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Synthesis and Pharmacologic Characterization of an Alkylating Analogue (Chlornaltrexamine) of Naltrexone with Ultralong-Lasting Narcotic Antagonist Properties

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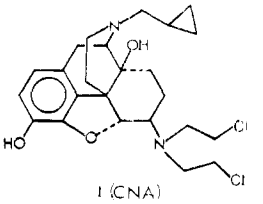
Chlornaltrexamine (CNA) produces ultralong-lasting (3–6 days) narcotic antagonism in mice and persistent stereospecific binding to rat-brain homogenate. Protection studies in mice suggest that CNA mediates its narcotic antagonist effects by interacting with the same receptors that are occupied by naloxone. A single icv dose of CNA also has been found to inhibit the development of physical dependence in mice for at least 3 days. These studies suggest that CNA exerts its sustained effects by selective covalent association with opioid receptors.

Narcotic antagonists are used extensively as pharmacologic tools for the investigation of opioid receptors.[1] Indeed, the recent research literature attests to the impact that such antagonists have made in this active research area.[2] However, the reversible nature of conventional narcotic antagonists (e.g., naloxone and naltrexone) is an inherent limitation to their utility, particularly with regard to the use of such compounds in the isolation and purification of opioid receptors. Ligands that specifically form covalent bonds with opioid receptors, therefore, would represent a major addition to the armamentarium of agents employed as investigational tools.

For this reason, considerable effort has been devoted to the design and synthesis of agents having this potential.[3-9] In this publication we describe our detailed studies concerning the first example of an alkylating agent which covalently associates with receptors which mediate narcotic antagonist activity in vivo and in vitro. We have named this ultralong-acting antagonist, chlornaltrexamine (CNA) 1.[10]



1 (CNA)

Design Considerations and Chemistry. Three factors will affect the efficiency of receptor alkylation once an affinity labeling agent[11] reaches the biophase. These are (1) the affinity of the ligand for the receptor, (2) the intrinsic chemical reactivity of the alkylating moiety, and (3) the proximity of the reactive moiety of the ligand to a receptor nucleophile in the drug-receptor complex. While criteria 1 and 2 can be met without much difficulty, criterion 3 is not easily attained because there is no information on the location of nucleophiles on or adjacent to the receptor.

Since previous studies involving the attachment of reactive moieties to the aromatic ring of the *N*-phenethyl group of anileridine[4,6] and *N*-phenethyl-3-hydroxymorphinan[9] gave inconclusive results, we decided to modify our approach by attaching the reactive moiety to a narcotic antagonist rather than an agonist. Also, the position of attachment was changed so as to explore a different receptor locus for the nucleophile.

Inasmuch as naltrexone[12] (2) is a relatively "pure" narcotic antagonist with high receptor affinity,[13] we selected it for modification. The C-6 center was chosen as the point of attachment for the bis(α-chloroethyl)amino group because a variety of substituents in this position do not destroy receptor affinity.[13-15]

CNA (1) was synthesized from naltrexone (2) by two different routes (see Scheme I). In the first route, reductive amination of 2 with $NaCNBH_3$[16] and diethanol-

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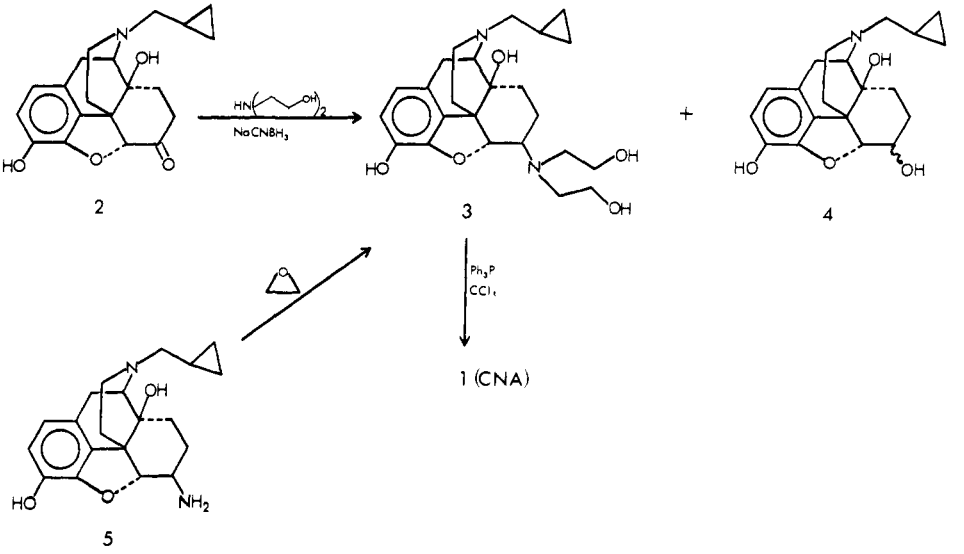
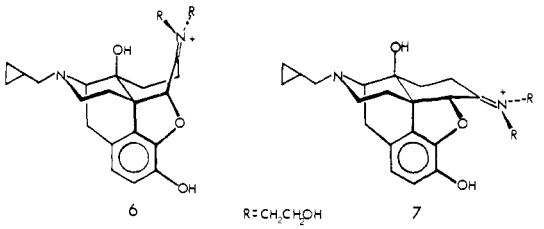
Scheme I



amine in methanol containing molecular sieves afforded the desired intermediate **3** together with the alcohol **4** in a 40:60 ratio. An optimum pH of 8.5 was determined for this reaction, and all attempts to increase the yield of **3** relative to **4** by varying the reaction conditions failed. Since the corresponding reaction with NH_3 afforded only a minor amount of **4**,[13] its preponderance in the present case is probably related to steric hindrance in the formation of the imonium intermediate in the reduction leading to **3**.

In contrast to the mixture of 6α- and 6β-amino isomers obtained in a 2:1 ratio[13] from **2** and NH_3, the corresponding reaction with diethanolamine afforded only one isomer (**3**) having the 6β configuration. The stereochemistry of **3** was determined from the NMR chemical shift and coupling constant, which are quite close to those of **5**.[13] The fact that **5** can be converted to **3** by treatment with oxirane confirms this assignment and also served as the second route to CNA.

The stereospecificity of the reduction leading to **3** can be explained in terms of a boat conformation of the C ring in the imonium intermediate **6**. This would render the



α face of ring C more accessible to hydride attack than the β face. The driving force for a preferred boat conformation **6** presumably arises from the steric hindrance between the CH_2CH_2OH group and the C-5 oxygen when the C ring is in the chair conformation **7**. There is considerable precedent for this type of steric hindrance in cyclohexane systems.[17]

In preliminary attempts to convert **3** to CNA, thionyl chloride was employed under a variety of conditions without success. Although it appeared that the primary hydroxyl groups of **3** were being replaced by chlorine, a competing elimination of the 14-OH occurred. We therefore used a modification of the procedure developed by Wiley et al.[18] which employs a mixture CCl_4 and Ph_3P. In order to minimize the reactivity of CNA formed during this reaction, the dihydrochloride salt of **3** was used.

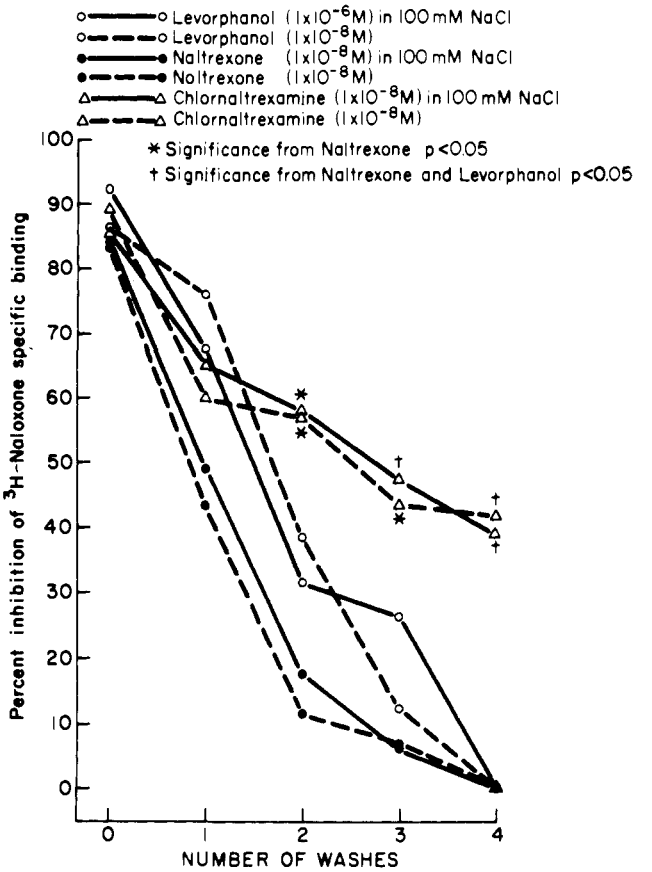


Figure 1. Inhibition of stereospecific [^{3}H]naloxone binding by levorphanol, naltrexone, and chlornaltrexamine (CNA).

The NMR coupling constant, $J_{5,6}$ = 7.5 Hz, of CNA is identical to that of it's precursor **3** and very similar to that of the corresponding primary amine **5**. This is consistent with the C ring of CNA having a flattened chair conformation,[19] and it seems likely that this distortion arises mainly from torsion introduced by the oxygen bridge.

The dihydrochloride salt of CNA is stable when refrigerated either in the solid state or in acidified ethanol solution. Chromatographic studies indicate that CNA is rapidly consumed when treated with potent nucleophiles such as iodide, thiosulfate, or azide.

Pharmacological Results. Inhibition of [^{3}H]naloxone binding by naltrexone, levorphanol, and CNA is shown in

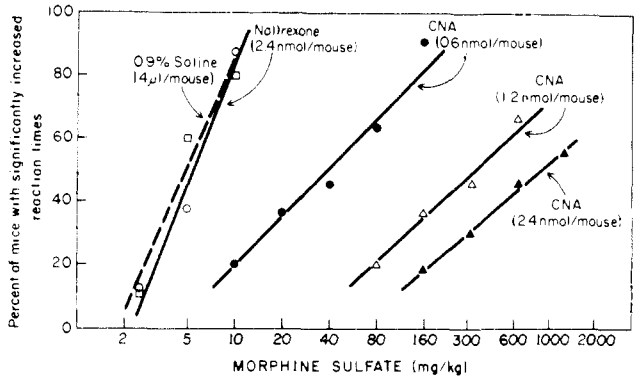


Figure 2. Dose-response curves for morphine sulfate (sc) after various doses of CNA (icv).

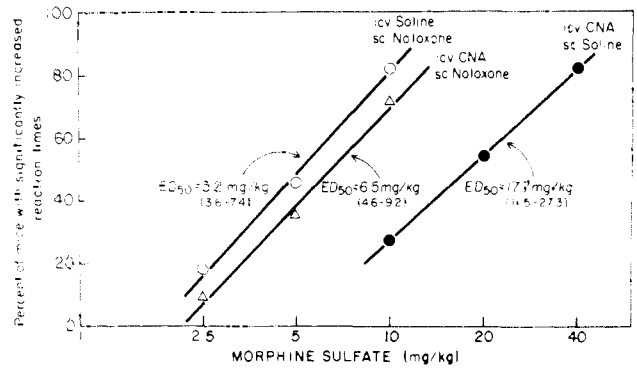


Figure 3. The effect of naloxone in blocking the antagonistic effect of CNA.

Figure 1. Brain homogenate preparations were preincubated for 5 min with a concentration of agonist or antagonist which initially inhibited about 85-90% of the [^{3}H]naloxone binding. Inhibition of [^{3}H]naloxone binding by naltrexone or levorphanol was rapidly lost following a series of washes; however, CNA continued to inhibit 40% of the binding throughout these washes. The initial percent inhibition of [^{3}H]naloxone binding caused by CNA was not significantly different from that caused by naltrexone or levorphanol. After the second wash, the CNA-treated homogenate preparation had a significantly greater ($p < 0.05$) percent of inhibition of [^{3}H]naloxone binding when compared to that of the naltrexone-treated homogenate preparation. A significant difference between levorphanol- and CNA-treated preparations was not evident until after the third wash. Neither naltrexone- nor levorphanol-treated preparations exhibited any inhibition of [^{3}H]naloxone binding after four washes, which was in contrast to the 40% inhibition of [^{3}H]naloxone binding remaining in the CNA-treated preparation after this number of washings. Further washes were not done due to the poor reproducibility of results obtained after more than four washes. There were no significant differences between the preparations containing NaCl and no NaCl with regards to the loss of inhibition of [^{3}H]naloxone binding by the washing procedure.

CNA had no analgesic effect of its own at doses of 0.6, 1.2, 2.4, and 4.8 nmol/mouse 2 h after an intracerebroventricular (icv) injection. A dose of 4.8 nmol/mouse of CNA did produce analgesia in 18% of the mice when tested 10 and 20 min after the injection. This analgesic effect was no longer apparent after 60 min. Saline injections given icv in the same volume had no effect. Chlorambucil, tested at a dose of 4.0 nmol/mouse, produced no analgesia at any time after the injection. CNA (4.8 nmol/mouse) also caused lethality in 12% of the animals, which was manifested within 1.5 h of the injection. Lethality was preceded by tonic convulsions. The only other dose with lethal effects was the 2.4 nmol/mouse dose which caused death in 2% of the animals tested.

Animals injected icv with chlorambucil (4.0 nmol/mouse) or CNA showed a lethargic behavior which increased with increasing dose. These animals retained a startle reflex to loud and abrupt auditory stimuli such as a hand clap. Locomotor activity was also decreased, but tail flick latencies were not altered in most cases.

Analgesia was measured 30 min after sc morphine injection and 2 h after icv injection of either 0.9% saline, naltrexone (2.4 nmol/mouse), chlorambucil (2.4 nmol/mouse), or CNA (0.6, 1.2, or 2.4 nmol/mouse). Two hours after icv injection of naltrexone there was no residual inhibition of morphine-induced analgesia (Figure 2), as evidenced by the similar morphine ED_{50} values of 5.0 (3.5-7.2) and 5.2 (3.0-9.1) for the saline- and naltrexone-treated animals, respectively. However, 2 h after icv injection of CNA there was a significant inhibition of morphine analgesia which was dose related. Doses of 0.6, 1.2, and 2.4 nmol/mouse increased the morphine ED_{50} to 38.5 (21.6-68.5), 330 (153-710), and 895 (398-2010) mg/kg, respectively. Slope functions of these curves were 4.84 (1.70-13.82), 6.00 (0.68-42.0), and 6.64 (0.78-56.44) for 0.6, 1.2, and 2.4 nmol/mouse doses of CNA, respectively. These slope functions were not significantly different from the saline control value of 2.02 (1.6-3.28). The slope functions did appear to increase with an increasing dose of CNA, as evidenced by the increasingly wider confidence limits of the morphine ED_{50} values and the slope functions. Chlorambucil was used as a nonspecific alkylator, and this compound had no significant effect on morphine analgesia [ED_{50} = 4.8 (3.3-6.9) mg/kg].

The inhibitory effect of CNA on morphine analgesia could be demonstrated[10] for at least 3 days. Significant inhibition of morphine analgesia was no longer present after 6 days. Inhibition after 2 h by this dose of CNA (1.2 nmol/mouse) resulted in a 66-fold increase in the morphine ED_{50}. Twenty-four hours after the injection, the ED_{50} of morphine was still increased by almost sixfold. At 72 h after the injection, a twofold increase of the morphine ED_{50} remained. Loss of inhibition seemingly followed a multiexponential curve.

Naloxone was capable of blocking the CNA inhibition of morphine-induced analgesia (Figure 3). Mice treated with saline sc and CNA icv displayed a significantly increased morphine ED_{50} of 17.7 (11.5-27.3) mg/kg 24 h after treatment when compared to the ED_{50} of 5.2 (3.6-7.4) mg/kg of animals treated with naloxone sc and saline icv. This was in contrast to the group of naloxone-treated animals given CNA icv which exhibited a morphine ED_{50} of 6.5 (4.6-9.2) mg/kg, which was not significantly different from that of icv saline-treated animals.

Naloxone ED_{50} values for the precipitation of withdrawal jumping in morphine-dependent mice were determined to see if icv injection of CNA could also inhibit the development of dependence on morphine (Table I). Simultaneous treatment of mice with morphine pellet implantation and an icv injection of naltrexone (2.4 nmol/mouse) resulted in naloxone ED_{50} values of 0.29 and 0.029 mg/kg, 24 and 72 h after treatment, respectively. These values were not significantly different from those of control animals of 0.52 and 0.033 mg/kg, 24 and 72 h after pellet implantation, respectively. Naloxone ED_{50} values of mice treated with icv CNA instead of naltrexone were significantly higher at both times after treatment; these values were 7.13 mg/kg at 24 h and 0.157 mg/kg at 72 h after treatment. Treatment of the mice with CNA and placebo

Table I. Effect of CNA on the Development of Physical Dependence to Morphine

icv treatment,[a] 2.4 nmol/mouse	pellet	time of testing, h	N^b	naloxone ED_{50} ± SE, mg/kg
saline	placebo	24 and 72	4	>100
naltrexone (2)	placebo	24 and 72	4	>100
CNA (1)	placebo	24 and 72	4	>100
saline	morphine	24	4	0.52 ± 0.09
naltrexone	morphine	24	5	0.29 ± 0.04
CNA	morphine	24	4	7.13 ± 1.68[c]
saline	morphine	72	3	0.033 ± 0.001
naltrexone	morphine	72	7	0.029 ± 0.005
CNA	morphine	72	4	0.16 ± 0.05[c]

[a] Mice were given icv injections of either saline, naltrexone (2.4 nmol/mouse), or CNA (2.4 nmol/mouse) immediately after sc implantation of either a placebo or morphine (50 mg) pellet. [b] Naloxone ED_{50} for withdrawal jumping was determined for a group of four or five mice and N represents the number of groups. [c] Significantly different from saline control ($p < 0.05$).

pellet implantation did not produce jumping in mice even with naloxone doses as high as 100 mg/kg. These animals were not different from placebo-pelleted animals treated with either saline or naltrexone icv.

Discussion

The persistent inhibition of [^{3}H]naloxone binding of CNA in the binding assay (Figure 1) is in marked contrast to that of naltrexone or levorphanol, both of which are easily removed by washing. Such sustained inhibition is consistent with specific covalent binding. The report[20] that phenoxybenzamine, at concentrations approximately four magnitudes higher than those used for CNA in these studies, does not produce significant effects on naloxone binding lends support to the idea that the avid binding of CNA is due to site-directed alkylation. Moreover, the specific, irreversible antagonist effect of CNA on the electrically stimulated guinea pig ileum[21] also supports the results of our binding studies.

CNA had no analgesic effect at doses lower than 4.8 nmol/mouse 2 h after icv injection. However, at 4.8 nmol/mouse, 18% analgesia was observed 10 and 20 min after administration, and this effect subsided within 1 h. The analgesia appears to be a specific effect of CNA, since chlorambucil, a nonspecific alkylating agent, had no effect at a comparable dose. Interestingly, naloxone and other narcotic antagonists share the ability to produce antinociception of short duration in mice.[22] Since a transient, naloxone reversible, agonist effect with CNA also has been observed[21] in the electrically stimulated guinea pig ileum, this is apparently a receptor-related event.

Narcotic antagonistic properties of CNA were demonstrated 2 h after an icv injection of 2.4 nmol/mouse (Figure 2). This group of animals had an ED_{50} of morphine that was increased by 179-fold compared to the saline-treated controls. By comparison, no antagonism was manifested 2 h after an icv injection of an equimolar dose of naltrexone or chlorambucil. Thus, the antagonism due to CNA is much longer lasting than that of naltrexone and the effect is not due to nonspecific alkylation by a nitrogen mustard compound. The antagonistic effect of CNA at doses of 0.6 and 1.2 nmol/mouse increased the morphine ED_{50} values by 7- and 66-fold, respectively, indicating a dose-related inhibition of morphine analgesia.

A nonequilibrium antagonist generally has the property of decreasing the maximum response to the agonist.[23] Due to a lack of complete inhibition by CNA of the lethal effects of morphine (preliminary study), a maximum response could not be obtained, since these responses required doses that exceeded the LD_{99} of morphine in the mice. The low dose of CNA (0.6 nmol/mouse) evidently did not promote an observable change in the maximum response to morphine (Figure 2); however, the change produced by this dose may have been missed due to its closeness to the control maximum response.

Another way to ascertain the type of antagonism produced by CNA is to study the slope functions of curves from treated animal groups and to compare these to those of saline-treated controls. Nonparallelism of the log dose–response curves would be indicative of nonequilibrium antagonism. Although the morphine dose–response curves tended to flatten with increasing doses of CNA (Figure 2), examination of these slope functions reveals that there is no significant difference between treatment and control groups. The nature of the statistical analysis is such that the flatter the curve, the wider will be the 95% confidence limits and thus obscure any differences that may be truly present. Usage of higher doses of CNA in an attempt to further antagonize morphine analgesia was not possible due to the toxic effects of the antagonist.

The fact that the concentration–effect relationship of morphine on electrically stimulated ileal strips pretreated with CNA did exhibit a reduced maximum and significantly lower slope functions[21] suggests that these effects were not readily observable in vivo due to the toxicity of morphine and CNA.

The antagonistic effect of CNA was apparent as long as 3 days after a single injection of the antagonist but could not be detected after 6 days.[10] In preliminary studies, CNA is also an effective antagonist when administered parenterally, and the effect lasts about the same duration as that after icv administration. It is interesting that the loss of inhibition of morphine analgesia with time appears to follow a multiexponential decay which can be factored into at least three components.[10] The fastest decay may be due to redistribution of CNA or its active transformation products. This also might include depletion of CNA in the brain through covalent bond formation with sites other than those that mediate the narcotic antagonist effect. The less rapid kinetic components may be a consequence of cleavage of the drug–receptor covalent bond and turnover of the alkylated receptor.

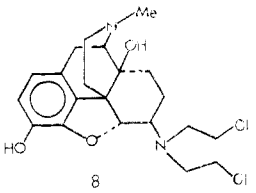
To show that CNA inhibits morphine analgesia by occupying the same sites that bind naloxone, receptor protection studies were carried out. Naloxone pretreatment was demonstrated to block the effect of icv injected CNA (1.2 nmol/mouse) on morphine analgesia (Figure 3), but the dose required was 150 mg/kg sc divided into three doses over a 30-min period. The high dose of naloxone which is required is related presumably to the nonequilibrium nature of CNA and to its higher receptor affinity (relative to naloxone) when it is in the equilibrium phase of binding.

CNA also was demonstrated to inhibit the development of dependence to morphine. Treatment of morphine-pelleted mice with CNA resulted in a higher naloxone ED_{50} for eliciting withdrawal jumping than the naltrexone- and saline-treated controls at either 24 or 72 h after pellet implantation (Table I). This inhibition increases the naloxone ED_{50} almost 14-fold at 24 h and more than five-fold at 72 h. The larger change at 24 h after pellet implantation and icv injection of CNA can be attributed to the greater effect of CNA during the first 24 h compared to that during the last 48 h.

Conclusions

The chemical and biological properties of CNA, namely, its chemical reactivity, ultralong narcotic antagonist activity, and avid binding in vitro, all strongly suggest that its sustained effects are due to covalent association with the same receptors that interact with naloxone or naltrexone.

Presumably, alkylation takes place via the aziridinium ion when the ligand is reversibly complexed with the receptor. Moreover, since the methyl analogue of CNA, chloroxymorphamine (COA) 8, exhibits a nonequilibrium



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agonist effect,[21] it is very likely a receptor alkylator as well. Thus, it appears that receptor alkylation does not alter the qualitative response seen for the reversible ligands, naltrexone and oxymorphone. This suggests the possibility that receptor occupation rather than the rate of ligand-receptor association[24] plays a more important role in the agonist effect.

The ability of both CNA and COA to exhibit nonequilibrium properties is particularly relevant to the mode of interaction[25] of agonist and antagonist ligands with receptors. Since the 14-OH group enhances the effects of agonists and antagonists,[26] it is likely that at least part of the molecular structure common to CNA and COA is oriented in a very similar or identical locus on the receptor(s). It therefore follows that the proximal nucleophile which reacts with the aziridinium group derived from CNA and COA is in a very similar or identical environment prior to receptor alkylation. Whether the nucleophiles are located on identical receptors or on similar but distinctly separate sites remains to be clarified.

Experimental Section

Melting points were determined with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by MHW Laboratories, Garden City, Mich., and were with in ±0.4% of the theoretical values. IR spectra were determined on a Perkin-Elmer 237B grating spectrophotometer and are consistent with the assigned structures. NMR data (δ) were obtained at ambient temperature using Me_4Si as internal standard using a T-60 and A-60D spectrometer. Mass spectra were obtained on an AEI MS-30 instrument. Naltrexone hydrochloride for these studies was provided by Dr. R. E. Willette of NIDA.

6β-[*N*,*N*-Bis(2-hydroxyethyl)amino]-17-cyclopropylmethyl-4,5α-epoxy-3,14-dihydroxymorphinan (3). Method a. A mixture of naltrexone hydrochloride (2.0 g, 5.3 mmol), diethanolamine (5.8 g, 55.2 mmol), and $NaBH_3CN$[16] (450 mg, 7.2 mmol) in methanol (10 mL) was adjusted to pH 8.5 with dry HCl along with thymol blue indicator and then was stirred in the presence of molecular sieves at ambient temperature for 90 h. Additional quantities of $NaBH_3CN$ were added over the time period to obtain a complete reaction. The reaction mixture was diluted with water, and the molecular sieves were removed. The aqueous mixture was made basic with excess NH_4OH and extracted (EtOAc). On removal of the solvent, the extract afforded a gummy residue (1.7 g) which contained an approximately 60:40 ratio of the alcohols[15] 4 and 3 [R_f 0.45 and 0.15, respectively; EtOAc–MeOH–NH_4OH (90:10:2); silica gel, 100 μm]. The mixture was subjected to dry column chromatography (DCC) on silica gel (200 g, Woelm for DCC), preequilibrated with 40 mL of EtOAc–MeOH–NH_4OH (90:10:4) for 2 h. The column was developed and eluted with the same solvent mixture to afford 0.970 g (44%) of 3: EIMS of base *m/e* 430 (7%, M^+), 399 (65%, $M^+ - CH_2OH$); NMR (base in $CDCl_3$) δ 6.55 (2 d, J = 8 Hz, Ar H), 6.18 (br s, C-14 OH), 5.47 (br s, –OH), 4.49 (d, J = 7.5 Hz, (C-5 H), 3.53 (br m, 4, NCH_2CH_2OH), 2.67 (br m, 4, NCH_2CH_2OH), 0.55 and 0.13 (2 m, 5, cyclopropane H). A portion of the base was converted to the dihydrochloride salt, which was crystallized from MeOH–Et_2O: mp 205–207 °C; $[\alpha]_D$ –133.4° (c 0.5, MeOH). Anal. ($C_{24}H_{36}N_2O_5Cl_2 \cdot 0.5CH_3OH$) C, H, N.

Method b. The base 5[13] which was liberated from 0.415 g (1 mmol) of its dihydrochloride salt was mixed with oxirane (5 mL, 100 mmol) and THF–CH_2Cl_2 (2 mL/0.5 mL) and was shaken in a low-pressure bomb at ambient temperature for 3 days. After the reaction mixture was chromatographed on silica gel using EtOAc–MeOH–NH_2OH (100:10:3) as eluant, there was obtained 0.332 g (77%) of 3 which is identical in all respects to the product obtained in method a.

6β-[*N*,*N*-Bis(2-chloroethyl)amino]-17-cyclopropylmethyl-4,5α-epoxy-3,14-dihydroxymorphinan (CNA, 1). This was prepared using a modification of the chlorination procedure of Wiley et al.[18] A mixture of 3·2HCl (0.252 g, 0.5 mmol) with Ph_3P (1.5 g, 5.9 mmol) and CCl_4 (46 g, 0.3 mol) in 30 mL of DMF was kept at 4 °C overnight, and the resulting solution was evaporated to dryness under reduced pressure. The residue was extracted with hot EtOAc to remove triphenylphosphine oxide, and then the base was subjected to dry column chromatography on silica gel using Et_2O–NH_4OH (98:2) as solvent. Pure CNA base (0.093 g, 42%): TLC [silica gel, 100 μm; Et_2O–NH_4OH (100:1)] R_f 0.63; NMR ($CDCl_3$) δ 6.45 (m, 2, Ar H), 5.73 (br s, 2 OH), 4.39 (d, J = 7.5 Hz, C-5 H), 3.38 (m, 4, NCH_2CH_2Cl), 2.95 (m, 4, NCH_2CH_2Cl), 0.55 and 0.13 (2 m, 5, cyclopropane H); EIMS *m/e* 468 (M^+). Treatment of the base in EtOAc with ethanolic HCl gave the dihydrochloride salt, which was isolated as a solid: mp 185–195 °C dec; $[\alpha]_D$ –126° (c 0.5, MeOH). CNA was observed to decompose rapidly as the hydrochloride salt in H_2O at room temperature but was stable for some time in the freezer as an acidified methanolic solution or dry powder. Anal. ($C_{24}H_{34}N_2O_3Cl_4$) C, H, N, Cl.

Solutions. CNA was stored in a solid form as the dihydrochloride salt at –92 °C. Stock solutions were made by dissolving the compounds in ethanol containing 0.05 N HCl in a concentration of 1 mg/mL. Purity and stability of this solution was ascertained by submitting it to TLC analysis. When the stock solution was kept at –15 °C the compound remained stable for more than a month. A working solution of CNA was made the day of the experiment by evaporating the stock solution under nitrogen and adding to the residue either saline (for icv injection) or water (for binding studies). The desired amounts of these compounds were administered icv in volumes of 4 μL in the analgesia and dependence studies. Solutions administered sc were made in saline solution such that 10 mL/kg was administered to the mice at each dose level.

Inhibition of Binding of [^{3}H]Naloxone. Inhibition of binding of [^{3}H]naloxone to putative opioid receptors by either naltrexone, levorphanol, or CNA was performed by a method similar to that of Pert and Synder,[27] as modified by Pasternak et al.[28] Slight modifications to this method have been made with the addition of a washing procedure. Two rat brains without cerebella were homogenized in 30 volumes (w/v) of cold Tris buffer (50 mM Tris-HCl, pH 7.7, at 25 °C), and the homogenate was centrifuged at 49 000*g* for 15 min. The pellets were resuspended in an equal volume of fresh buffer and incubated at 37 °C for 30 min, followed by repetition of the centrifugation and resuspension procedure. The homogenate preparation was then divided into equal parts. To one portion, enough NaCl was added to make a 100 mM NaCl solution. The prepared homogenate (either with or without NaCl) was then divided into three parts (see flow chart, Figure 4). Either test drug (agonist or antagonist), water, or levallorphan (1×10^{-6} M final concentration) was added to these three portions of homogenate in volumes that allow equal dilution of each portion. Then all homogenate mixtures were incubated at 25 °C for 5 min. Aliquots of each homogenate mixture were then tested for [^{3}H]naloxone binding. [^{3}H]Naloxone binding was determined in duplicate by incubating 2.1 mL of homogenate mixture with 0.1 mL of [^{3}H]ualoxone (final concentration of 4.3×10^{-9} M) at 25 °C for 15 min. After incubation, these mixtures were filtered under suction over glass-fiber filters (Whatman GF/B) and washed twice with 5 mL of the proper Tris buffer (with or without 100 mM NaCl), and the radioactivity was counted in

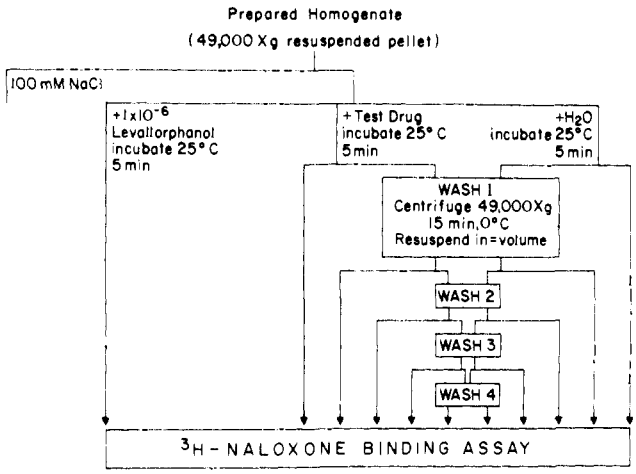

Figure 4. Flow diagram illustrating the washing sequence in the [^{3}H]naloxone binding assay. The same flow diagram was followed with preparations containing 100 mM NaCl.

15 mL of Aquasol II (New England Nuclear) by liquid scintillation spectrometry. The remaining water- and drug-homogenate mixtures were subjected to a wash procedure which consisted of centrifugation and resuspension of the pellet in buffer as described above. After wash, aliquots were again taken for the [^{3}H]naloxone binding assay. The inhibition of specific binding of [^{3}H]naloxone was calculated as follows:

$$\%\ \text{inhibition of [}^3\text{H]naloxone binding} = \frac{\left[\begin{array}{l}[^3\text{H]naloxone binding}\\ \text{in the presence}\\ \text{of drug (wash N)}\end{array}\right] - \left[\begin{array}{l}[^3\text{H]naloxone binding}\\ \text{in the presence}\\ \text{of levallorphan}\end{array}\right]}{\left[\begin{array}{l}[^3\text{H]naloxone binding}\\ \text{in the absence}\\ \text{of drug (wash N)}\end{array}\right] - \left[\begin{array}{l}[^3\text{H]naloxone binding}\\ \text{in the presence}\\ \text{of levallorphan}\end{array}\right]}$$

Analgesia. Male Swiss Webster mice (Biolab, White Bear, Minn.) weighing 20 to 25 g were used in all experiments. The tail-flick assay of D'Amour and Smith[29] which was modified[30] for mice was used to assess the analgesic potency of morphine at various times after icv injection of CNA, naltrexone, saline, or chlorambucil. Agonistic activity of CNA and chlormabucil after an icv injection was also assessed by this method. At least 24 animals were used to determine each dose–response curve and ED_{50} value. The ED_{50} values with 95% confidence limits were estimated by the method of Litchfield and Wilcoxon.[31] The inhibition by naloxone of CNA antagonism of morphine analgesia was carried out with sc injections of naloxone (50 mg/kg) at 20 and 10 min prior and 10 min after CNA (1.2 nmol/mouse) icv. Morphine analgesia was measured 24 h after the icv injection. Control animals for this experiment were given either sc naloxone and icv saline or sc saline and icv CNA. Analgesia was measured 30 min after sc injection of morphine.

Dependence. Male Swiss Webster mice weighing between 20 and 25 g were injected icv with saline, CNA, or naltrexone immediately after sc implantation of mice with 50-mg pellets of morphine.[32] Twenty-four and seventy-two hours later, the degree of dependence exhibited by these mice was assessed by the method of naloxone-precipitated withdrawal jumping described by Way et al.[32]

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