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OPIOID ACTIVITY OF A BENZOMORPHAN LACKING AN OXYGEN ATOM

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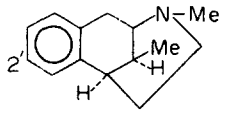
Abstract—A benzomorphan without an oxygen atom had typical opiate activity in the guinea pig myenteric plexus-longitudinal muscle preparation and in the opiate receptor binding assay. This activity was shown not to be due to an impurity or to hydroxylation during the assay procedures. Although phenolic oxygen enhances potency, an oxygen atom is not essential for interaction of an opioid agonist with the opiate receptors.

Among the potent opiates related to morphine, including those in the benzomorphan series, a phenolic hydroxyl group is frequently present. In compounds of the methadone or meperidine type, molecular models in appropriate orientation place an oxygen atom close to the position that would be occupied by phenolic *O* in morphine. The naturally occurring opioid pentapeptides containing the enkephalin sequence [Tyr-Gly-Gly-Phe-(Met) (Leu)] [1] have phenolic *O* at the appropriate distance from a basic *N* (the free α-NH_2 of tyrosine). In synthesizing a heptapeptide with typical, albeit weak, opioid activity, we argued that *O* at the position corresponding to that of phenolic *O* in morphine was essential [2].

When analgesia in a whole animal is the test for opioid activity, bioconversion of a small fraction of a non-phenolic compound to a more potent hydroxylated derivative cannot easily be ruled out, and therefore, reports of analgesic activity in compounds lacking an *O* atom can not be decisive. Inoue *et al.* [3] reported weak analgesic activity of such a benzomorphan, 2,9-β-dimethyl-6,7-benzomorphan (NIH 8933):



NIH 8933

We decided to test this compound and its 2'-hydroxylated derivative in two *in vitro* assay systems that are widely used as indicators of typical opioid activity, the guinea pig myenteric plexus-longitudinal muscle preparation and the opiate receptor binding assay [4–7]. In the myenteric plexus assay, field stimulation elicits a muscle twitch every 10 sec. Inhibition by an IC_{50} concentration of an opioid can usually be observed within 20 sec, although 1 min or more may be required to attain the full 50 per cent inhibition. Bioconversion of an inactive to an active compound is suggested by an unusually slow and progressive development of inhibition, as we have shown for β-lipotropin [8].

MATERIALS AND METHODS

The benzomorphan NIH 8933 (as the hydrochloride) and its 2'-hydroxyl derivative (as the hydrobromide) were kindly furnished by Dr. Everette L. May. Naloxone hydrochloride was a gift of Endo Laboratories. Levorphanol was generously donated by Hoffmann-La Roche, Inc. [^{3}H]naloxone (20 Ci/m-mole) was purchased from New England Nuclear, [^{3}H]etorphine (30 Ci/m-mole) from Amersham-Searle. All other reagents were Baker, reagent grade.

The guinea pig myenteric plexus-longitudinal muscle preparation was used as described by Schulz and Goldstein [5]. For testing the sensitivity of the muscle strip to acetylcholine, the electrical stimulation was interrupted, as described elsewhere [9]. The binding assay was carried out in 2.0 ml final volume, with washed membranes from guinea pig brain, [^{3}H]etorphine (2.5×10^{-9} M) or [^{3}H]naloxone (6×10^{-9} M) as primary ligand, levorphanol (10^{-6} M) as competing ligand. Incubation with the test compound was at 23° for 15 min, followed by incubation with the radioactive ligand for another 15 min. Filtration and washing on Whatman GF/C glass fiber filters was followed by standard scintillation counting. Inhibition of muscle twitch in the bioassay or of stereo-specific binding in the binding assay was plotted against log concentration of test compound on probit paper, and IC_{50} values were obtained from the resulting linear plots. Normorphine hydrochloride was used as a standard reference compound. In both assays, the slopes of the two benzomorphans were indistinguishable from that of normorphine.

RESULTS

Does NIH 8933 have typical opioid activity? Figure 1 shows typical dose–response curves for NIH 8933 and normorphine in the two assays, and

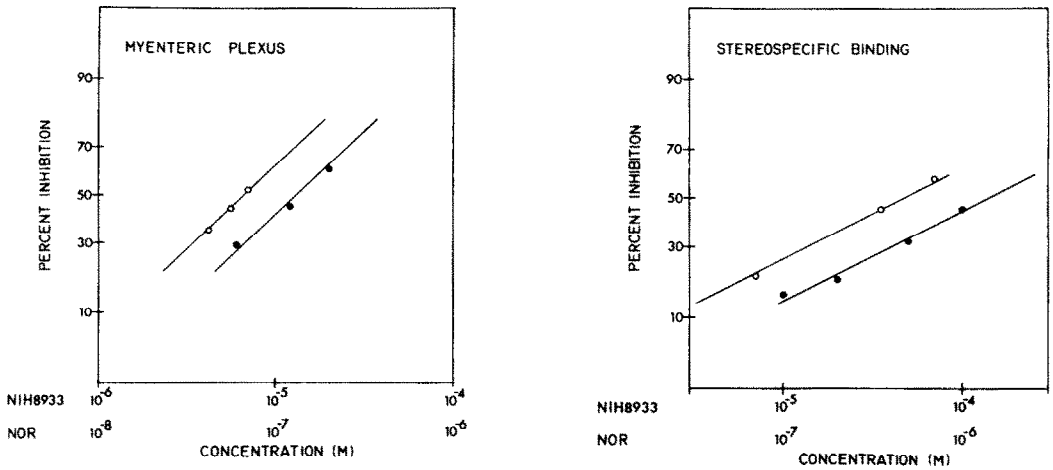


Fig. 1. Dose–response relationships in the two assays. Open symbols = normorphine; solid symbols = NIH 8933. In the binding assay, [^{3}H]etorphine was the primary ligand. For details of assay procedures, see text.

Table 1 summarizes our data on the potencies of NIH 8933 and its 2′-hydroxy derivative. The unsubstituted benzomorphan was about two hundred times less potent than normorphine and approximately 10–50 times less potent than its 2′-hydroxy derivative.

Reversal and blockade by naloxone is a criterion of typical opioid effect in bioassays. Figure 2 shows the typical inhibitory effect of NIH 8933 and the reversal (left) and blockade (right) by naloxone. Consistently, only about one-half of the total inhibition was antagonized by naloxone at concentrations that are known to completely reverse and block comparable inhibitions due to normorphine (Table 2).

In order to determine if the naloxone-insensitive inhibition was due to a direct effect on the muscle, responses to acetylcholine were measured over a wide range of concentrations, without electrical stimulation, in the absence and presence of NIH 8933 (20 μM, somewhat greater than the EC_{50}). There was a parallel shift of the acetylcholine log dose–response curve to the right, resulting in a change in the acetylcholine EC_{50} from 39 nM to 70 nM. Thus, an atropine-like effect of NIH 8933 is evident. This probably accounts for the naloxone-insensitive part of the benzomorphan inhibition.

In the opiate receptor binding assay, when an antagonist is used as primary ligand, the inhibition of binding produced by opioid agonists is typically reduced by sodium ions [10, 11]. This characteristic "sodium effect" is contributory evidence that an observed inhibition of [^{3}H]naloxone binding is a specific opiate receptor interaction. The effects of Na^+ ion on the potencies of NIH 8933, its 2′-hydroxy derivative, and normorphine are shown in Fig. 3. For all three compounds, the presence of Na^+ caused a marked parallel shift of the log dose–response curve to the right. The sodium effect with NIH 8933 was as great as that observed with the HO-benzomorphan or with normorphine. With [^{3}H]etorphine (not shown), NIH 8933 behaved identically to normorphine, both compounds displaying a very slight sodium effect, while the HO-benzomorphan showed none.

Is the opioid activity of NIH 8933 due to a potent impurity? It is evident from Table 1 that an impurity with the potency of normorphine would only need to be present at 1 part in 200 to account for all the opioid activity of NIH 8933.

NIH 8933 was purified by thin-layer chromatography on silica gel plates (Polygram SilG 0.25 mm) developed in ethanol:dioxane:benzene:ammonia (5:40:50:5). A guide strip was exposed to I_2 fumes for visualization of spots. The major spot was at R_f 0.78; a very faint spot was observed at R_f 0.65. Strips corresponding to both spots were cut from the main plate and eluted with a mixture of methanol

Table 1. Potencies of the benzomorphans in two assays of opioid activity*

	Myenteric plexus		Binding	
	IC_{50} (μM)	Relative potency (normorphine = 1)	IC_{50} (μM)	Relative potency (normorphine = 1)
NIH8933	12.2 ± 2 (5)	0.0063	83, 140	0.0043
2′-OH NIH8933	1.2	0.063	2.3	0.21

* IC_{50} = concentration producing 50 per cent inhibition of muscle twitch or of stereo-specific [^{3}H]etorphine binding. When more than two experiments were done; the number is given in parentheses. For details of the two assays, see Methods. Normorphine IC_{50} was 0.076 ± 0.005 in six experiments with the myenteric plexus preparations, 0.485 ± 0.061 in three experiments with the binding assay.

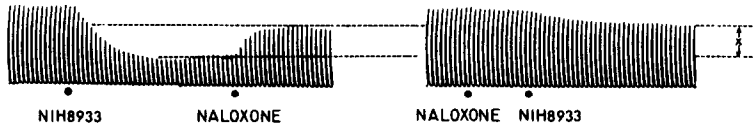


Fig. 2. Benzomorphan (NIH 8933) in the myenteric plexus bioassay. Isometric twitches of the longitudinal muscle, field stimulation every 10 sec. Naloxone reversal is shown at left, naloxone blockade (same preparation) at right. NIH 8933, 10 μM; naloxone, 0.1 μM. x indicates naloxone-reversible or naloxone-blocked inhibition. In Table 2, values of x are given as percent of total twitch amplitude.

Table 2. Naloxone reversibility of benzomorphan effect on myentric plexus preparation*

Experiment	Naloxone reversible inhibition (%)	Naloxone blocked inhibition (%)
1	45, 47	46
2	41, 44	53, 57
3	49	51
4	57	67
5	49	54

* In experiments one to five, concentrations of NIH8933 were 16, 8, 10, 10, 20 μM, respectively. Naloxone concentration was 0.1 or 0.2 μM. Method of computing naloxone reversible and naloxone blocked inhibition is given in legend to Fig. 3.

and 1 N HCl (9 : 1, v/v) and assayed in the myenteric plexus preparation. The major spot contained 93 per cent of the applied activity. We concluded that the opioid activity of NIH 8933 was probably not due, to any significant extent, to an impurity. NIH 8933, purified in this manner, was used for the following experiments.

Is the opioid activity of NIH 8933 due to bioconversion to a potent derivative? Bioconversion (e.g. hydroxylation) of a very small amount of NIH 8933 to a much more potent derivative, during the bioassay and binding assay procedures, could possibly account for the observed opioid activity.

It was noted that inhibition of the muscle twitch developed rather slowly, as compared with normorphine. If this inhibition were due to a gradual bioconversion of NIH 8933 to an active compound, a fresh strip exposed to the same bath fluid might be inhibited much more quickly. The experiment was carried out, and the rate of onset in the second strip was the same as in the first.

Next, NIH 8933, at a concentration sufficient to produce 70–80 per cent inhibition, was left in contact with the myenteric plexus preparation in four 5-ml tissue baths for 1 hr at 37°, following which the bath solutions were lyophilized, the dried powder was extracted with a mixture of ethylene dichloride and an equal volume of conc. NH_4OH. The extraction was repeated, the extracts were pooled, the aqueous phase was discarded, and the organic phase was reduced in volume under N_2. Controls consisted of NIH 8933 in bath fluid (Krebs–Ringer solution) that had previously been in contact with the tissue preparation for 1 hr and the same fluid without any added drug. The extracts were streaked on TLC plates and chromatographed as described above. Guide strips from bath fluid that contained NIH 8933 (whether or not the drug had been in contact with tissue) showed only a single spot, at R_f 0.74. Strips from the main plates were cut horizontally from the origin to the solvent front, eight in all (each 1.7 × 13 cm) so that the entire diameter of the spot on the guide strip was included in one strip of the main plate. All strips were eluted as described above, and assayed. Opioid activity was recovered quantitatively in the strip corresponding to the spot at R_f 0.74, and no other strip contained detectable activity. Moreover, the rate of onset of inhibition in the bioassay was

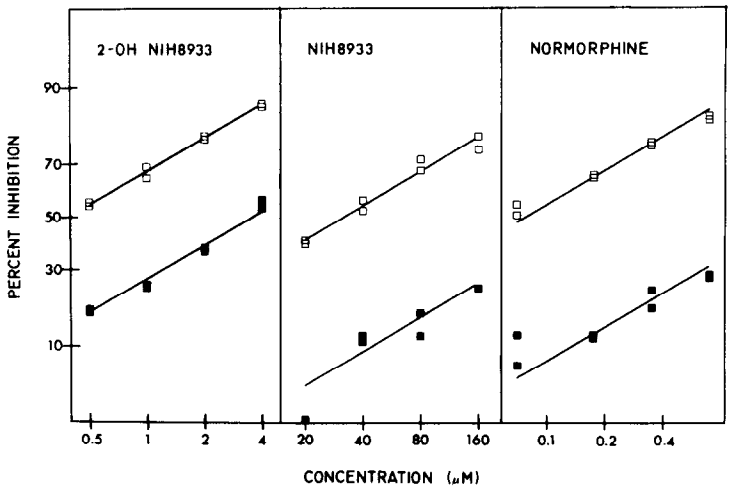


Fig. 3. Effect of sodium ion on inhibition of stereospecific [^{3}H]naloxone binding by benzomorphans. For details of assay procedure, see text. Open symbols: 100 mM Tris buffer. Solid symbols: Tris buffer + NaCl, 100 mM.

the same as observed with fresh NIH 8933. We concluded that the opioid activity of NIH 8933 could not be attributed to a bioconversion product.

To satisfy ourselves that the procedure was capable of separating the 2'-hydroxy derivative from NIH 8933 and extracting it quantitatively if it had been present, we carried out a similar experiment with the 2'-hydroxy derivative in the same bioassay preparation. The guide strip yielded a single spot at R_f 0.57, and 89 per cent of the opioid activity was recovered from a single strip of the main plate corresponding to this R_f. No opioid activity was detected in any other strip.

DISCUSSION

These results show unequivocally that NIH 8933 has typical opioid activity, and that this activity is not due to an impurity or to bioconversion to a more potent derivative. In analgesia assays, Inoue *et al.* [3] found that the 2'-hydroxy derivative of NIH 8933 was ten times more potent than the parent compound, about the same potency ratio as found in the myenteric plexus preparation here [3]. It is probable, therefore, that the analgesic effect of NIH 8933 is not due to bioconversion *in vivo*.

While these experiments were in progress, several groups reported on the potency of Phe^1-Met^5-enkephalin as compared with Met^5-enkephalin (which contains Tyr in position 1). In the myenteric plexus bioassay as well as in the binding assay, the effect of removing the phenolic hydroxyl group was to reduce potency by a factor of about 500 [12–15]. Thus, this functional group makes a much greater contribution to the receptor affinity of the peptide ligands than of the benzomorphan ligands. For both classes of ligand it is clear that a phenolic oxygen atom is not an absolute requirement for opioid activity, but it does make a major contribution to potency.

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