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IRREVERSIBLE OPIATE AGONISTS AND ANTAGONISTS: THE 14-HYDROXYDIHYDROMORPHINONE AZINES[1]

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Abstract

Further investigations into the molecular actions of the 14-hydroxydihydromorphinone hydrazones (naloxazone, oxymorphazone, and naltrexazone) have suggested that their irreversible actions can be explained by the formation of their azines. These azines, naloxonazine, naltrexonazine, and oxymorphonazine, irreversibly block opiate binding *in vitro* 20- to 40-fold more potently than their corresponding hydrazones, naloxazone, naltrexazone, and oxymorphazone. The blockade of binding by naloxonazine shows the same selectivity for high affinity, or μ_1, sites as naloxazone.

The advantages of irreversible drugs in both the *in vivo* and *in vitro* evaluation of opiate action have led to the recent development of a number of irreversible opiate agonists and antagonists (Caruso et al., 1979; Portoghese et al., 1978, 1979, 1980; Schultz and Goldstein, 1975; Winter and Goldstein, 1972). One compound, naloxazone, has been particularly useful in characterizing opiate receptors and opiate actions because of its ability to inhibit selectively the high affinity, or μ_1, binding sites and to block opiate analgesia with little effect on other classes of binding sites or on opiate-induced lethality (Pasternak et al., 1980a, b; Pasternak, 1980, 1981; Zhang and Pasternak, 1981). The need for high doses of naloxazone both *in vivo* and *in vitro* to inactivate these high affinity, or μ_1, binding sites raised many questions and suggested that naloxazone's activity might result from its conversion to a more active compound. In addition, the molecular structure of naloxazone did not give a clear insight into the mechanism of its irreversible actions. We now report the isolation, synthesis, and chemical characterization of a series of compounds formed from naloxazone, naltrexazone, and oxymorphazone which may be responsible for their irreversible actions both *in vitro* and *in vivo*.

Materials and Methods

[^{3}H]Dihydromophine, D-Ala^2-D-Leu^5-[^{3}H]enkephalin, D-Ala-[^{3}H]enkephalinamide, and Formula 963 scintillation fluor were purchased from New England Nuclear Corp., Inc. (Boston, MA); naloxone, naltrexone, and oxymorphone were from Endo (Garden City, NY); and naloxazone, naltrexazone, and oxymorphazone were synthesized as previously described (Pasternak and Hahn, 1980).

The azine derivatives of naloxone, naltrexone, and oxymorphone (Fig. 1) were synthesized by reacting a 3-fold molar excess of the parent alkaloid in ethanol with naloxazone, naltrexazone, and oxymorphazone, respectively. The individual reactions which were monitored by thin layer chromatography (tlc) (silica gel, $CHCl_3$:CH_3OH:NH_4OH, 90:10:1) were complete within 3 hr. After evaporation of the solvent *in vacuo*, the products were purified by preparative tlc using the above solvent system. The isolated compounds had an R_f identical with the product in the unpurified reaction mixture, suggesting that rearrangement did not occur during purification. The individual alkaloids, naloxonazine, naltrexonazine, and oxymorphonazine, were characterized further by their mass, nuclear magnetic resonance (nmr), and infrared (ir) spectra. Mass spectroscopy using chemical ionization detection showed M+1 ions at 651, 679, and 599, respectively. In the nmr spectrum, the downfield shift in the C-5 hydrogen absorption recorded for the hydrazone derivatives (Pasternak and Hahn, 1980) also was observed for the azine derivatives. The absence of a carbonyl absorption in the ir spectra of the individual compounds further confirmed reaction at C-6 in each of

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the 14-hydroxydihydromorphinone derivatives. Microanalysis (CHNO, Rockefeller University Microanalytical Laboratory) of each compound also verified the proposed structures. The absence of a free NH_2 group was indicated by a negative 2,4,6-trinitrobenzenesulfonate (TNBS) test (Pasternak and Hahn, 1980). In contrast to the parent hydrazones which produced a reddish brown color on standing with TNBS, no color change was seen with any of the azine derivatives.

Radiolabeled [^{3}H]naloxazone (4 mCi/mmol) was synthesized as previously described (Pasternak et al., 1980b). [^{3}H]Naloxonazine (2 mCi/mmol) was prepared by reacting naloxazone with [^{3}H]naloxone (2 mCi/mmol) and separating the products by thin layer chromatography as described above. The purity of all radiolabeled compounds was established by co-migration with authentic compounds on thin layer chromatography, comparing both radioactivity scans of the plates and UV absorption of the ^{3}H compounds.

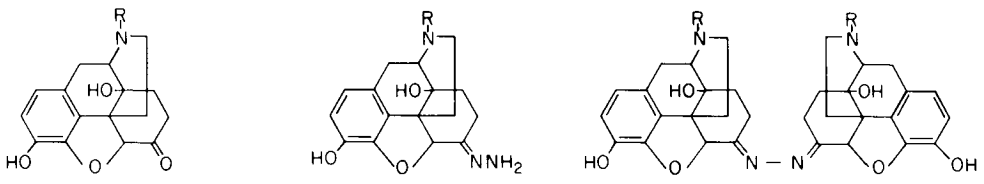
Binding experiments were performed as previously described using 20 mg/ml of tissue (Pasternak et al., 1975). In brief, brain membranes were prepared and triplicate samples were incubated with ^{3}H-labeled ligands and designated drugs for 30 min at 25°C and then filtered over Whatman GF/B filters. The filters then were counted in Formula 963 scintillation fluor. Specific binding is defined as the difference in binding in the presence and absence of levallorphan (1 μM). Irreversible inhibition of opiate receptor binding was established by the persistence of binding inhibition despite extensive washes which did reverse any inhibition by the same concentration of naloxone. Each tissue wash included incubation for 10 min at 37°C, centrifugation, and resuspension. Irreversibility experiments included four washes before binding assays were performed unless otherwise stated.

Results

Chemistry. The synthesis of monosubstituted hydrazones, such as naloxazone, oxymorphazone, and naltrexazone, may be complicated by the formation of azines (Fig. 1). To avoid this problem, a large excess of hydrazine was used in the synthesis. Analysis of the compounds, including nuclear magnetic resonance, mass and infrared spectroscopy, thin layer chromatography, CHNO analysis, and chemical titration of free —NH_2 groups with TNBS (Pasternak and Hahn, 1980), confirmed the formation of a single product and excluded the presence of an azine in each case. However, we soon discovered that solutions of the 14-hydroxydihydromorphinone hydrazones rapidly undergo a reaction, particularly in acidic aqueous solutions. Isolation and characterization of the product suggested the formation of azines (Fig. 2, *scheme I*). The direct synthesis of the various 14-hydroxydihydromorphinone azines showed that they were identical with the products formed in the various hydrazone solutions. To quantitate the extent of azine formation, we incubated 2 μM [^{3}H]naloxazone (4 mCi/mmol) at 25°C in 1% acetic acid, the conditions used in previous studies to inhibit μ_1 or high affinity sites *in vitro*. Prior to the incubation, the [^{3}H]naloxazone was pure, with no evidence of any [^{3}H]naloxonazine as determined by radioscans of thin layer chromatograms. After 15 min in the aqueous acetic acid solution, approximately 35% of the [^{3}H]naloxazone was converted to [^{3}H]naloxonazine, with the remaining 65% of the [^{3}H]naloxazone unchanged as determined by integrating the areas under the radioscan peaks. After 3 hr, the conversion to [^{3}H]naloxonazine was still 35%, implying that the conversion was complete after 15 min. Since 1 mol of naloxonazine is formed from 2 mol of naloxazone, the actual concentration of naloxonazine formed by 2 μM naloxazone under these conditions is approximately 300 nM. Presumably, the reaction does not proceed further due to the formation of free hydrazine. Hydrazine can convert naloxonazine back to naloxone (Fig. 2, the reverse of *scheme I*).

We next investigated the stability of naloxonazine in aqueous solutions (Fig. 2, *scheme II*). [^{3}H]Naloxonazine (2 mCi/mol; 1 mg/ml of 1% acetic acid) was incubated at room temperature for 60 min and then run on a thin layer chromatogram. A single peak was found which comigrated with authentic naloxonazine, suggesting little, if any, dissociation of the azine into the hydrazone and ketone under these conditions.

To investigate further whether naloxonazine is actually in equilibrium with naloxazone and naloxone (Fig. 2, *scheme III*), we incubated equimolar amounts of naloxonazine (2 mg/ml) with [^{3}H]naloxone (2 mCi/mmol; 1 mg/ml) in aqueous 1% acetic acid at room temperature for 3 hr. If the naloxonazine were truly in equilibrium with naloxazone and naloxone, some [^{3}H]naloxone should



R	14-Hydroxydihydromorphinone Series	14-Hydroxydihydromorphinone Hydrazone Series	14-Hydroxydihydromorphinone azine Series
$-CH_3$	oxymorphone	oxymorphazone	oxymorphonazine
$-CH_2-CH=CH_2$	naloxone	naloxazone	naloxonazine
$-CH_2-CH<\begin{matrix}CH_2\\ \vert \\ CH_2\end{matrix}$	naltrexone	naltrexazone	naltrexonazine

Figure 1. Structures of the 14-hydroxydihydromorphinones, their hydrazones, and their azines.

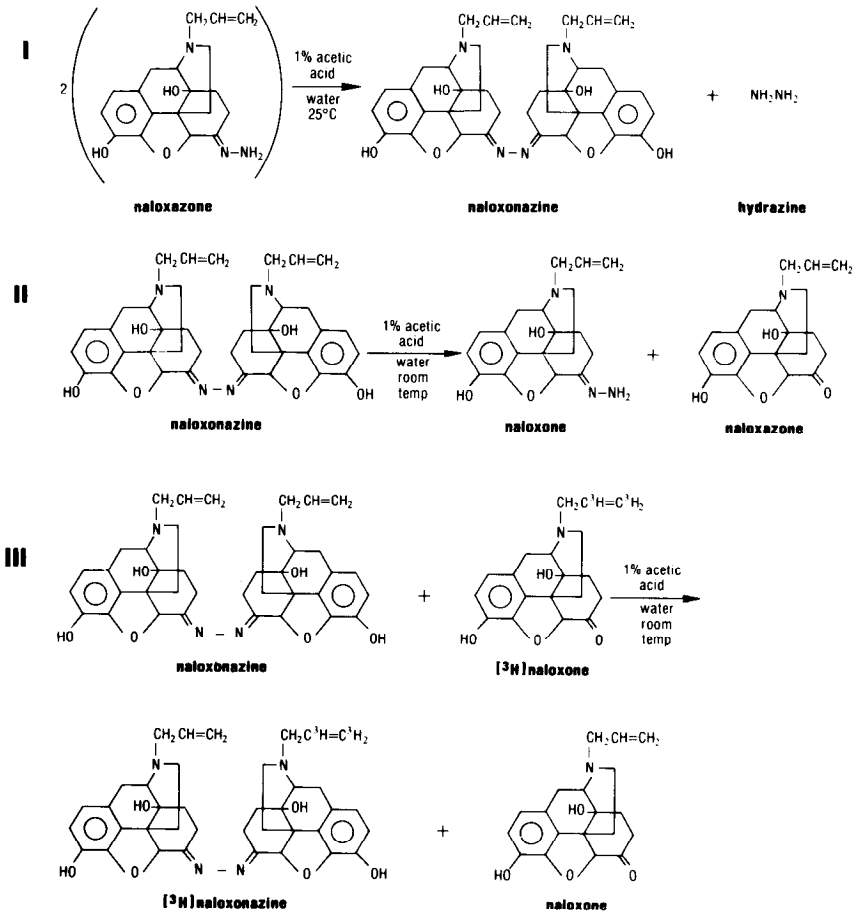


Figure 2. Reaction schemes.

be incorporated into naloxonazine. Radioscans of thin layer chromatograms of the solutions demonstrated no detectable incorporation of [^{3}H]naloxone into naloxonazine. Thus, naloxonazine appears to be quite stable under these conditions. Having isolated and established the structures and the stability of the azines, we then investigated whether they were responsible for the irreversible actions of naloxazone, naltrexazone, and oxymorphazone.

Pharmacological comparisons between the 14-hydroxydihydromorphinones, their hydrazones, and their azines. We first tested the affinity of naloxone and naltrexone, their hydrazones, and their azines for opiate receptors (Table I). Interestingly, the hydrazones and azines displace [^{3}H]dihydromorphine binding less potently than their parent ketones. In both groups, however, the azine is more potent than the hydrazone. This study does not address the question of irreversibility since the displacer was not washed away.

In an effort to determine which compounds did irreversibly bind to the receptor, we incubated membranes with the various drugs (2 μM) and then washed the free and reversibly bound drug away (Table II). Special care was taken with the hydrazones to use conditions under which little or no azine formation could be detected. Under these conditions, neither naloxazone nor oxymorphazone irreversibly inhibited the binding of either D-Ala^2-D-Leu^5-[^{3}H]enkephalin or [^{3}H]dihydromorphine. The small amount of inhibition by naltrexazone reflects the difficulty in totally eliminating azines from its solutions. Making concentrated naloxazone solutions using acetic acid to dissolve the free base as previously reported (Childers and Pasternak, 1981) results in a significant amount of azine formation (about 300 nM) and does irreversibly block the specific binding of both ^{3}H-ligands. However, the far greater potency of the azines suggests that they are the active component.

Since naloxonazine at 2 μM eliminated over 90% of [^{3}H]dihydromorphine and D-Ala^2-D-Leu^5-[^{3}H]enkephalin binding, we next examined its irreversible effects at a variety of concentrations (Fig. 3). After incubating membranes with the specified drug, the membranes were

TABLE I

Direct inhibition of [^{3}H]dihydromorphine binding by naloxone, naltrexone, and their derivatives

Rat brain homogenates were prepared as described and four concentrations of the drugs listed below were incubated with [^{3}H]dihydromorphine (0.8 nM) for 45 min and filtered. IC_{50} values were determined by least square fits of a log-probit curve. The results are the means ± SEM of three IC_{50} values determined in separate experiments.

Compound	IC_{50}
	nM
Naloxone	4.4 ± 0.7
Naloxazone	16.3 ± 4.9
Naloxonazine	5.4 ± 1.3
Naltrexone	0.77 ± 0.05
Naltrexazone	1.7 ± 0.2
Naltrexonazine	1.16 ± 0.3

TABLE II

Irreversible inhibition of receptor binding by naloxone, naltrexone, oxymorphone, and their derivations

Rat brain membranes were prepared and incubated with the above drugs (2 μM) for 30 min at 25°C and the tissue then was washed four times as described in the text to remove reversibly bound material. The results represent the means ± SEM of three separate experiments utilizing D-Ala^2-D-Leu^5-[^{3}H]enkephalin (1 nM) and [^{3}H]dihydromorphine (0.8 nM). With the exception of naloxazone and acetic acid, all drugs were dissolved in absolute ethanol immediately before dilution and addition to tissue. Under these conditions, no azine formation could be detected for naloxazone and oxymorphazone as demonstrated by thin layer chromatography. A trace amount of azine was present in the naltrexazone solutions. The naloxazone and acetic acid sample (10 mg/kg) was dissolved in water/acetic acid (1%). After 15 min, the solution was added to tissue. Under these conditions, a significant portion of the naloxazone had reacted to its azine.

Compound	Change in Binding	
	D-Ala^2-D-Leu^5-[^{3}H]Enkephalin	[^{3}H]Dihydromorphine
	%	%
Naloxone	—[a]	—
Naloxazone	15 ± 5	2 ± 11
Naloxazone + acetic acid	−48 ± 11	−41 ± 13
Naloxonazine	−93 ± 4	−89 ± 4
Naltrexone	—	—
Naltrexazone	−17 ± 19	−31 ± 26
Naltrexonazine	−97 ± 3	−88 ± 12
Oxymorphone	—	—
Oxymorphazone	3 ± 24	3 ± 21
Oxymorphonazine	−59 ± 3	−71 ± 4

[a] —, no change.

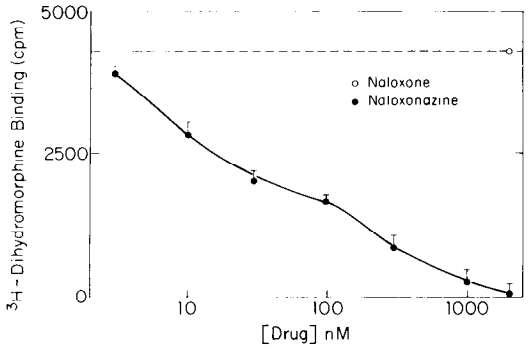


Figure 3. Irreversibility of naloxone and naloxonazine binding in rat brain membranes. Rat brain membranes were prepared and incubated with naloxone (○) or naloxonazine (●) at the stated concentration for 30 min at 25°C, then washed four times, and finally assayed with [^{3}H]dihydromorphine (1.2 nM). The results are from a single experiment which has been replicated three times.

washed extensively and a binding assay with [^{3}H]dihydromorphine was performed. As expected, naloxone at high concentrations does not inhibit binding irreversibly. Naloxonazine potently inhibits binding in a dose-dependent manner. The inhibition curve appears biphasic, suggesting more than one site with differing sensitivities to naloxonazine's irreversible actions. Although it is difficult to determine IC_{50} values accurately without computer analysis, one site appears to be quite sensitive to naloxonazine (IC_{50}, ~10 to 15 nM), while the other requires concentrations approximately 40-fold higher (IC_{50}, 400 to 500 nM).

Previous studies have demonstrated naloxazone's selective inhibition of high affinity, or μ_1, binding sites (Pasternak et al., 1980a, b; Zhang and Pasternak, 1981; Childers and Pasternak, 1981). To determine whether naloxonazine demonstrated the same selectivity described with naloxazone treatment, we incubated tissue with either naloxone or naloxonazine (50 nM), washed the tissue, and examined morphine's displacement of D-Ala^2-Met^5-[^{3}H]enkephalinamide binding (Fig. 4). Morphine has the same biphasic displacement in naloxone-treated tissue as previously published (Chang and Cuatrecasas, 1978; Zhang and Pasternak, 1981). The initial morphine displacement represents D-Ala^2-Met^5-[^{3}H]enkephalinamide binding to high affinity, or μ_1, receptors (Wolozin and Pasternak, 1981; Zhang and Pasternak, 1981). Treating the tissue with naloxonazine eliminates this initial displacement, yielding results virtually the same as those using naloxazone.

We also performed saturation studies with [^{3}H]naloxone in control tissue and tissue treated with naloxonazine (50 nM) and then washed. Previous studies using naloxazone (2 μM) clearly demonstrated the loss of the high affinity, or μ_1, binding sites. Similarly, naloxonazine treatment irreversibly inhibited the high affinity, or μ_1, sites with little effect on low affinity binding. Thus, naloxonazine has the same selectivity but far greater potency than naloxazone.

Discussion

Naloxazone has proven very useful in our understanding of opiate receptor subtypes and their correlation with analgesia (Pasternak et al., 1980a, b, 1981; Pasternak and Hahn, 1980; Zhang and Pasternak, 1981; Hazum et al., 1981; Pasternak, 1980), even though its mechanism of

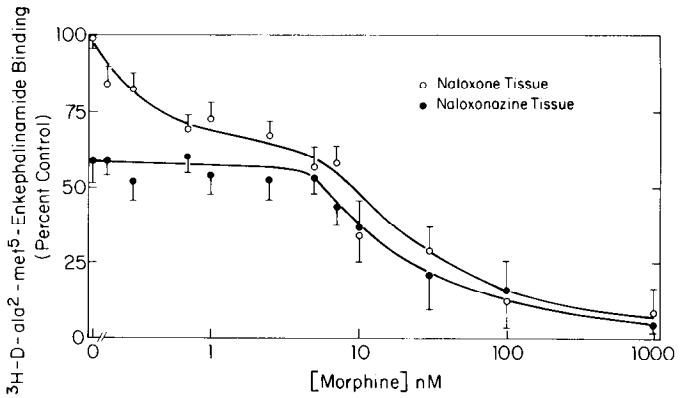


Figure 4. Morphine displacement of D-Ala^2-Met^5-[^{3}H]enkephalinamide binding in naloxone- and naloxonazine-treated tissue. Rat brain membranes were prepared and incubated with either naloxone (50 nM) or naloxonazine (50 nM) for 30 min at 25°C and washed three times. The binding of D-Ala^2-Met^5-[^{3}H]enkephalinamide (1.5 nM) then was measured in the absence and presence of various concentrations of morphine sulfate. The values are expressed as the percentage of binding in the naloxone-treated tissue in the absence of morphine and represent a single experiment which has been replicated three times.

action was not known. The high concentrations and doses required for irreversible activity suggested that it was converted to a more active component. This active compound appears to be the azine. It has the same pharmacological specificity for the μ_1, or high affinity, sites as naloxazone. Although the naloxazone used was chemically pure, it rearranged to the azine in solution, explaining, in part, the low potency of naloxazone. First, only a portion of naloxazone (about 35%) is converted to its azine and, second, the remaining unchanged naloxazone probably competitively inhibits naloxonazine binding. Thus, the irreversible blockade of opiate receptor binding requiring high doses of naloxazone (2 μM) is reproduced by low (50 nM) doses of naloxonazine.

Merely demonstrating that the azines have a very high potency does not prove that they are actually the molecular species responsible for the irreversible inhibition of binding sites. However, naloxonazine does appear to be stable chemically in our system. More important, we still do not know the mechanism of the irreversible inhibition of binding. Many irreversible compounds form covalent linkages with the binding site, and this possibility still must be considered for the azines. However, it should be stressed that irreversibility in these studies is being determined experimentally. A noncovalently bound drug which dissociates very slowly might appear to be irreversible in a practical sense. An example would be the binding of α-bungarotoxin to nicotinic acetylcholine receptors (Changeux et al., 1970). Neither possibility can be dismissed. Since the azines might be considered bifunctional molecules, they might bind to two different sites at once, greatly enhancing the affinity and decreasing the rate of dissociation. Further characterization of the mechanism of azine action is needed but will have to wait for the development of radiolabeled azines of high specific activity.

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