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AFFINITY LABELS FOR OPIOID RECEPTORS

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INTRODUCTION

The concept of multiple opioid receptors and multiple modes of interaction with a single receptor emanated from two convergent lines of research. This proposal was based originally on a detailed analysis of a wide spectrum of structure-activity relationships among analgesic ligands (1, 2). Very soon, pharmacological studies complemented and provided a more detailed framework for this concept (3–5). More recently, the multiple receptor concept has been advanced through a number of in vitro and in vivo biological assays and the opioid receptor binding assay. Data from such studies have been summarized in a number of reviews (6–13).

Based on a wide range of pharmacological tests in the chronic spinal dog, Martin and coworkers (14, 15) suggested the designation μ, κ, or σ for those receptors at which morphine, ketazocine, and SKF 10,047 (N-allyl-normetazocine) respectively are postulated to interact. The notion of different receptors for morphine-like and ketazocine- or nalorphine-like compounds has been strengthened by the use of the pA_2 concept (16) both in vitro (17–19) and in vivo (5) and by various other testing methods in vivo (20–22). The δ and ε receptor through which the enkephalins and β-endorphin respectively are thought to mediate their effects have been identified in vitro (19, 23, 24). The δ receptor in vivo has been implicated in a number of physiological functions

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(25–27). In addition, recent evidence suggests that there may be subtypes of certain of these types of opioid receptors (28–30).

While opioid antagonists such as naloxone and naltrexone have been used extensively as pharmacologic tools (31, 32), these agents are not highly selective for the types of opioid receptors they block. Despite their vast utility in opioid-receptor research, a great deal more useful information might be obtained if more highly selective ligands were available. In some instances ligands of the nonequilibrium type would be preferable for use as pharmacologic tools in vitro and in vivo. For example, the use of a compound that bonds covalently and specifically with opioid receptors would be far more advantageous than a reversible ligand for receptor-isolation studies. The advantage would be increased further if the ligand were specific for a particular type of opioid receptor. Such specific, nonequilibrium ligands could be used in sorting out the various opioid receptor types. The interaction of a ligand with a specific type of receptor would avoid cross-reactivity with other types of receptors, as is the case with the presently available reversible antagonists. Precise information can be obtained by the use of specific ligands in receptor binding experiments as well as in mapping the location and distribution of type-specific sites. Specific agonists and antagonists could also be used to determine the relative involvement of a certain receptor type in a particular opiate-induced pharmacologic effect. Finally, nonequilibrium ligands are inherently long-acting and as such may find clinical utility, e.g. an ultralong-lasting narcotic antagonist.

It is the purpose of this article to review the availability of affinity labels for opioid receptors, discuss their selectivity for the various types of opioid receptors, and point out their utility as pharmacologic tools in opioid-receptor research.

Nomenclature

It is important at the outset to define the manner in which certain nomenclature is used in this review. Affinity labels refer to ligands that have very high affinity for receptors such that the interaction is essentially nonequilibrium. The ligands may or may not be covalently bonded to receptors.

As suggested by Avram Goldstein and Hans Kosterlitz (personal communication), opioid receptors such as μ, κ, and δ are designated as receptor types. Subclassifications of these types of receptors are called subtypes. It should be borne in mind, however, that the designation of different receptors as subtypes of a population rather than of types can be quite arbitrary in the absence of definitive criteria for classification. Ultimately, whether different opioid receptors (e.g. μ_1 and μ_2) are more correctly designated as types or subtypes awaits their chemical and biochemical characterization. Presently, it appears that the chemical reaction of μ-type receptors with the μ-specific

affinity label, β-funaltrexamine (β-FNA) (33), is diagnostic for this opioid-receptor system.

A distinction between selectivity and specificity deserves comment. A selective ligand possesses a preferential affinity for a certain type or subtype of receptor, but will have affinity for one or more other types or subtypes. The selectivity of these agents may be relatively high or low at the various receptor types or subtypes. On the other hand, a specific ligand has exclusive affinity for one particular type or subtype of receptor. An agent may possess such high selectivity for a certain type of receptor that interactions at other types are undetectable, in which case the agent could be considered specific.

FACTORS CONTRIBUTING TO SELECTIVITY

By definition, affinity labels take part in a recognition process that leads to a selectively or specificially bound recognition site (34). Their usefulness as pharmacologic and biochemical tools resides with their extremely low degree of dissociation from the site. For these reasons, many affinity labels for opioid receptors possess chemical groups that are sufficiently reactive to form covalent linkages. These groups may be intrinsically reactive and are usually electrophilic in nature, or they may require an activation step that leads to a reactive chemical species. The most common example of the latter involves the photoconversion of the reversibly bound photoaffinity label to a highly reactive intermediate, most often a nitrene or carbene, that covalently binds the opioid receptor.

A ligand with exceptionally high affinity, but without covalent binding capacity, may also meet the criteria for an affinity label if it is sufficiently selective. In this regard, a K_d of not greater than 1×10^{-12}M might be required (assuming an association rate of $10^6 M^{-1}$ sec^{-1}) for such a ligand to remain firmly attached to the receptor for a useful duration (35).

With affinity labels that contain an electrophilic moiety, high selectivity for opioid receptors is dependent on four parameters. These are: (*a*) the affinity of the receptor for the ligand, (*b*) the receptor selectivity of the ligand, (*c*) location of the electrophilic center in the ligand, and (*d*) the reactivity and chemical selectivity of the electrophile. If any one of these parameters is unfavorable, it may have a profound effect on the usefulness of the affinity label as a probe. The studies of Baker (36) on the design of active site–directed irreversible inhibitors of enzymes have considered the importance of some of these parameters.

In considering each of these parameters, it is evident that electrophilic affinity labels are involved in two consecutive recognition processes that lead to the covalent binding of its receptor (33). The first recognition step is

reflected by receptor affinity, and the second recognition step involves the proper alignment of the electrophilic center (attached to the reversibly bound ligand) with a compatible, proximal, receptor-based nucleophile. Because two recognition steps rather than one lead to the covalent binding of the affinity label, enhanced receptor selectivity (recognition amplification) is attainable. This recognition amplification may be particularly evident with chemically selective electrophiles.

When the affinity label contains a highly reactive electrophilic group, such recognition amplification is either minimal or absent. The promiscuous nature of such an electrophile (e.g. aziridinium ion derived from a nitrogen mustard group) enables it to alkylate almost any nucleophile within covalent bonding distance on the opioid receptor. In such cases, the covalent binding selectivity is determined primarily by its affinity for the receptor (first recognition step).

This is particularly the case for photoaffinity labels where there is essentially one recognition step due to the high reactivity of the photolyzed intermediate. Such intermediates are so reactive that they can bind covalently to any amino acid sidechain. Consequently, the ability of a photoaffinity label to covalently bind to a particular opioid receptor type is conferred by its selectivity as a reversible ligand. Because the high reactivity of the intermediate ensures covalent binding to a variety of groups on the receptor, the location of the activated center in the photoaffinity label is not critical. It therefore follows that the covalent selectivity of a photoaffinity label is no better than its reversible binding selectivity. This is not the case for affinity labels that contain a chemically selective electrophile.

In view of the very high selectivity, and perhaps specificity, that can be conferred by the intervention of a second recognition step leading to covalent bonding, an electrophilic affinity label is more capable of sorting out a single opioid receptor type among multiple types than are other classes of affinity labels. Thus, if each opioid receptor type contains an unique array of nucleophiles that differ with respect to reactivity and accessibility, then specific covalent binding will depend upon the nature and orientation of the electrophilic center in the reversibly bound affinity label (33). This is illustrated schematically in Figure 1, where three types of receptors (**A, B, C**) with similar topographic features all are capable of associating reversibly with the affinity label. The electrophilic group **X** is sufficiently chemically selective to permit a reaction with the receptor nucleophile $\mathbf{G}^1$ only when it is within covalent bonding distance. This occurs with type **A** receptors, but not with types **B** and **C.** This is because the nucleophiles in the latter are either within covalent bonding distance but insufficiently reactive (type **B**), or beyond the distance required for an efficient reaction (type **C**). As can be noted, an electrophilic affinity label need not form a highly selective, reversible receptor complex to ensure specific covalent binding. Receptor type **A** is specifically covalently

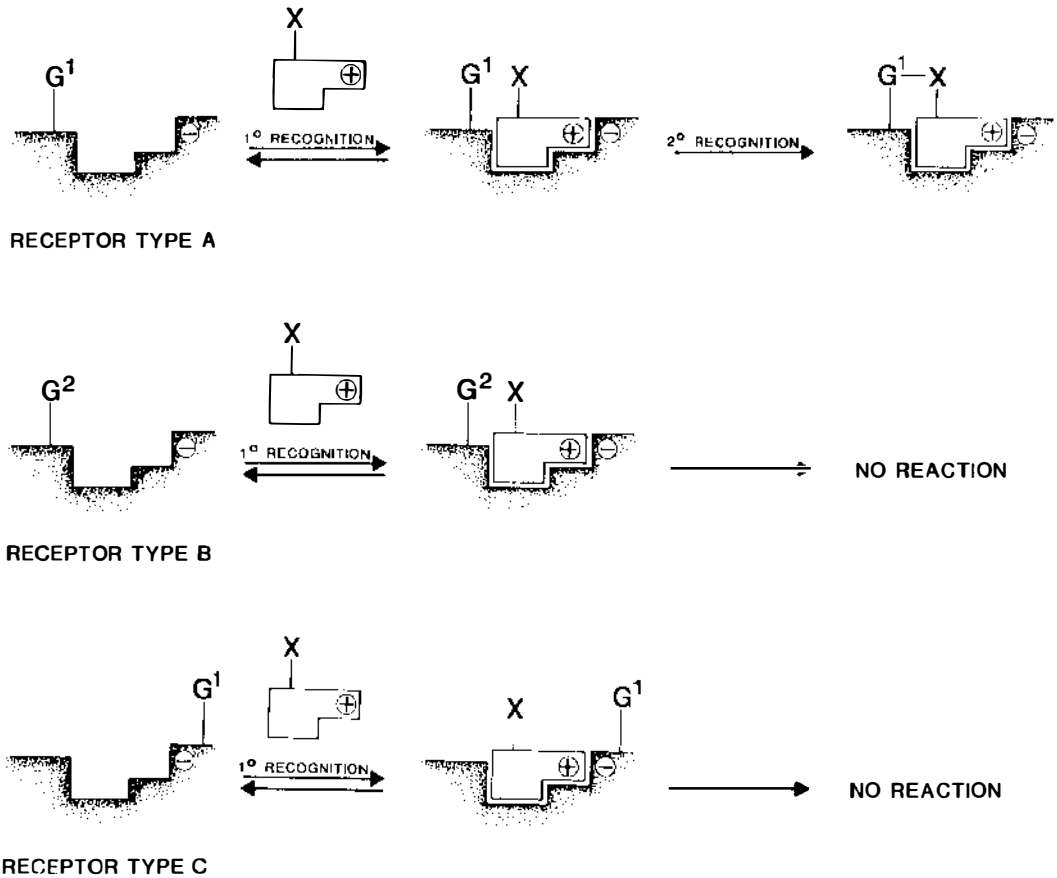


Figure 1 A schematic illustration of the principle of recognition amplification in the covalent binding of receptor type **A** by an affinity label containing a group-selective electrophile **X.** Note that receptor types **A–C** have similar topographic features that lead to reversible binding (1° recognition). However, the receptor types differ with respect to the reactivity (G^1 versus G^2 in **A** and **B**) and location (G^1 in **A** and **C**) of nucleophiles. Only in **A** is the nucleophile G^1 reactive with respect to **X** and within covalent binding distance (2° recognition).

bound, despite recognition of the ligand by types **B** and **C.** Of course, the efficiency with which covalent binding takes place at receptor **A** also is dependent on the residence time (affinity) of the ligand at that site.

PHOTOAFFINITY VERSUS ELECTROPHILIC AFFINITY LABELS

On account of the high reactivity of the photoactivated intermediate relative to most electrophiles (e.g., Michael acceptor groups, isothiocyanate, etc), it is easier to design a photoaffinity label than an electrophilic affinity label. Thus, the location of the photoreactive group in the molecule is not critical for covalent attachment, as is the case for an electrophilic group. This offers a distinct advantage to designing photoaffinity labels that are selective for an opioid receptor type. However, there are practical reasons that make electrophilic affinity labels the agents of choice as probes to investigate opioid receptors.

One should be aware of the effect of modest fluxes of irradiation on biological material when short-wavelength ultraviolet light is used for photoactivation. Large losses of opioid receptor–binding capacity have been reported after irradiation at 254 nm for relatively short time periods (e.g. 7.5 minutes) (37, 38). Further, it has been shown that opiates (morphine, etorphine) appear to bind covalently under such conditions, and it has been pointed out that protection experiments using such ligands should be interpreted with extreme care. Because of these unwanted reactions, groups that can be photoactivated at longer wavelengths offer greater likelihood of success in photoaffinity labeling.

Because photoaffinity labels are useful only under conditions amenable to photolysis, they cannot be employed in vivo or under other conditions that preclude photoactivation. The fact that electrophilic affinity labels do not require activation means that they may be employed both in vitro and in vivo. This advantage makes electrophilic agents much more versatile as tools in opioid research.

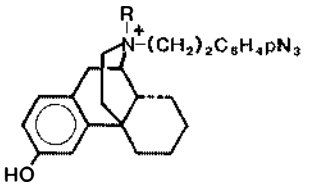
The utility of an electrophilic affinity label in vivo depends on (*a*) the biophase concentration required for effective covalent binding to opioid receptors, and (*b*) ready access of the ligand to opioid receptors. The first requirement is met if a significant fraction of opioid receptors become covalently bound at a concentration of affinity label that is not toxic to the animals. For example, if a concentration in the μM range is required for covalent binding within a time-frame of one half hour in vitro, it seems unlikely that this can occur in vivo without toxic effects. With regard to the second requirement, the distribution of the affinity label should be sufficiently favorable to penetrate the opioid receptor compartment.

PHOTOAFFINITY LABELS

The earliest attempt at making a photoaffinity label for opioid receptors was that of Winter & Goldstein (39), who synthesized a [^{3}H]norlevorphanol derivative (APL) (Figure 2). APL acted as a typical reversible agonist in mice and in the guinea pig ileum longitudinal muscle preparation (GPI) before photoactivation. When APL was photolyzed in the presence of either mouse brain particulate fraction or the GPI, irreversible binding occurred, but levorphanol failed to effectively block the binding. As the authors suggest, extensive nonspecific binding probably occurred with this agent. Indeed, even bovine serum albumin was readily bound by APL. The N-methyl quaternary derivative of APL, MAPL, was subsequently synthesized and tested on the GPI (40). Upon photoactivation, MAPL displayed irreversible activity in decreasing acetylcholine output from the preparation. Although the selectivity of this agent was not examined, this agonist effect did show that the rate of ligand-receptor dissociation (41) was not important for agonist activity in the GPI.

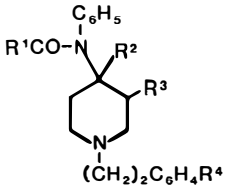
A number of fentanyl derivatives (Figure 3) have been synthesized (42). These compounds were generally unsuccessful as affinity labels, although the diazoketone **3** and the arylazide **5** appeared upon photolysis to produce a moderate irreversible inhibition of [^{3}H]naloxone binding to opioid receptors. Related ligands with electrophilic R^1 substituents similarly were unsuccessful as affinity labels.

In another attempt at obtaining useful photoaffinity probes, the synthesis and properties of a nitro-azido derivative of 14β-aminonormorphinone (NAM)



	R
APL	H
MAPL	CH_3

Figure 2



	R^1	R^2	R^3	R^4
1	CHN_2	H	H	H
2	CHN_2	CO_2Me	H	H
3	CHN_2	H	CH_3	H
4	CH_2N_3	H	H	H
5	Et	CH_2OMe	H	p-N_3

Figure 3

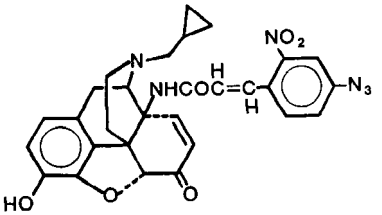


Figure 4

(Figure 4) has been reported (43). NAM was a pure antagonist in both the GPI and the mouse vas deferens preparation (MVD) with a slow association and dissociation rate from opioid receptors. In binding studies, photoactivation of NAM in the presence of rat brain membranes inhibited irreversibly the binding of [^{3}H][D-Ala^2,MePhe^{4}Gly-ol^5]enkephalin (DAGO). Although NAM appeared to bind selectively to μ opioid receptors, it has been deemed unsuitable for labeling opioid receptors due to its slow receptor kinetics. An important observation from this study was that ultraviolet irradiation of rat brain membranes alone decreased their binding activity, as reported earlier by others (37).

Attempts to photoaffinity label opioid receptors also have been carried out with enkephalin analogues (Table 1). Photolysis of analogue **1** (44) inhibited irreversibly the binding of [^{3}H][D-Ala^2,Met^5]enkephalinamide (DAME) to membrane fractions of neuroblastoma × glioma hybrid cells (NG 108–15), which are known to contain only δ opioid receptors (45). Although δ receptors are implicated in this interaction, no further experiments on the selectivity of this ligand have been performed. Analogue **2** (46) inhibited [^{125}I][D-Ala^2,D-Leu^5]enkephalin (DADLE) binding to brain membranes, and upon photoactivation it appeared to bind covalently. The selectivity of the covalent association has not been examined. In a related series of azide enkephalin analogs **(3–5)** (38), the results of the receptor binding assay showed that opioid receptors in bovine caudate nucleus were inactivated. Since [^{3}H]etorphine was used in the opiate binding assay and etorphine is known to possess equal affinity at most types of opioid receptors (47), the selectivity of these photoaffinity labels has not been established. These peptides possessed agonist activity in the GPI, but photoactivation was not possible because ultraviolet irradiation alone eliminated completely and irreversibly the electrically stimulated contractions of the GPI. Recently, two other photoaffinity labels **(6, 7)** that contain a 2-nitro-4-azidophenyl group linked to [D-Ala^2,Leu^5]enkephalin by ethylenediamine or ethylenediamine-β-alanine spacers have been described (48, 49). Both compounds had high affinity in displacing [^{3}H]DAME from rat brain membranes, in inhibiting contractions of the MVD, and in inhibiting opiate-sensitive adenylate cyclase of NG 108-15 hybrid cell membranes. The latter two effects were reversed by naloxone. When photoactivated in the presence of brain membranes, both compounds bound irreversibly to about

Table 1 Photoaffinity enkephalin analogues

Compound number	Analogue
1	Tyr·D-Ala·Gly·Phe·Met·Tyr-NH$(CH_2)_2$NH-C_6H_4(2-NO_2,4-N_3)
2	Tyr·D-Ala·Gly·Phe·Leu-NHCH$(CO_2H)(CH_2)_4$NH-C_6H_4(2-NO_2,4-N_3)
3	Tyr·D-Ala·Gly-NH$(CH_2)_3C_6H_4$ *p*-N_3
4	Tyr·D-Ala·Gly·Phe-NH$(CH_2)_3C_6H_4$ *p*-N_3
5	Tyr·D-Ala·Gly·DL-(*m*-N_3)Phe·Leu-NH_2
6	Tyr·D-Ala·Gly·Phe·Leu-NH$(CH_2)_2$NH-C_6H_4(2-NO_2,4-N_3)
7	Tyr·D-Ala·Gly·Phe·Leu-NH$(CH_2)_2$NH·CO$(CH_2)_2$NH-C_6H_4(2-NO_2,4-N_3)
8	Tyr·D-Thr·Gly·(*p*-N_3)Phe·Leu·Thr
9	Tyr·D-Ala·Gly·Me-(*p*-N_3)Phe·Gly-ol

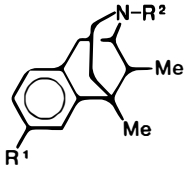
50% of the binding sites. However, when membranes of NG 108-15 cells were used, up to 80% of the receptors were inactivated. Since DAME binds equally well to μ and δ receptors present in brain membranes, and NG 108-15 cells are known to contain only δ receptors, the investigators concluded that these ligands were relatively selective for δ receptors. They also found with [^{3}H]**7** that much of the label bound selectively to opioid receptors, although this labeling of brain membranes was not specific. An important departure in the photoactivation procedure in this study was the use of purely visible light for photolysis rather than the usual ultraviolet irradiation. Thus, these investigators avoided the destruction of opioid receptors associated with short-wavelength ultraviolet irradiation (37). More recently, the azido derivatives of two selective peptides have been synthesized (50). They are derivatives of DAGO (a μ-selective ligand) and Tyr·D-Thr-Gly·Phe·Leu·Thr (DTLET) (a δ-selective ligand). Upon photoirradiation of brain membranes in the presence of azido-DTLET **(8),** binding of [^{3}H]DTLET was inhibited without any effect on the binding of [^{3}H]DAGO. Excess DTLET was capable of completely protecting δ binding sites from alkylation by azido-DTLET. At high concentrations of azido-DTLET, photoinactivation of some μ sites also occurred. The authors did not rule out the possibility of inactivating κ sites with azido-DTLET. Analogous photoactivation experiments with azido-DAGO (**9**) have not been described. If azido-DTLET proves to have high selectivity in receptor binding as well as in biological systems, it may find utility in characterizing δ receptors in the future.

ELECTROPHILIC AFFINITY LABELS

Agonists

May et al (51) have described the first attempt to design electrophilic ligands for opioid receptors. These compounds, which are two N-2-bromoalkyl substi-

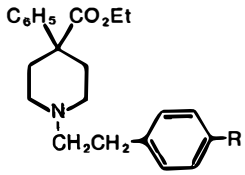
tuted benzomorphans **(1–3)** (Figure 5), produced prolonged central depression together with a low degree of analgesic activity in mice. However, the opioid nature and the irreversibility of the pharmacologic effects have not been demonstrated. Thus, it is uncertain whether these derivatives interacted with opioid receptors in a nonequilibrium manner. Very recently, another potential benzomorphan affinity label **(4)** (52) has been reported to inhibit irreversibly the binding of [^{3}H]DAME to opioid receptors on NG 108-15 hybrid cells. Although δ receptors are implicated here, the extent of its selectivity has not been explored.



	R¹	R²
1	H	CH_2CH_2Br
2	H	$CH_2CHBrCH_3$
3	H	$CH_2CHBrC_6H_5$
4	OH	$(CH_2)_2C_6H_4pNHCOCH_2Br$

Figure 5

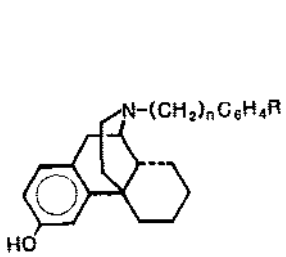
An early effort to design affinity labels with the anileridine pharmacophore afforded equivocal results (53). Derivatives containing electrophilic moieties attached to the anilino nitrogen (Figure 6) all possessed analgesic activity in mice, but only the fumaramido ethyl ester **(2)** appeared to cause fairly long-lasting (> 6 hours) blockade of morphine analgesia. Pretreatment of mice with naloxone prevented the analgesic activity of **2** and protected opioid receptors from long-lasting blockade. It was suggested that the initial interaction of **2** with opioid receptors gave rise to a reversible complex that resulted in analgesia, while the subsequent interaction arose from the alkylation and inactivation of opioid receptors. Further studies on the GPI indicated that compounds in this series interacted with μ opioid receptors (54). Unexpectedly, however, **2** did not display any antagonist activity in this preparation. This observation suggests that central opioid receptors may differ from those in the ileum.



	R
1	$NHCOCH_2X$, X = Br or I
2	$NHCOC(H)=C(H)COOEt$
3	$NHCOC(H)=C(H)COOEt$
4	

Figure 6

A similar rationale has been employed in the utilization of 3-hydroxymorphinan as a pharmacophore (Figure 7) (55). Many of these derivatives exhibited analgesic activity in mice and some have been found to be modest inhibitors of morphine-induced analgesia. The most potent antagonist among these compounds was the maleimide derivative (**4**). Studies with naloxone revealed that it blocked the analgesic action of **4** and protected the receptors from interaction with this ligand. Although this compound contained an electrophilic moiety, additional pharmacologic data suggested that the blockage of opioid receptors in vivo may not involve covalent association.



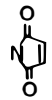
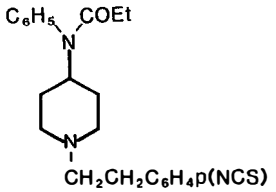
	R (o, m, or p)
1	$NHCOCH_2Br$
2	$NHCOC(H)=C(H)COOEt$
3	$NHCOC(H)=C(H)COOEt$
4	

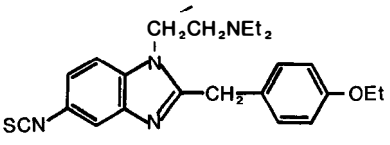
Figure 7

In another effort to obtain selective affinity labels, the isothiocyanate derivatives of fentanyl (FIT) and etonitazene (BIT) and the fumaramido derivative of endoethenotetrahydrooripavine (FAO) have been synthesized (Figure 8) (56). In the opioid receptor binding assay using both rat brain membranes and NG 108-15 hybrid cells, FIT and FAO appeared to be highly selective alkylators of δ receptors and BIT appeared to alkylate μ receptors selectively. These labels have been reported to possess no cross-reactivity between these two types of receptors. The possible interaction of these ligands with κ receptors and their activities in biological systems in vitro or in vivo have not been investigated. Employing NG 108-15 hybrid cells, [^{3}H]FIT has been used to identify a Mr 58,000 subunit of opioid receptors (57). The binding of [^{3}H]FIT to this subunit withstood extensive washing, and levorphanol, but not dextrorphan, prevented this binding. From its adsorption on and specific elution from wheat germ agglutinin, the subunit has been thought to be a glycoprotein. [^{3}H]FIT also labels a number of proteins and phospholipids nonspecifically.

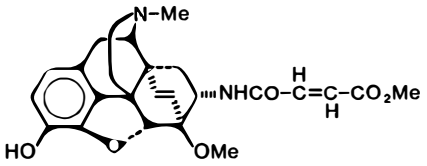
Morphinone (**1**) (Figure 9) has been reported to irreversibly inhibit [^{3}H]naloxone binding to opioid receptors in brain membranes (58). Since dihydromorphinone did not inhibit binding, it is possible that the α,β-unsaturated ketone system in morphinone may not react with a receptor nucleophile. The authors of this report have also demonstrated that the parenteral



FIT



BIT

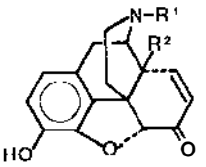


FAO

Figure 8

administration of morphinone to mice for 1–3 days inhibited markedly the analgesic effect of morphine. Recently, cyclopropylmethylnormorphinone **(2)** as well as morphinone have been prepared and tested on the GPI, MVD, and in mice (59). Morphinone behaved as a reversible agonist and cyclopropylmethylnormorphinone as a reversible antagonist in the in vitro and in vivo assays. No evidence for sustained agonism or antagonism has been observed with either compound. Since in the earlier report, morphinone was administered parenterally and the latter study employed the intracerebroventricular (icv) route, a direct comparison of the two studies is difficult with regard to antagonism of morphine analgesia. However, if morphinone were capable of antagonizing morphine analgesia, it would have been more easily detected by the icv route. Another morphinone derivative **3** and its morphine derivative have been described (60). Both compounds had modest irreversible inhibitory activity against [^{3}H]naloxone binding to opioid receptors in brain membranes. A rigorous test for selectivity or experiments in biological systems have not been performed.

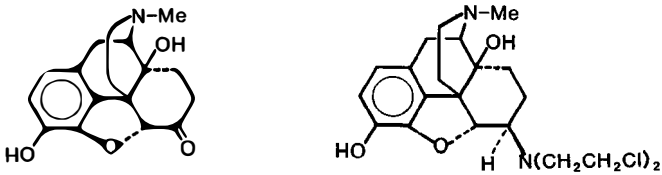
The nitrogen mustard derivative of oxymorphone, β-chloroxymorphamine



	R¹	R²
1	Me	H
2	CH_2-cyclopropyl	H
3	Me	$NHCOCH_2Br$

Figure 9

(β-COA) (Figure 10) has been shown to be an irreversible agonist in the GPI (61, 62). This observation suggests that receptor occupation rather than rate of ligand-receptor dissociation (41) is important for agonist activity in the GPI (40). β-COA also appeared to bind irreversibly opioid receptor sites in brain membranes, as indicated by the fact that exhaustive washing and eight hours of dialysis of β–COA treated membranes did not eliminate the inhibition of [^{3}H]naltrexone binding. In mice, β-COA produced analgesia with a duration four times that of its reversible analogue, oxymorphone. This β–COA induced analgesia was inhibited by pretreatment of mice with naloxone. In addition, β-COA given icv had long-lasting antagonist effect (~ 6 days) against morphine-induced analgesia. The antagonism of morphine-induced analgesia at spinal sites was even more pronounced when β-COA was given intrathecally (it), as the antagonism in this case lasted more than 21 days (63). The inhibition of [^{3}H]naltrexone binding to brain membranes of β–COA treated animals also persisted for about six days. These results indicated that β-COA was covalently bound to opioid receptors in vivo.



Oxymorphone β–COA *Figure 10*

With the success of obtaining the irreversible agonist β-COA, which has the nitrogen mustard group at the 6β position of the opiate, placement of the electrophilic group at other positions in the opiate molecule, such as at the C-8 position, has been investigated in order to explore the accessibility of other nucleophiles on opioid receptors to alkylation (64). An opiate with the nitrogen mustard group in the C-8 position (Figure 11) has been found to be a weak, reversible, partial agonist in both the GPI and MVD without any significant irreversible agonist or antagonist activity. It also has been found that a small moiety, such as an azide substitution at C-8, did not seriously impair reversible agonist activity, but a larger moiety, such as the precursor diol of the nitrogen mustard derivative, was devoid of any reversible agonist or antagonist activity. These observations led the authors to conclude that this nitrogen mustard derivative has low affinity for opioid receptors and that the C-8 position cannot accommodate large electrophilic groups without compromising the primary recognition step for ligand-receptor interaction.

The first reported peptide electrophilic affinity label was the chloromethyl ketone derivative of [D-Ala^{2}Leu^5]enkephalin (DALECK) (Figure 12), which

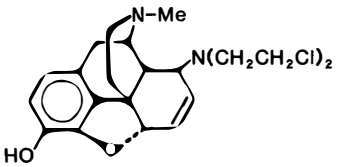


Figure 11

was initially synthesized by Pelton et al (65) and subsequently by two other groups (66, 67). The binding of this agent to opioid receptors in the receptor binding assay using [^{3}H]DADLE, [^{3}H]etorphine, or [^{3}H]naloxone appeared to be irreversible. However, the agonist effects of DALECK in the GPI, MVD, and analgesic assays were all fully reversible by naloxone. Thus, it is uncertain whether DALECK possesses selectivity or if covalent association of opioid receptors is possible with this agent in biological systems. In another approach to obtain a potential peptide label, [D-Ala^2,Leu^5]enkephalin (DALA) was extended with the methyl ester of the nitrogen mustard drug melphalan (Mel) at the C-terminus (68). This compound, DALA-Mel-OMe, displayed high affinity in displacing [^{3}H]DALA and [^{3}H]naloxone from brain membranes. Although DALA-Mel-OMe appeared to block irreversibly the binding of [^{3}H]naloxone, the fact that naloxone and DALA afforded only partial protection suggests that nonspecific labeling also occurred.

Tyr·D-Ala·Gly·Phe·Leu-R

	R
DALA	OH
DALECK	CH_2Cl
DALA-Mel-OMe	$NHCH(COOH)CH_2C_6H_5pN(CH_2CH_2Cl)_2$

Figure 12

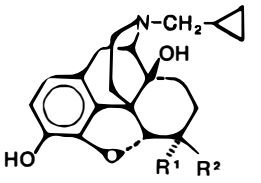
Antagonists

It is obvious from the preceding discussion that agonist electrophilic affinity labels in general have not yielded compounds with wide utility. In investigations of pharmacologic effects and characterizations of opioid receptor types, antagonists, especially those without agonist activity, have been extremely useful, as exemplified by the contribution that naloxone and naltrexone have made in this field as pharmacologic tools. However, the reversible nature of such antagonists and their cross-reactivity with the various opioid receptor types are inherent limitations to their use as receptor probes. Thus, there have been a number of efforts to develop highly selective antagonists of the non-equilibrium type.

The first successful antagonist affinity label synthesized was a nitrogen mustard derivative of naltrexone, β-chlornaltrexamine (β-CNA) (Figure 13) (69, 70). β-CNA proved to be a potent affinity label highly selective for opioid receptors both in vitro and in vivo. β-CNA inhibited irreversibly the binding of either [^{3}H]naloxone or [^{3}H]naltrexone to brain membranes. Support for opioid receptor alkylation by β-CNA has been indicated by the fact that neither exhaustive washing nor eight hours of dialysis dissociated β-CNA from opioid receptors, while the reversible ligands, naltrexone and oxymorphone, were completely removed by these procedures. Nonopioid nitrogen mustards, chlorambucil and phenoxybenzamine, had no affinity for specific opioid sites in this regard (62). In both the GPI and the MVD preparations, β-CNA produced irreversible antagonism that could be prevented but not reversed by naloxone (61, 71). β-CNA displayed classical nonequilibrium antagonism in that the agonist concentration-response curve was shifted to the right with a decrease in the maximum effect. Although β-CNA blocked all opioid receptor types, i.e. antagonism of the effects of morphine (μ) and ethylketazocine (κ) in the GPI and additionally DADLE (δ) in the MVD, it did not antagonize the effects of norepinephrine in the GPI. The observation that β-CNA failed to block cholinergic, prostaglandin, or benzodiazepine binding sites (72) provided further evidence for the selectivity of β-CNA for opioid receptors. The nonopioid nitrogen mustards, chlorambucil and phenoxybenzamine, possessed no antagonist activity against morphine in the GPI. The facility with which β-CNA irreversibly blocked different receptor types was $\mu > \kappa > \delta$, which parallels its affinity for these sites in the reversible association phase preceding covalent binding. This is in accordance with the idea that little or no secondary recognition is operative in affinity labels that contain highly reactive and indiscriminate electrophiles (73).

In mice, β-CNA produced ultralong antagonism (> 3 days) of morphine-induced analgesia after a single icv injection. By comparison, antagonism by naltrexone icv lasted less than two hours (62). β-CNA displayed very weak analgesia that dissipated within 30 minutes. In line with the long duration of action, a single icv administration of β-CNA to mice inhibited the development of physical dependence on morphine during the 72-hour observation period. When given it, β-CNA produced even longer antagonism against it morphine analgesia (> 13 days) (63). The antagonism by β-CNA could be prevented by prior administration of naloxone, indicating that opioid receptors were involved. Significantly, the specific [^{3}H]naltrexone binding capacity of brain membranes of animals treated with β-CNA was significantly decreased for the same length of time as β-CNA antagonism, which suggests that covalent bonding had occurred in vivo as well.

[^{3}H]β-CNA has been used to isolate opioid receptor components from brain membranes (74). Covalently bound [^{3}H]β-CNA complex has been solubilized,



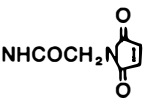
		R¹	R²
1	β-CNA	H	$N(CH_2CH_2Cl)_2$
	α-CNA	$N(CH_2CH_2Cl)_2$	H
2	β-FNA	H	NHCOC(H)=C(H)COOMe
	α-FNA	NHCOC(H)=C(H)COOMe	H
3		H	NCS
		NCS	H
4		H	NHCOC(H)=C(H)COOMe
5		H	$NHCOCH{=}CH_2$
6		H	NHCOC(Cl)=CH_2
7		H	NHCOC(H)=C(H)COMe
8		H	NHCOC≡CH
9		H	
10		H	
11		H	$NHCOCH_2{-}S{-}S{-}C_6H_5o(NO_2)$
12		H	$NHCOCH_2HgCl$
13		H	$NHCOCH_2I$
14		H	$NH{-}COCO{-}C_6H_5$

Figure 13

dialyzed, and chromatographed. The elution profile suggests four selective [^{3}H]β-CNA complexes. At least two of these complexes migrated in a single large peak, which has been calibrated to be 590,000 daltons. One of the complexes eluted at the elution volume and was dialyzable, while putative aggregates of these complexes eluted at the void volume. Because of the high reactivity of β-CNA, these complexes may represent multiple forms of opioid receptors.

Since β-CNA is highly selective for opioid receptors, it has been used for typical protection studies. For example, in the MVD, β-CNA has been used with Tyr·D-Ser·Gly·Phe·Leu·Thr (DSLET), a highly selective δ agonist, as the protector to irreversibly block μ and κ receptors. Such preparations contain a near-homogeneous population of δ receptors and have been used in assessing the δ activity of agonists (75). In the GPI, β-CNA has been used with DADLE, which interacts with μ receptors in this preparation, and dynorphin$_{(1-13)}$ as protectors to selectively alkylate κ and μ receptors respectively (76, 77). Further studies using ethylketazocine and normorphine as additional protectors suggested that the relative potencies of dynorphin$_{(1-13)}$ in these blocked preparations are very similar to those of ethylketazocine. This observation, together with the fact that naloxone had similar K_e values in antagonizing dynorphin$_{(1-13)}$ and ethylketazocine, has led investigators to conclude that dynorphin is a specific endogenous κ agonist. Further work in the GPI has revealed that low concentrations of β-CNA selectively alkylated μ and κ receptors without inactivating δ receptors (78). In addition, increasing concentrations of β-CNA have been employed to progressively block dynorphin receptors in the GPI and MVD (79). A concentration of β-CNA that lowered substantially the maximum effect of dynorphin in the MVD caused a parallel shift of the concentration-response curve in the GPI. It has been concluded that the difference in potency of dynorphin in the two preparations is due to the presence of more spare receptors in the GPI than in the MVD. The dissociation constant of the normorphine-receptor complex in the GPI has been estimated by using β-CNA to partially block a fraction of the receptor population (80). The estimate of the dissociation constant has been accomplished by the principle laid down by Furchgott & Bursztyn (81). Since the dissociation constants of normorphine in naive and morphine-tolerant ilea were not significantly different, the authors concluded that changes in affinity at opioid receptors do not occur with the development of tolerance and that tolerance may be related to events after the receptor interaction. However, in relating this to the whole animal, it has already been demonstrated that in chronic studies opioid receptors in the GPI may not be an appropriate model for those in the central nervous system (82).

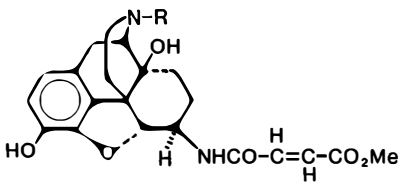
Using the protection procedure, β-CNA has been used to enrich specific binding sites in brain membranes (83). Sufentanil, DADLE, and dynorphin A have been used as protectors for μ, δ, and κ sites respectively. However, as in

all protection studies, the protection of selected receptor sites is only as good as the selectivity of the protector. Thus, except for the dynorphin-protected sites, the enriched preparations were still not completely homogeneous. Nevertheless, β-CNA pretreatment procedure affords better estimates of binding selectivity than the conventional method. Highly selective agonists for the various opioid receptor types have been identified: DAGO, sufentanil, and morphiceptin for μ sites; [D-Pen^2,D-Pen^5]enkephalin and [D-Pen^2,L-Pen^5]enkephalin for δ sites; and tifluadom and Trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]benzeneacetamide (U50,488) for κ sites.

β-CNA also has been used in vivo as a pharmacologic tool in a variety of studies. In an early study, β-CNA was used to study the opiate nature of the effects of Δ^9-tetrahydrocannabinol (THC) in rats (84). After a single icv injection of β-CNA, the analgesia, hypothermia, hypothermic tolerance, and physical dependence produced by THC were all inhibited. The results suggested that there are some common features between THC and opiates and that some actions of THC may be mediated by an opioid-related mechanism. β-CNA has been employed in several behavioral studies. The long-lasting opioid antagonism of β-CNA has been confirmed in studies involving climbing behavior in mice (85), suppression of autoshaped behavior in rats (86), and separation-induced distress vocalization in chicks (87). Opioid selectivity of β-CNA also has been demonstrated by the ability of β-CNA to antagonize morphine, but not amphetamine, in suppressing autoshaped behavior (86). In an effort to study the role of the spinal cord in the development of tolerance and physical dependence, β-CNA has been used to block spinal opioid receptors (88). β-CNA treatment it antagonized the analgesic action of morphine, blocked the development of tolerance, and attenuated several characteristic signs of precipitated withdrawal. The authors concluded that opioid receptors in the spinal cord play a significant role in the development of tolerance and physical dependence induced by systemically administered opiates.

Although β-CNA has proved to be selective for opioid receptors, it was not able to distinguish readily between various receptor types. The very high reactivity of the aziridinium ion generated from β-CNA facilitates the alkylation of opioid receptors but has made this step less selective because this electrophile can react with a variety of nucleophiles. As discussed earlier, the selectivity of the probe for a given opioid receptor type may depend on the reactivity of the attached electrophile and the proximity of the correct nucleophile within that opioid receptor type. In an effort to obtain affinity labels that have more selectivity, electrophilic groups that are less reactive and more selective than the nitrogen mustard group have been attached to the C-6 position of naltrexone (73, 89). One of the most selective ligands in this series was the fumaramate methyl ester derivative β-funaltrexamine (β-FNA) (Figure

14) (90). The analogous derivative of oxymorphone, β-fuoxymorphamine (β-FOA), also has been synthesized in order to compare the interaction of an antagonist with a closely related agonist affinity label. In the GPI, both β-FNA and β-FOA were reversible agonists (90, 91). Using naloxone as the antagonist, pA_2 analyses revealed that β-FOA interacted with μ receptors, whereas β-FNA interacted with κ receptors to manifest agonism. Remarkably, in the case of β-FNA but not β-FOA, a concurrent irreversible blockage of μ sites occurred. Thus, after the agonism of β-FNA was terminated by thorough washing of the GPI, a persistent antagonism of the effect of morphine was observed. The degree of irreversible antagonism was concentration- and time-dependent. That this antagonism was selective for μ sites was revealed by the lack of inhibition of κ agonists such as nalorphine and ethylketazocine. In addition, β-FNA antagonized the agonist effect of β-FOA (μ sites) but not its own agonist effect (κ sites). In contrast to β-CNA, which displayed a typical irreversible antagonism, the inhibition of morphine by β-FNA was manifested as a parallel shift of the concentration-response curve. No diminution of the maximum response has been observed because morphine also is a full agonist at κ receptors (75). In the MVD, β-FNA also displayed a reversible agonist action that upon pA_2 analysis with naloxone appeared to be mediated through κ receptor interaction (71). β-FNA displayed all the irreversible antagonist features that it exhibited in the GPI. In addition to having little or no effect on the activity of the κ-agonists, nalorphine and ethylketazocine, β-FNA did not inhibit the effects of the δ-agonists, met-enkephalin, leu-enkephalin, and DADLE. Moreover, IC_{50} of μ-agonists, morphine and methadone, were increased substantially, and the IC_{50} of δ-agonists, leu- and met-enkephalin, were unchanged in vas deferentia taken from mice treated with β-FNA forty-eight hours prior to testing. These observations showed the specificity of β-FNA for μ receptors in this preparation. However, as with any affinity label, at very high concentrations, β-FNA may interact with receptors other than the μ type.



	R
β-FNA	CH_2–◁
β-FOA	Me

Figure 14

In the opioid receptor binding assay, β-FNA bound to mouse brain membranes in both a reversible and irreversible manner (92). When membranes were treated with β-FNA followed by thorough washing, the binding capacity for [^{3}H]morphine and [^{3}H]naltrexone was reduced markedly, whereas binding

of [^{3}H]met-enkephalin, [^{3}H]DADLE, and [^{3}H]ethylketazocine was decreased to a lesser extent. In brain membranes from mice treated with β-FNA forty-eight hours prior to sacrifice, the binding sites for [^{3}H]morphine were reduced by about 50%, whereas those for [^{3}H]met-enkephalin were unaffected. The receptors in the NG 108-15 hybrid cells were also unaffected by treatment with β-FNA (K.-J. Chang, personal communication). The ability of various unlabeled ligands to inhibit the reversible binding of [^{3}H]β-FNA resembled the relative ability of the same ligands to inhibit the binding of [^{3}H]ethylketazocine (92). The binding characteristics of β-FNA appeared to be consistent with its profile in isolated tissues in that the irreversible portion of β-FNA binding demonstrated selectivity for μ over δ binding sites, while the reversible portion of β-FNA binding exhibited a selectivity for κ over μ or δ binding sites.

In vivo, β-FNA demonstrated analgesic activity in mice of short duration (93). This analgesic effect was antagonized by naloxone and upon pA_2 analysis appeared to be mediated by κ opioid receptors. In contrast, the antagonist action of β-FNA was of remarkably long duration and selective toward μ agonists. For example, antagonism of morphine-induced analgesia by β-FNA subcutaneously (sc) lasted over four days. The locus of action of β-FNA has been thought to be central because sc administration of β-FNA antagonized the action of morphine given icv and vice versa. The selectivity of the antagonism of β-FNA was exemplified by its lack of antagonism of analgesia elicited by either nalorphine or β-FNA when mice were tested forty-eight hours after β-FNA treatment. This activity profile is consistent with that in vitro.

One of the initial uses of β-FNA has been to deplete the GPI of functional μ receptors (75). Under appropriate conditions, all the μ receptors can be irreversibly blocked by β-FNA, thereby affording a near homogeneous population of κ receptors. This preparation is useful in assessing the κ activity of agonists. It has been shown in this blocked preparation that morphine was a full agonist at κ receptors, i.e. the pA_2 value of morphine-naloxone changed to that resembling ethylketazocine-naloxone. A whole range of so-called μ agonists have been tested in this β-FNA-treated GPI and they all exhibited κ activity at high concentrations (A.E. Takemori, P. S. Portoghese, unpublished data). The use of a specific irreversible antagonist such as β-FNA obviates the necessity for protection experiments involving μ receptors. This is particularly advantageous in view of the cross-reactivity to κ receptors by μ agonists at the high concentrations used for protection. In this connection, the β–FNA treated GPI has been used to characterize dynorphin as a κ agonist (94). β-FNA also has been used in the tolerant GPI to demonstrate that selective opiate tolerance is not associated with selective dependence, i.e. induction of a selective tolerance at μ receptors produces cross-dependence at κ receptors and vice versa (95). Although μ agonists (normorphine and morphine) and δ agonists such as the enkephalins and their derivatives are presumed to interact at μ receptors in the

GPI (pA_2 values with naloxone are similar), recently it has been reported that in the β–FNA treated GPI the pA_2 values of the μ agonists decreased to values resembling κ agonists, whereas the pA_2 values of the peptides remained unaltered (96, 97). One group has suggested that the peptides were interacting with δ sites uncovered by β-FNA, while another investigator has proposed that the peptides interacted with a μ receptor subtype. Very recently, in a protection experiment involving the same conditions under which the MVD was converted into a relatively pure δ preparation (DSLET + β-CNA) (75), DSLET failed to protect against alkylation by β-CNA in the GPI (A. E. Takemori, P. S. Portoghese, unpublished data). Thus, whatever these β-FNA insensitive sites are called, they do not appear to be of the δ type found in the MVD.

Since β-FNA interacts reversibly at κ receptors and irreversibly at μ receptors, and β-FOA interacts reversibly only at μ receptors, the GPI has been used to study the possibility that μ agonists and antagonists may interact at different sites (98). A series of opioid agonists and antagonists have been evaluated for their ability to protect against the irreversible antagonism of the action of morphine by β-FNA. Antagonists afforded excellent protection against irreversible blockage by β-FNA, whereas most of the agonists were relatively poor protectors. Moreover, the ability of the compounds to protect against β-FNA antagonism appeared to correlate with their antagonist potencies (K_e) but not with their agonist activities (IC_{50}). These results suggest that agonists and antagonists may interact at separate sites on the μ receptor system. Additionally, the fact that both β-FNA and β-FOA contain an identical electrophilic moiety at the C-6 position, with only β-FNA alkylating opioid receptors, suggests that β-FOA and β-FNA may interact at different sites as an agonist and antagonist respectively. This is supported by the fact that β-FOA was unable to protect against β–FNA induced irreversible antagonism. A model consistent with the above findings has been proposed that consists of a μ subunit with which agonists have high affinity and a regulatory ρ subunit with which antagonists have high affinity (98). Occupation of the ρ site has been proposed to produce an unidirectional coupling (allosteric coupling) to the μ site such that a decrease in affinity of agonist interaction at the μ site occurs. The ρ site may be occupied by agonists but only after the μ site is occupied, whereas antagonists interact selectively at the ρ site. The ρ sites are envisaged as regulatory sites for some endogenous μ agonist ligand. A similar dual site opioid receptor model has been proposed by others (99).

A number of studies in vivo have made use of β-FNA to study the relative involvement of μ receptors in various opiate actions. The μ receptor involvement in opiate-induced respiratory depression (100), decrease in gastrointestinal transit (101), cardiovascular effects (102, 103), prolactin secretion (104; L. Krulich, personal communication), spinal analgesia (105, 106), and antidiuresis (107) has been investigated. β-FNA also has been used to demonstrate the

noninvolvement of μ receptors in certain opioid actions such as endotoxic shock (108) and post-ictal analgesia (109), which are thought to be mediated by δ receptors, and diuresis (107), which is thought to involve κ receptors. The μ receptor involvement in analgesia produced by κ and δ agonists has also been investigated using β-FNA (110–114).

An important use of β-FNA has been to determine if the phenomena of tolerance and physical dependence are associated with μ opioid receptors. In rats, the physical dependence produced by a continuous intraperontoneal (ip) infusion of morphine was completely blocked by β-FNA (115). When β-FNA was given it, the tolerance and dependence produced by sc morphine pellets was markedly inhibited (116). These results suggest that μ receptors in the central nervous system, including the spinal cord, play a prominent role in the development of tolerance and physical dependence in rodents. β-FNA sc promptly precipitated withdrawal signs in morphine-dependent monkeys that were still evident thirty hours later in spite of the fact that morphine was being administered every six hours (115). In contrast, naloxone-precipitated withdrawal lasted ninety minutes. In another study, β-FNA sc precipitated withdrawal in morphine-dependent monkeys that lasted seventy-two hours (117). When β-FNA was administered icv it was much more potent in precipitating withdrawal, and the syndrome was more severe and longer-lasting than that produced upon abrupt withdrawal of morphine. It is evident that these findings strongly implicate μ opioid receptors in the development of physical dependence in primates. It is also important to note that β-FNA was about 20,000 times more effective when administered icv than sc, indicating that β-FNA may have encountered some difficulty distributing to the brain (117). These investigators also have studied the surmountability by morphine of the antagonist-induced withdrawal in morphine-dependent monkeys. Naltrexone-precipitated withdrawal and abrupt withdrawal were completely suppressed by a relatively moderate dose of morphine sc. However, extremely high doses of morphine did not completely alleviate the monkeys from β-FNA precipitated withdrawal, which strongly suggests the covalent nature of the binding of β-FNA to opioid μ receptors.

In order to determine whether the orientation of the electrophile is important for covalent bonding to opioid receptors, a series of epimeric pairs of naltrexone derivatives (**1–3**) that contain an electrophilic group at the 6α- or 6β-position have been investigated (73) (Figure 13). All compounds were active as reversible agonists in the GPI, but only the 6β-isomers of the fumaramate ester (β-FNA) and isothiocyanate (**3**) displayed selective irreversible antagonism of the μ agonist, morphine, without affecting κ agonist activity. The 6α-isomer (α-FNA) acted reversibly and was able to protect the receptors against irreversible blockage by β-FNA, indicating that the two epimers bind to the same μ site. These results suggest that proper orientation of the electrophilic substituent with a proximal nucleophile in μ receptors is necessary for covalent

bonding to occur. Moreover, the lack of covalent bonding to κ receptors by ligands in this series indicated that sufficiently reactive nucleophiles were not within covalent bonding distance at the κ site. In the MVD, β-FNA, but not α-FNA, irreversibly antagonized morphine, whereas neither isomer antagonized the δ agonist, DADLE. In contrast, both isothiocyanate epimers **(3)** irreversibly blocked μ and δ receptors. Because a more reactive electrophile is less efficient in its ability to distinguish between different types of nucleophiles, the isothiocyanate epimers, which are presumably more reactive and less selective than the fumaramates, displayed less of a difference in their capacity to covalently bind receptors. With an extremely reactive electrophile such as the nitrogen mustard group in α- and β-CNA, the difference between the receptor covalent-binding capacity of the two epimers was even less pronounced. Thus, in cases of highly reactive electrophiles, the chirality at C-6 becomes less important in the secondary recognition process.

In addition to the importance of the 6β stereochemistry in ligands that contain moderately reactive and relatively selective electrophiles, it appears that trans geometry of the double bond in the Michael acceptor group is required for irreversible blockage of μ receptors. Thus, it has been found that ligands that contain cis double bonds (**4** and **9**) (Figure 13) do not irreversibly block the effects of morphine, while β-FNA and **7** are effective in this regard (73). It is conceivable that the role of geometry is one of orienting the electrophilic center within covalent bonding distance of a compatible (e.g. sulfhydryl group) receptor-bound nucleophile. In this connection, the lack of correlation between irreversible μ antagonism and the chemical reactivity of the Michael acceptor ligands supports this notion. Several of these compounds **(6, 7)** (Figure 13) acted like β-FNA in the GPI and MVD, i.e. reversible agonism at κ receptors and irreversible antagonism at μ, but not κ or δ receptors. Some derivatives **(8, 14)** were considerably more potent as κ agonists than was β-FNA. These compounds, as well as the maleimidoacetamide **(10)** and chloromercuriacetamide **(12)** derivatives, exhibited irreversible agonism. It is noteworthy that **10** and the iodoacetamide **(13)** were very different from β-FNA in that the irreversible μ antagonism that they displayed was substantially greater in the MVD than in the GPI. α-CNA, in addition to covalently binding μ, κ, and δ receptors, also displayed concurrent irreversible agonist activity in the GPI but not in the MVD (118). These observations suggest that the two preparations contain different proportions of opioid μ receptor subtypes.

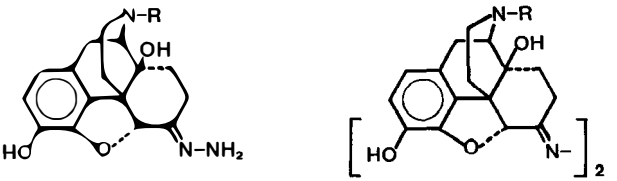
MISCELLANEOUS AFFINITY LABELS

It sometimes may be difficult to distinguish between tight noncovalent association and covalent binding of a ligand with its receptor. In the case of ligands that do not contain intrinsically reactive groups, apparent nonequilibrium binding is

most likely a consequence of very high affinity, but other mechanisms also are possible. For example, entrapment of a ligand in a tissue compartment or membrane domain that interfaces with opioid receptors might lead to apparent irreversible binding or sustained activity if washing does not readily remove the ligand (89). This mechanism implies that the ligand-receptor interaction is comprised of two consecutive steps: i.e. the partitioning of ligand into a tissue compartment followed by association with the receptor (119). In this case, a locally high concentration may result in an apparent high affinity. Sustained activity can arise from inefficient removal of ligand from the compartment rather than from high receptor affinity. A combination of entrapment and high affinity also will afford sustained activity. It is conceivable that the slow offset of action of the μ partial agonist, buprenorphine (120), in the GPI and its slow dissociation from rat brain receptors arise from such factors.

Nonequilibrium binding and ultralong actions have been reported for hydrazone derivatives of naloxone, naltrexone, and oxymorphone (Figure 15) (121, 122). However, subsequent studies (123) have suggested that the nonequilibrium actions of these hydrazones are due wholly or in part to the corresponding azines as the result of a disproportionation reaction of the hydrazone in solution. Presently, the mechanism of the persistent effect of the azines at opioid receptors is not known. These azines blocked opiate binding in vitro 20-40 times more potently than did their corresponding hydrazones. It is therefore possible that the irreversible action of the hydrazones might be due to conversion of a small fraction of the hydrazone to azine. These azines appear to block a high affinity morphine binding site, designated as μ_1, in brain membranes. A low affinity site, named μ_2 by the researchers, was not permanently blocked. Since it was found that the effects of the azines were reversible in both binding and pharmacologic assays on the GPI, it has been suggested that morphine mediates its effects through a different μ receptor subtype (μ_2) in this preparation (124). More recently, the fact that naloxonazine inhibited the high affinity binding sites for [^{3}H]dihydromorphine and [^{3}H]DADLE far more potently than for the lower affinity sites prompted the proposal for a common high affinity binding site for opiates and opioid peptides (125). Naloxazone and naloxonazine have been employed as biochemical and pharmacological tools in a variety of studies to sort out different opioid receptor types and subtypes (126).

Still another mechanism that may lead to nonequilibrium binding of an unreactive ligand is enzymatic bioactivation. Because of the noncatalytic nature of the opioid receptor, such bioactivation would occur in the biophase. The reactive ligand then would covalently bind after recognition by the receptor. Rice et al have attempted the design of such a compound (127) in the synthesis of N(2,4,5-trihydroxyphenethyl)normetazocine (Figure 16). Their rationale was based on 6-hydroxydopamine, which is known to undergo facile



R
$CH_2CH{=}CH_2$
CH_2-cyclopropyl
CH_3

Figure 15

oxidation to an electrophilic intermediate that reacts covalently with catecholamine receptors. However, the normetazocine analogue was not active, possibly because of its low affinity for opioid receptors.

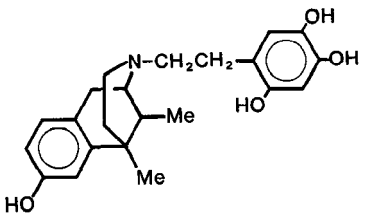


Figure 16

SUMMARY AND CONCLUSIONS

A variety of affinity labels have been synthesized as pharmacologic and biochemical probes for opioid receptors. Their usefulness as affinity labels is related to their receptor selectivity and pharmacologic characteristics. High selectivity or, better still, specificity for a single receptor type or subtype is an important feature. The potential for obtaining specific electrophilic affinity labels is greater than for other classes of affinity labels because two consecutive recognition steps lead to the amplification of selectivity (Figure 1). Indeed, of the presently available affinity labels, the electrophilic antagonist affinity labels have proved to be the most useful opioid receptor probes for pharmacologic studies. The fact that electrophilic affinity labels can be employed in studies both in vitro and in vivo represents a distinct advantage over photoaffinity labels, which can be activated only in vitro.

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