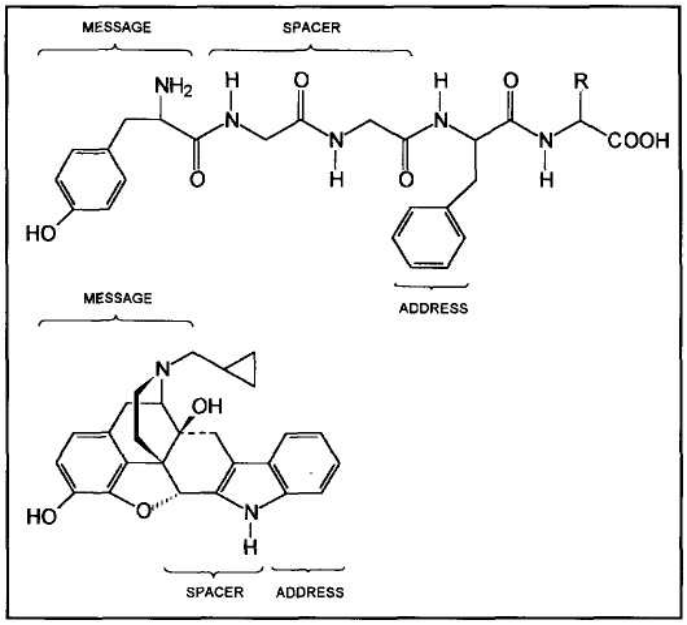

Fig. 8. Schematic comparison of functional units in enkephalin (above) and naltrindole (below).

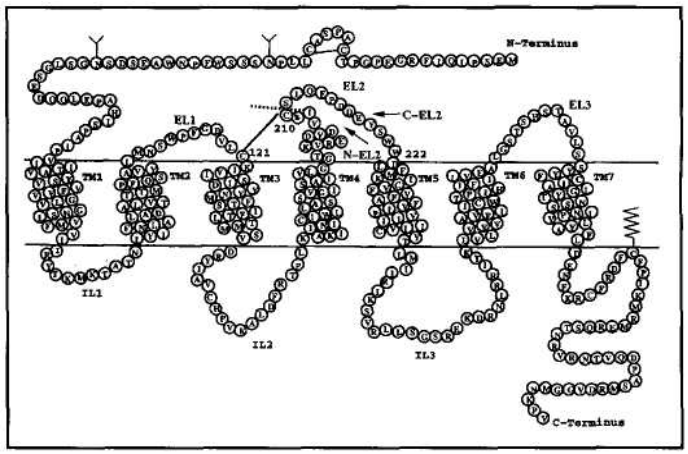

Fig. 9. Serpentine model of the kappa receptor. The horizontal lines represent the membrane boundary, the dashed lines separate the N-EL2 and C-EL2 regions. Glycolsylation sites on the terminal and palmitoylation site on the C-terminus are also shown. TM = transmembrane region; EL = extracellular loop; IL = intracellular loop; N-EL2 = fragment 197-210; C-EL2 = fragment 211-222.

in 1993-94 through cloning(18). The delta, kappa, and mu opioid receptors are approximately 60 percent homologous with respect to amino acid identity and belong to the rhodopsin family in the superfamily of G protein-coupled receptors. The number of amino acid residues for the opioid receptors ranges from 372 to 398. The feature that is common to the opioid receptors and other members of this superfamily is that their hydropathy profiles are consistent with structures that have seven transmembrane (TM) helices (Figure 9). The possible existence of additional opioid receptor types and subtypes have been reported based on binding and pharmacology, but confirmation of their presence awaits cloning)(18).

An acidic amino acid residue in TM3 of opioid receptors is a feature that is conserved in aminergic receptors (*e.g.*, catecholamine, histamine, and serotonin receptors) and muscarinic receptors(19). On this basis, it has been proposed that ion-pair-

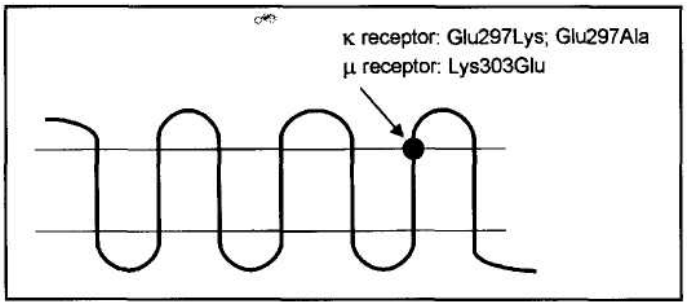

Fig. 10. A schematic illustration of the equivalent location ofGlu297 in the κ receptor andLys303 in the/u receptor. Mutation ofGlu297 to Lys or Ala in the κreceptor leads to reduced binding ofnorBNI. In the μ receptor, mutation ofLys303 to Glu greatly enhances the binding of norBNI.

ing with the cationic group (protonated amine or quaternery ammonium) of the agonist ligand may be involved in binding. The finding that receptors whose TM3 acidic residue has been mutated to an uncharged residue usually exhibit greatly reduced agonist affinity is consistent with this proposal, although it does not rule out other possibilities for the change in binding.

The cloning of opioid receptors has opened a new chapter on the structure-activity relationship studies of opioid ligands because molecular recognition can be investigated based on the structure of both the ligand and the receptor. The impressive diversity and number of ligands that are recognized, together with the high homology of the three major opioid receptor types, represents an advantage not shared by many other G protein-coupled receptors. For these reasons, analysis of the interaction of ligands with chimeric and mutant opioid receptors should provide insight into residues at the receptor site that are involved in molecular recognition.

ANALYSIS OF THE INTERACTION OF LIGANDS WITH CLONED KAPPA OPIOID RECEPTORS: A CASE STUDY

Recognition of Norbinaltorphimine (norBNI) at the Kappa Opioid Receptor

As was discussed earlier, structure-activity relationship studies of the kappa antagonist, norBNI, led to the proposal that it can be viewed as an antagonist pharmacophore connected through a scaffold to an "address" moiety (the N-17' basic nitrogen). The scaffold was postulated to direct the cationic form of the "address" to a subsite on the receptor that contains an acidic amino acid residue and thereby enhance ligand affinity via an ionic interaction(20). With the cloning of the kappa receptor and the availability of technology to construct mutants and chimera, it became possible to test and refine the model. I will use this as an example to illustrate how the combined use of molecular biology and three-dimensional modeling can provide insight into the recognition ofnorBNI by kappa receptors.

A group of ten kappa-mu chimeras were constructed and their affinities for norBNI were compared with those of the wild-type kappa and mu receptors(21). Since wild-type kappa receptors bind norBNI with over three orders of magnitude greater affinity relative to wild-type mu receptors, it was possible by analysis of the chimera-affinity relationship to localize the segment of the receptor that contributes to binding of norBNI. The critical segment was found to be in the vicinity of extracellular loop three or at the top of TM6. Since structure-activity relationship studies originally had suggested that kappa selectivity is conferred by interaction of the norBNI

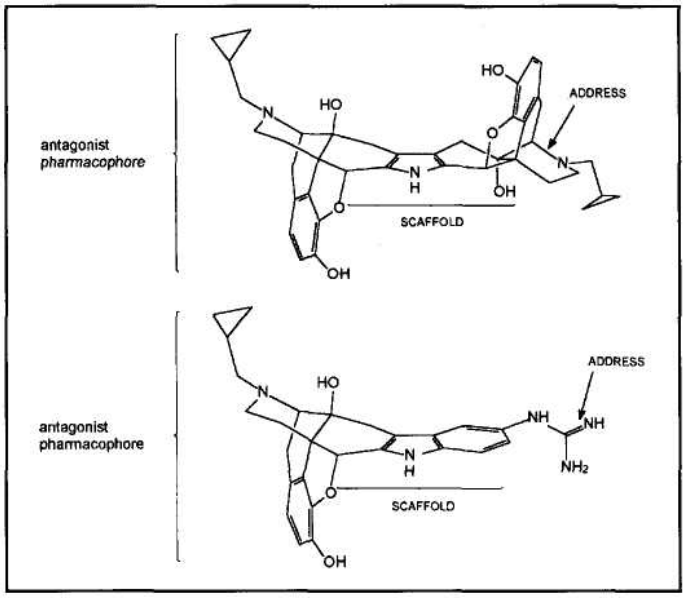

Fig. 11. Three-dimensional structures of norBNI (above) and GNTI (below). Note that the basic address moiety is in a similar orientation with respect to the antagonist pharmacophore.

"address" with a putative acidic group(20), the only acidic amino acid residue (Glu297) in the critical segemnt was mutated to either Lys (Glu297Lys) or Ala (Glu297Ala) to eliminate the negative charge (Figure 10)(21). The finding that the mutant kappa receptors possessed greatly reduced affinity for norBNI supported the idea that Glu297 interacts as a counterion with the cationic +HN-17' "address" moiety. Significantly, when a residue (Lys303) in a position equivalent to Glu297 in the mu opioid receptor was mutated to Glu (Lys303Glu), the binding of norBNI also was greatly enhanced, suggesting that the cavities of the mu and kappa receptors that accommodate the antagonist pharmacophore are highly homologous(22). Thus, it appears that a unique "address" moiety (Glu297 carboxylate anion) is the primarary determinant responsible for the relatively higher affinity of norBNI for kappa relative to mu receptors.

Structure-activity relationship studies of opioid ligands on wild-type and mutant kappa receptors have supported similar conclusions concerning the role of the address(22). The delta antagonist, naltrindole, has been converted to a highly selective and potent kappa antagonist, GNTI, merely by the attachment of a hightly basic guanidine group to the 5'-position of its indole moiety (Figure 11). In this case, the kappa selectivity of GNTI is due to the the indole moiety functioning as a scaffold to hold the guanidine group in a position very similar to that of the N-17' basic group in norBNI. The N-cyanoguanidine analogue of GNTI (CN-GNTI) possessed lower kappa selectivity, but its affinity for wild type kappa receptors was reduced only marginally. This was surprising because the cyanoguanidine group is not basic due to the strong electron withdrawing effect of the cyano group. It is possible that the small reduction of high affinity binding of the cyano derivative to the wild-type kappa receptor may be due to hydrogen bonding of the neutral guanidine group with Glu297 without the assistance of ionic attraction. This is supported by the finding that the norBNI analogue whose N-17' was rendered neutral through amidation and has no donor hydrogen bonding potential, has greatly reduced kappa receptor affinity and selectivity(23).

Molecular Modeling of Ligand Binding to Kappa Opioid Receptors

Three-dimensional molecular modeling using experimentally-derived constraints has been useful in visualizing the interaction of norBNI and GNTI with the kappa opioid receptor site. Molecular dynamics simulations have placed the antagonist pharmacophore in the cavity created by the TM7 bundle and the "address" moiety (N-17' for norBNI or the guanidinium group for GNTI) in proximity to the Glu297 position of the kappa receptor. The binding mode included possible interactions of the protonated N-17 group and the phenolic hydroxyl group of the antagonist pharmacophore (N-cyclopropylmethyl-tyramine moiety) with Aspl38 (TM3) and His291 (TM6), respectively(24). Both of the proton-bearing groups in this pharmacophore may act as hydrogen bonding donors to the receptor-based proton acceptor groups (carboxylate anion and imidazole) in the cavity. As has been suggested(19) for other aminergic receptors that contain a TM3 acidic residue, the Asp 138 anion may be involved in ionic bonding with the cationic N-17 group. The modeling indicates that the kappa and mu receptor cavities that accommodate the antagonist pharmacophore are highly homologous, given that ~80 percent of the residues within six angstroms of the bound ligand are identical. This is consitent with the aforementioned enhanced affinity of norBNI for the mutant mu receptor (Lys303Glu)(22).

While the enhanced affinity of norBNI and GNTI for the kappa receptor may be due to ion-pairing and/or hydrogen bonding between the "address" and Glu297, the high selectivity of norBNI and GNTI arises from a combination of increased affinity for the kappa receptor and reduced affinity for mu and delta receptors. The reduced affinity at the mu recognition site is due to Lys303 which is located in the same position as Glu297 in the kappa receptor. The protonated epsilon-amino group of Lys303 apparently hinders the binding of the kappa antagonists through ionic repulsion of the cationic "address'Vmoiety. With regard to the delta receptor, the same position contains Trp284 whose hydrophobic and bulky character also reduces binding.

Although the studies have demonstrated that a single residue (Glu297) functions as a recognition subsite for the norBNI "address" (N-17'), it has been found that mutation of Glu297 does not affect the binding of the endogenous opioid peptide, dynorphin[2]. The fact that kappa receptor chimeras with an exchanged second extracellular loop (EL2) have been reported(25) to possess low affinity for dynorphin has suggested that the negatively charged EL2 functions as the "address" recognition locus for this opioid peptide, particularly since the putative "address" segment of dynorphin contains five cationic residues that could be the basis for enhanced affinity through electrostatic attraction.

Homology modeling has led to the conclusion that a segment of EL2 (residues 197-208) of the kappa receptor possesses a helical motif with amphiphilic character(26). A similar motif was not predicted for EL2 of either the mu or delta opioid receptors. The helical motif of EL2 for the kappa receptor was shown to be complementary to the amphiphilic helical segment(27) (residues 4 - 9) of the dynorphin "address". The recognition between EL2 and the "address" involved interaction between the hydrophobic faces of the helices (Phe4Leu5Ile8 of dynorphin and Val201Val205Val207Ile208 of EL2) rather than the bonding between counterions located on the hydrophilic faces of the helices. Molecular dynamics simulations of dynorphin that was complexed to the kappa receptor afforded structures in which the tyramine moiety or "message" component was bound within a conserved region of the cavity formed by the TM domain, while the helical segment of the "address" made contact with the helical portion of EL2(27).

The proposed docking mode of dynorphin exemplifies the shortcoming of analyzing structure-activity relationships in the absence of a credible three-dimensional model receptor, inasmuch as the anions in EL2 and the cations in the "address" are apparently not directly involved in ionic bonding. In contrast to the dynorphin binding mode, interaction between the cationic "address" of norBNI and the Glu297 residue is essential for high affinity binding to the kappa receptor. The possible involvement of different "address" subsites for norBNI and dynorphin implies that there may be different modes of interaction interaction for agonists and antagonists or for peptides and nonpeptides. Also, it serves to illustrate the danger in assuming that the structural determinants for agonists and antagonists are necessarily similar.

SUMMARY AND CONCLUSIONS

The impact of recombinant DNA technology and computers on the practice of medicinal chemistry has been enormous. It is changing the paradigm for drug design and the mode of analyzing the relationship between molecular structure and biological activity. In this presentation, I have attempted to illustrate how these changes have influenced my research on the investigation of molecular recognition of ligands at opioid receptors. The cloning of opioid receptors and the availability of technology to construct mutants and chimeras have permitted us to view molecular recognition from the perspectives of both the opioid receptor and the ligand. While this provides an additional dimension to the traditional approach which has focused only on the structure of the ligand, I hasten to mention that molecular modeling does not have the resolving power of the "structure-based design" approach that employs an X-ray structure of a crystalline macromolecular complex. Nevertheless, the melding of molecular biology with three-dimensional molecular modeling for the analysis of the interaction of a series of ligands with G protein-coupled receptors in the rhodopsin family has provided considerable insight into the recognition process.

This is exemplified in the structure-activity (SAR) analysis of the kappa opioid antagonists, norBNI and GNTI, which were evaluated on mutant opioid receptors to reveal that a nonconserved glutamate (Glu297) on the kappa receptor is a key contributor to binding. Significantly, modeling studies involving the docking of antagonists to opioid receptors suggest that selectivity is dependent upon ionic attraction between the N-17' group of norBNI and Glu297, and on repulsive interactions with residues at an equivalent site in the mu and delta receptors. The identification of key amino acid residues that contribute to the binding or repulsion of ligands at opioid receptors represents a paradigm shift, as earlier studies have relied only on the SAR analysis of the ligand without knowledge of the receptor-based residues involved in ligand recognition.

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