MOLECULAR PHARMACOLOGY, 23:629–640



# High-Affinity Dextromethorphan Binding Sites in Guinea Pig Brain

## II. Competition Experiments

GALE L. CRAVISO[1] AND JOSÉ M. MUSACCHIO[2]

*Department of Pharmacology, New York University Medical Center, New York, New York 10016*

Received August 13, 1982; Accepted December 2, 1982

SUMMARY

Binding of dextromethorphan (DM) to guinea pig brain is stereoselective, since levomethorphan is 20 times weaker than DM in competing for DM sites. In general, opiate agonists and antagonists as well as their corresponding dextrorotatory isomers are weak competitors for tritiated dextromethorphan ([$^3$H]DM) binding sites and display $IC_{50}$ values in the micromolar range. In contrast, several non-narcotic, centrally acting antitussives are inhibitory in the nanomolar range ($IC_{50}$ values for caramiphen, carbetapentane, dimethoxanate, and pipazethate are 25 nM, 9 nM, 41 nM, and 190 nM, respectively). Other antitussives, such as levopropoxyphene, chlophedianol, and fominoben, have poor affinity for DM sites whereas the antitussive noscapine enhances DM binding by increasing the affinity of DM for its central binding sites. Additional competition studies indicate that there is no correlation of DM binding with any of the known or putative neurotransmitters in the central nervous system. DM binding is also not related to tricyclic antidepressant binding sites or biogenic amine uptake sites. However, certain phenothiazine neuroleptics and typical and atypical antidepressants inhibit binding with $IC_{50}$ values in the nanomolar range. Moreover, the anticonvulsant drug diphenylhydantoin enhances DM binding in a manner similar to that of noscapine. Preliminary experiments utilizing acid extracts of brain have not demonstrated the presence of an endogenous ligand for DM sites. The binding characteristics of DM sites studied in rat and mouse brain indicate that the relative potencies of several antitussives to inhibit specific DM binding vary according to species. High-affinity, saturable, and stereoselective [$^3$H]DM binding sites are present in liver homogenates, but several differences have been found for these peripheral binding sites and those described for brain. Although the nature of central DM binding sites is not known, the potent interaction of several classes of centrally acting antitussives with DM sites suggests that they may be related to the mechanism of action of this drug.

### INTRODUCTION

Several of the findings presented in the preceding paper (1) suggest that central [$^3$H]DM[3] binding sites are not a subclass of opiate receptors. In regional distribution studies, DM sites have been found in brain regions which are relatively devoid of opiate receptors (e.g., cerebellum). Furthermore, it has been shown that the subcellular distribution patterns for opiate receptor binding and DM binding are dissimilar and that the number of DM sites is 10-fold greater than the number of opiate receptors. It has also been demonstrated that sodium does not exert a significant effect on DM binding as it does on opiate receptor binding.

The purpose of the present study was to extend our characterization of DM binding sites by examining the ability of opiate agonists and antagonists to interact with DM sites. In attempting to correlate DM binding with antitussive activity, we have also studied the effects of non-narcotic, centrally acting antitussives at DM sites. Finally, additional competition experiments have been carried out to determine whether DM binding is related to any of the known or putative central neurotransmitters or to sites labeled by other drugs with central nervous system actions.

This work was supported by United States Public Health Service Grants DA-02013 and MH-29591. It was presented by G. L. C. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology at New York University.

[1] Recipient of a Pharmaceutical Manufacturers Association Predoctoral Fellowship.

[2] Recipient of Research Scientist Award MH-17785.

[3] The abbreviations used are: DM, dextromethorphan; DPH, diphenylhydantoin; GABA, γ-aminobutyric acid; QNB, quinuclidinyl benzilate; 5-HT, serotonin.

0026-895X/83/030629-12$02.00/0

## MATERIALS AND METHODS

*[$^3$H]DM binding assay.* [$^3$H]DM binding was determined in guinea pig, rat and mouse lower brain stem homogenates using the routine binding assay method described in the preceding paper (1). For competition studies, most drugs were dissolved in water and diluted in 50 mM Tris-Cl buffer (pH 7.7) before addition to incubation samples. When ethanol was used to dissolve and dilute various drugs, the maximal concentration of ethanol in the incubation tubes did not exceed 1%. This concentration had no effect on specific or nonspecific [$^3$H]DM binding. In all studies, the concentration of [$^3$H]DM used was 4 nM, which mostly reflects binding of DM to high-affinity sites. Samples were assayed in duplicate or triplicate, and all experiments were performed at least twice. $IC_{50}$ values were determined by log-probit analysis.

*Preparation of guinea pig liver homogenates and subcellular fractions.* Fresh guinea pig livers were rinsed in ice-cold saline, minced with scissors, and homogenized with 100 volumes of 50 mM Tris-Cl buffer (pH 7.7) using a Polytron tissue disruptor (speed 5, 1 min). Binding assays were carried out as described for brain, using the equivalent of 0.5–1.0 mg of liver (wet weight) per incubation tube. [$^3$H]DM binding was also measured in subcellular fractions of guinea pig liver prepared as described by Snady and Musacchio (2).

*Preparation of brain extracts for endogenous ligand studies.* The pons-medulla and cerebellum from guinea pig brain were homogenized in 9 volumes of ice-cold 0.1 N HCl using a Polytron tissue disruptor (60 sec, full speed). The homogenate was centrifuged at 100,000 × *g* for 1 hr, and aliquots of the supernatant corresponding to 0.3 g or 0.8 g of brain (wet weight) were applied to a Sephadex G-50 column (2.49 × 40.5 cm) equilibrated in 50% acetic acid. Fractions (6 ml) were collected, and tissue equivalents of 4 mg or 11 mg were assayed for inhibition of [$^3$H]DM binding using 4 nM [$^3$H]DM and a washed homogenate of guinea pig lower brain stem (39,000 × *g*, 40 min). Incubations were carried out for 75 min at 0°, and [$^3$H]DM binding was determined as described for the routine binding assay procedure.

*Drugs.* Drugs were generously supplied by the following companies: Ayerst Laboratories, dimethoxanate, (+)- and (−)-butaclamol, (+)- and (−)-propranolol, and practolol; Boehringer Ingelheim, clonidine; Boehringer Sohn, dipyridamole; Bristol Laboratories, (+)- and (−)-butorphanol, (−)-3-methoxybutorphanol, (−)-moxazocine, and (−)-3-methoxymoxazocine; Burroughs-Wellcome, butoxamine and methoxamine; Ciba-Geigy, carbamazepine, choripramine, antistine, desipramine, imipramine, maprotiline, metoprolol, phentolamine, opipramol, terbutaline, and tolazoline; Endo Laboratories, naloxone; Fisons Corporation, noscapine; Hoffmann-La Roche, dextromethorphan, dextromethorphan analogues; levomethorphan, dextrorphan, and Ro 20-1724; Eli Lilly, nortriptyline, fluoxetine, and (+)- and (−)-propoxyphene; Mead Johnson, trazodone; Merck Sharp & Dohme, benztropine, protriptyline, amitriptyline, pyrilamine, and cyproheptadine; Pfizer Laboratories, doxepin and carbetapentane; Riker Laboratories, chlophedianol and orphenadrine; A. H. Robins, doxapram; Sandoz Pharmaceuticals, dihydroergokryptine; Schering Corporation, fluphenazine, (+)- and (−)-chlorpheniramine, trihexyphenidyl, and perphenazine; Smith Kline & French Laboratories, trimeprazine, pipazethate, metiamide, cimetidine, caramiphen, chlorpromazine, chlorpromazine sulfoxide, and prochlorperazine; E. R. Squibb & Sons, cinanserin; Sterling-Winthrop, (+)- and (−)-pentazocine, (+)- and (−)-cyclazocine; Upjohn, prostaglandins $E_1$ and $E_2$; Wyeth Laboratories, iprindole, indoramin, promazine, and promethazine. The following compounds were also generous gifts: (+)-morphine, (+)-codeine, (+)-2′-methoxymetazocine, (+)-2′-methoxyphenazocine (Dr. Arthur Jacobson, National Institutes of Health); phencyclidine, ketamine, dexoxadrol, levoxadrol (Dr. James H. Woods, University of Michigan Medical School); fominoben (Dr. Herbert Geller, Rutgers Medical School). All other drugs were obtained from commercial sources.

## RESULTS

***Effect of opiate agonists, antagonists, DM analogues, and other structurally related compounds on [$^3$H]DM binding.*** Opiate agonists and antagonists as well as their corresponding non-analgesic optical isomers were the first groups of drugs tested for their ability to compete for DM binding sites. The results of these studies are presented in Table 1, where several observations can be made.

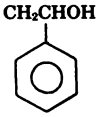
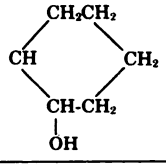
First, binding of [$^3$H]DM to brain homogenate was stereoselective. Levomethorphan, the (−)-isomer of DM,

TABLE 1

*Effect of opiate agonists, antagonists, DM analogues, and other structurally related compounds on [$^3$H]DM binding*

The inhibition of specific binding of [$^3$H]DM (4 nM) to guinea pig brain homogenate was determined with five or six concentrations of competing drugs assayed in duplicate. The mean inhibitory concentration ($IC_{50}$) values are the mean of two separate experiments. In studies investigating the effects of methionine and leucine enkephalin, binding assays were conducted for 40 min at 0° using a brain homogenate preparation which had been centrifuged at 39,000 × *g* for 40 min and resuspended in fresh 50 mM Tris-Cl buffer (pH 7.7). Under these conditions, the opiate peptides were not degraded as determined by bioassay in the guinea pig ileum preparation.

| Compound | $IC_{50}$ |
|---|---|
| | nM |
| **Opiate agonists and antagonists** | |
| Levomethorphan | 500 |
| Levorphanol | 10,000 |
| (−)-Methadone | 2000 |
| (−)-Cyclazocine | 3025 |
| (−)-Pentazocine | 5390 |
| (−)-Morphine | >10,000 |
| (−)-Codeine | >10,000 |
| (−)-Etorphine | >10,000 |
| (+)-Propoxyphene | >10,000 |
| (−)-Butorphanol | >10,000 |
| (−)-Naloxone | 4000 |
| Met-Enkephalin | >10,000 |
| Leu-Enkephalin | >10,000 |
| **Optical isomers and analogues** | |
| Dextromethorphan | 25 |
| Dextrorphan | 2500 |
| (+)-Methadone | 2100 |
| (+)-Cyclazocine | 1325 |
| (+)-Pentazocine | 585 |
| (+)-Morphine | >10,000 |
| (+)-Codeine | >10,000 |
| (−)-Propoxyphene | >10,000 |
| (+)-Butorphanol | >10,000 |
| (+)-2′-methoxymetazocine | 135 |
| (+)-2′-methoxyphenazocine | 65 |
| (−)-3-Methoxybutorphanol | 400 |
| (−)-Moxazocine | >10,000 |
| (−)-3-Methoxymoxazocine | 2000 |

| DM analogues | R group[a] | $IC_{50}$ |
|---|---|---|
| Ro 21-6713 | $CH_2CH_2CN$ | 775 |
| Ro 21-6830 | $CH_2COCH_3$ | 420 |
| Ro 21-6952 | $CH_2CCH_3$ | 27 |
| Ro 21-7212 | $CH_2CHOH$<br>$CH_3$ | 30 |
| Ro 21-7214 | $CH_2CHOH$  | 235 |
| Ro 21-7215 |  | 105 |

[a] Represents substitution on the tertiary nitrogen atom.

displayed an $IC_{50}$ value (500 nM) 20-fold greater than the $IC_{50}$ value for DM.

Second, opiate agonists and antagonists, in general, were weak competitors for [$^3$H]DM binding sites and displayed $IC_{50}$ values in the micromolar range. Although codeine closely resembles the structure of DM, it was clearly not a potent inhibitor of binding. The butorphanol analogue (−)-3-methoxybutorphanol was the most potent displacing agent ($IC_{50}$ = 400 nM) of all the levorotatory compounds tested. The enkephalin pentapeptides had little effect on binding at 10 μM.

Third, DM binding was not potently inhibited by the majority of the dextrorotatory isomers. Dextrorphan was 100-fold less potent than DM, and (+)-codeine was without significant effect at 10 μM; levopropoxyphene, a non-analgesic compound reported to have antitussive activity in both animals and humans (3), was also a poor inhibitor of binding. The (+)-isomers of benzomorphans displayed slightly higher affinity for DM binding sites, since (+)-pentazocine and (+)-cyclazocine had $IC_{50}$ values of 600 and 1300 nM, respectively. Moreover, the ability of (+)-benzomorphans to inhibit specific [$^3$H]DM binding was greatly enhanced when these compounds were converted to their corresponding methyl ethers; (+)-2′-methoxyphenazocine and (+)-2′-methoxymetazocine had $IC_{50}$ values of 65 and 135 nM, respectively.

Fourth, replacement of the methyl group on the tertiary nitrogen of DM by various chemical substituents could alter the affinity of DM for DM binding sites. Two analogues exhibited affinities similar to those of the parent compound, whereas in all of the other cases affinity for DM sites was greatly decreased.

*Effect of non-narcotic antitussives on [$^3$H]DM binding.* The structures of the more important and most commonly used, clinically effective, centrally acting non-narcotic antitussives are shown in Fig. 1. In contrast to the results obtained with opiate analgesics, several of these drugs inhibited specific [$^3$H]DM binding, with $IC_{50}$ values in the nanomolar range (Fig. 2). The phenylcyclopentylalkylamine antitussives caramiphen and carbetapentane had $IC_{50}$ values of 25 nM and 9 nM, respectively, whereas the $IC_{50}$ values for the phenothiazine antitussives dimethoxanate and pipazethate were 41 nM and 190 nM, respectively. Although the structures of these compounds are quite unrelated to the morphinan structure of DM, their displacement curves were parallel to that of DM, suggesting a competitive interaction with DM binding sites. Scatchard analysis of specific [$^3$H]DM binding in the presence of these drugs, as illustrated in Fig. 2 for caramiphen, also indicated that the interaction with DM binding sites is competitive in nature since only the apparent dissociation constant ($K_d$) was decreased and not the maximal number of binding sites ($B_{max}$).

Not all of the non-narcotic antitussives examined were potent inhibitors of specific [$^3$H]DM binding. Chlophedianol, a phenylalkylamine, had an $IC_{50}$ value of 1300 nM, and levopropoxyphene, as mentioned previously, had no significant effect on binding at 10 μM. Fominoben, at 10 μM, decreased binding by only 20%. However, an interesting finding was that the phthalideisoquinoline antitussive, noscapine, increased the high-affinity component of specific DM binding to brain homogenate. The effect was concentration-dependent, with the maximal enhancement of binding (30–50%) at 5–10 μM noscapine; at higher noscapine concentrations, the increase of binding was inhibited (Fig. 3). It was also a time-dependent effect and reached a maximum after 2–2.5 hr at 0°. The enhanced binding was not an artifact of the filtration procedure,

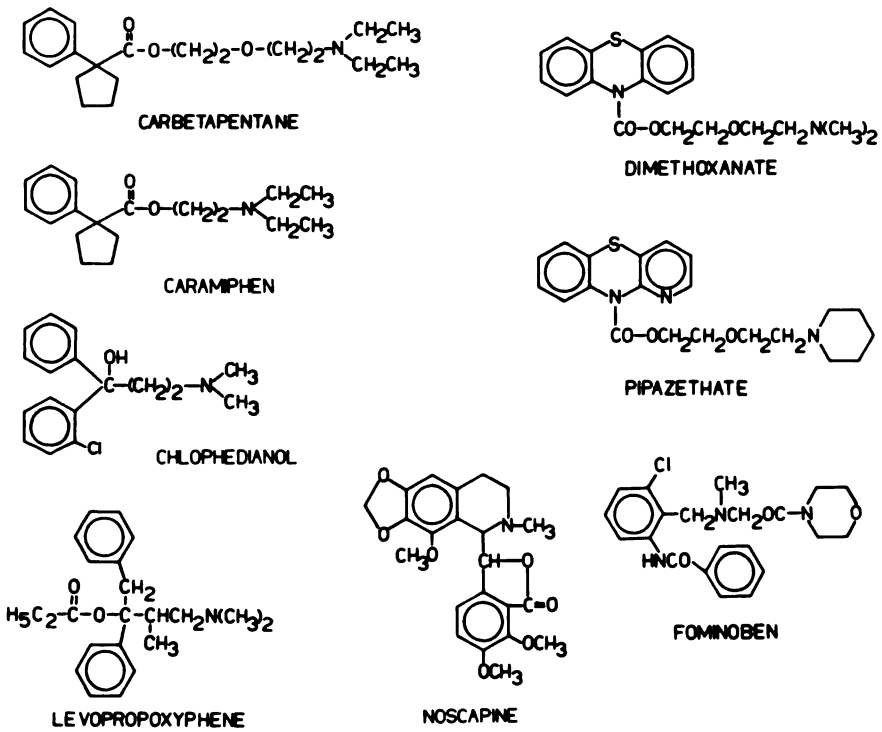



FIG. 1. *Non-narcotic antitussives*

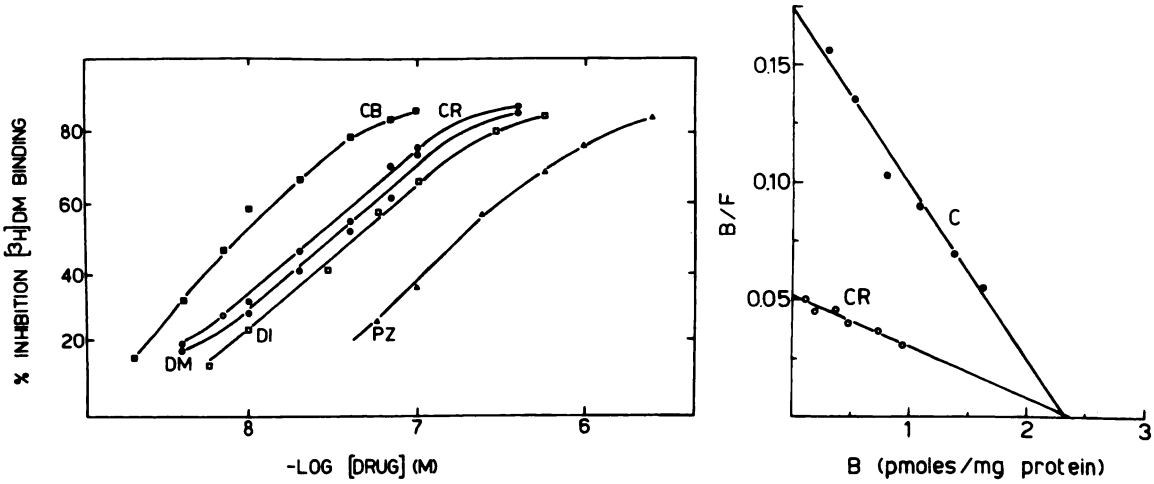



FIG. 2. *Inhibition of specific [$^3$H]DM binding to guinea pig brain stem homogenate by non-narcotic antitussives*

Left, Brain stem homogenate was incubated with 4 nM [$^3$H]DM and increasing concentrations of carbetapentane (■), caramiphen (○), dimethoxanate (□), pipazethate (▲), and unlabeled DM (●) under standard assay conditions. The results shown are representative of two experiments performed in duplicate. Right, Scatchard analysis of [$^3$H]DM binding in the presence and absence of caramiphen. Brain stem homogenate was incubated with increasing concentrations of [$^3$H]DM (2–30 nM) in the absence (●) and presence (○) of $2 \times 10^{-8}$ M caramiphen. Values were obtained from triplicate determinations. Control (no drug): $K_d$ = 13.32 nM; $B_{max}$ = 2.31 pmoles/mg of protein. In the presence of caramiphen: $K_d$ = 47.24 nM; $B_{max}$ = 2.44 pmoles/mg of protein.

since the amount of [$^3$H]DM bound to the glass-fiber filters was the same in the presence or absence of noscapine. Nonspecific tissue binding was also not altered by noscapine. By conducting various centrifugation and dilution experiments, it was also determined that the elevated levels of DM binding were not due to a change in the free radioligand concentration. For example, if noscapine were reducing nonspecific adsorption or low-affinity binding of [$^3$H]DM to the tissue during the incubation, there would be an increased concentration of free [$^3$H]DM, thus leading to greater amounts of DM bound at the nonsaturating concentrations of [$^3$H]DM used in the binding assay.

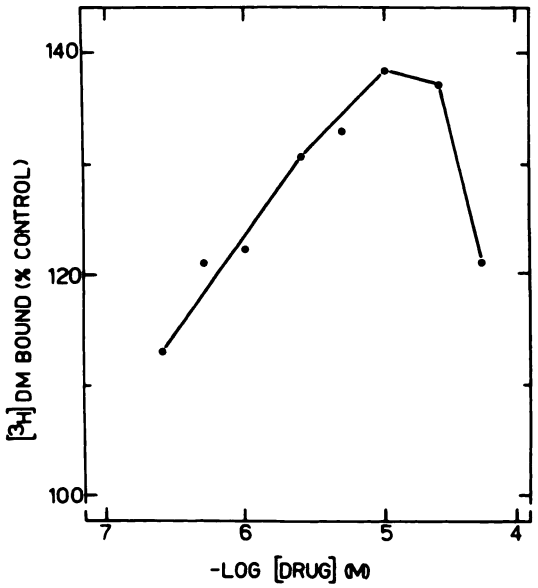



FIG. 3. *Enhancement of specific [$^3$H]DM binding to guinea pig brain stem homogenate by noscapine*

Increasing concentrations of noscapine were incubated with brain homogenate and 4 nM [$^3$H]DM under standard assay conditions. The results shown are representative of two experiments performed in duplicate.

When [$^3$H]DM saturation binding studies were carried out in the presence of noscapine, Scatchard analysis of the data indicated that the affinity of DM for DM binding sites was increased while the number of DM sites was not altered (Fig. 4). The Hill coefficients ($n_H$) in the presence and absence of noscapine were 1.02 and 1.00, respectively. As Fig. 4 illustrates, noscapine increased the affinity of DM by significantly decreasing the dissociation rate of DM. This effect on the DM off-rate did not produce any irreversible [$^3$H]DM binding since increasing the temperature from 0° to 25° during the dissociation reaction allowed a complete displacement of all remaining bound DM. Moreover, the effects of noscapine were reversible; when brain homogenate was preincubated with 10 μM noscapine and then washed by centrifugation and resuspension in drug-free buffer, binding of [$^3$H]DM was the same as in control samples in which brain homogenate was preincubated without noscapine and subjected to the same washing procedure. In subcellular fractionation studies, increases in specific [$^3$H]DM binding by noscapine were seen almost exclusively in the microsomal fraction, the same fraction in which high-affinity [$^3$H]DM binding had been localized. Monovalent or divalent ions did not affect the enhancement of [$^3$H]DM binding by noscapine.

Figure 5 illustrates the structural formulae for several noscapine-related compounds. Even though these substances have weak or negligible antitussive activity (3), it was interesting to determine what effect they have on DM binding. As the results in Table 2 demonstrate, the ability to increase DM binding was not a property common to all of these drugs. Hydrastine, a close analogue of noscapine, was the only other compound which increased

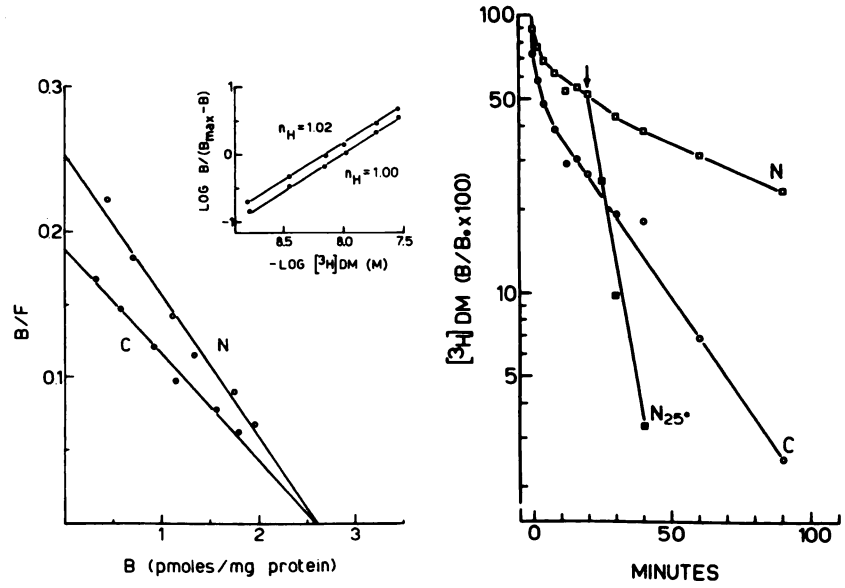



FIG. 4. *Effect of noscapine on [³H]DM binding*

Left, Scatchard analysis of [³H]DM binding in the presence and absence of noscapine. Brain stem homogenate was incubated with increasing concentrations of [³H]DM (2–30 nM) in the absence (●) and presence (○) of 5 μM noscapine. Values were obtained from triplicate determinations and are representative of two experiments. Control (no drug): $K_d$ = 13.72 nM; $B_{max}$ = 2.62 pmoles/mg of protein. In the presence of noscapine: $K_d$ = 10.37 nM; $B_{max}$ = 2.62 pmoles/mg of protein. Inset, Hill plot of the same data. Right, Dissociation of specific [³H]DM binding in the presence and absence of noscapine. Unlabeled DM (10 μM) was added to samples incubated to equilibrium and the dissociation of [³H]DM was determined at 0° in the presence (□) and absence (○) of 5 μM noscapine. At the *arrow*, the incubation temperature was increased to 25°. The data shown are representative of two experiments.

binding. However, the enhancement was only 10–20% at 10 μM. Narceine and papaverine have little effect on DM binding, and hydrocotarnine was inhibitory in the concentration range of 0.1–10 μM.

*Effect of central neurotransmitter receptor ligands and other centrally active drugs on [³H]DM binding.* Competition studies using agonists and antagonists specific for known and putative neurotransmitter receptor-binding sites in the central nervous system (Table 3) indicated that there was no correlation of DM binding with muscarinic and nicotinic cholinergic receptors, *alpha-* and *beta*-noradrenergic receptors, 5-HT receptors, dopamine, or histamine receptors. DM binding was also not related to tricyclic antidepressant binding sites or amine uptake sites. However, it was clearly evident from our results that various drugs competed for DM binding

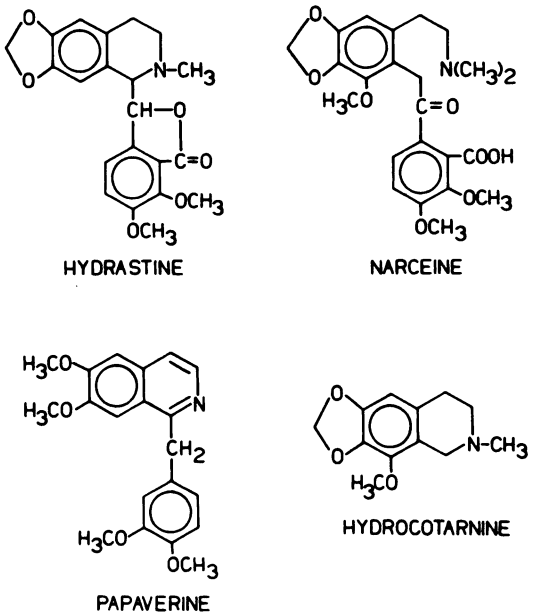



FIG. 5. *Compounds related to noscapine*

TABLE 2

*Effect of isoquinoline alkaloids and related substances on specific [³H]DM binding*

Specific [³H]DM binding was measured as described under Materials and Methods using 4 mM [³H]DM and the appropriate drug at the concentration indicated. Each value represents the mean of two separate experiments performed in duplicate.

| Drug | Concentration | [³H]DM binding |
|---|---|---|
| | μM | % of control |
| Noscapine | 0.1 | 109 |
| | 1.0 | 130 |
| | 10.0 | 137 |
| Narceine | 0.1 | 96 |
| | 1.0 | 99 |
| | 10.0 | 93 |
| Hydrastine | 0.1 | 104 |
| | 1.0 | 99 |
| | 10.0 | 128 |
| Papaverine | 0.1 | 100 |
| | 1.0 | 92 |
| | 10.0 | 74 |
| Hydrocotarnine | 0.1 | 87 |
| | 1.0 | 58 |
| | 10.0 | 23 |

TABLE 3

*Effect of central neurotransmitter receptor ligands on [$^3$H]DM binding*

The inhibition of specific binding of [$^3$H]DM (4 mM) was determined with five or six concentrations of competing drugs assayed in duplicate. The mean inhibitory concentration ($IC_{50}$) values were determined by log-probit analysis and are the mean of two separate experiments. The $IC_{50}$ or $K_i$ value of each drug for its respective neurotransmitter receptor site is included for comparison. NE means no inhibition was observed at the highest concentration tested, which was 100 μM for histamine and 10 μM for all other drugs.

| Drug | [$^3$H]DM $IC_{50}$ | [$^3$H]Ligand $IC_{50}$ or $K_i$ | |
|---|---|---|---|
| | *nM* | *nM* | |
| α-Noradrenergic receptor | | [$^3$H]WB-4101[a] | [$^3$H]Clonidine[b] |
| Clonidine | >10,000 | 620 | 5.7 |
| Methoxamine | >10,000 | 11,000 | 940 |
| Dihydroergokryptine | >10,000 | 2.4 | 7 |
| Yohimbine | >10,000 | 480 | 150 |
| Tolazoline | >10,000 | 2,100 | 180 |
| Phentolamine | >10,000 | 3.6 | 22 |
| Indoramin | 4,000 | 5.9 | 6,500 |
| β-Noradrenergic receptor | | [$^3$H]Dihydroalprenolol[c] | |
| Isoproterenol | NE | 46 | |
| Terbutaline | NE | >10,000 | |
| *l*-Propranolol | 135 | 2.1 | |
| *d*-Propranolol | 145 | 400 | |
| Metoprolol | 2,000 | — | |
| Practolol | NE | 880 | |
| Butoxamine | 1450 | — | |
| Nicotinic receptor | | [$^3$H]Nicotine[d] | |
| Carbachol | NE | 2,400 | |
| DMPP | 6,000 | 1,800 | |
| Lobeline | 1,500 | 300 | |
| *d*-Tubocurarine | NE | 150,000 | |
| Muscarinic receptor | | [$^3$H]QNB[e] | |
| Atropine | 5,000 | 0.4 | |
| Scopolamine | >10,000 | 0.3 | |
| Trihexyphenidyl | 1,000 | 0.6 | |
| Benztropine | 36 | 1.5 | |
| Oxotremorine | >10,000 | — | |
| Dopamine receptor | | [$^3$H]Dopamine[f] | [$^3$H]Haloperidol[g] |
| Apomorphine | >10,000 | 8.6 | 51 |
| (+)-Butaclamol | 1,000 | 80 | 0.54 |
| (−)-Butaclamol | >10,000 | >10,000 | 700 |
| Chlorpromazine | 35 | 900 | 10.2 |
| Perphenazine | 3 | — | — |
| Fluphenazine | 7 | 230 | 0.88 |
| Trifluoperazine | 12 | 740 | 2.1 |
| Prochlorperazine | 20 | — | — |
| Thioridazine | 100 | 1,800 | 15 |
| α-Flupenthixol | 25 | 180 | 0.98 |
| Haloperidol | 90 | 920 | 1.4 |
| Spiroperidol | 285 | 1,400 | 0.25 |
| Chlorpromazine sulfoxide | >10,000 | — | — |
| 5-HT receptor | | [$^3$H]5-HT[h] | [$^3$H]Spiroperidol[i] |
| 5-HT | >10,000 | 3.8 | 2,700 |
| 5-Methoxy-tryptamine | >10,000 | 11 | 2,700 |
| Methysergide | >10,000 | 88 | 2.6 |
| Cyproheptadine | 620 | 1,500 | 2.0 |
| Cinanserin | 76 | 1,800 | 18 |
| Spiroperidol | 285 | 730 | 0.5 |
| Histamine receptor | | [$^3$H]Mepyramine[j] | |
| Diphenhydramine | 990 | 17 | |
| Pyrilamine | 300 | 4.5 | |
| Antistine | 2,250 | 610 | |
| d-Chlorpheniramine | 1,600 | 8 | |
| l-Chlorpheniramine | 1,125 | 700 | |
| Tripelennamine | 510 | 35 | |
| Trimeprazine | 54 | 1.3 | |
| Promethazine | 290 | 2.9 | |
| Histamine | NE | 25,000–40,000 | |
| Metiamide | NE | 100,000 | |

TABLE 3—*continued*

| Drug | [$^3$H]DM $IC_{50}$ | [$^3$H]Ligand $IC_{50}$ or $K_i$ | |
|---|---|---|---|
| | *nM* | *nM* | |
| Cimetidine | NE | — | |
| Antidepressant binding sites | | [$^3$H]Imipramine[k] | [$^3$H]Desipramine[l] |
| Imipramine | 100 | 7 | 360 |
| Desipramine | 250 | 18 | 30 |
| Protriptyline | 50 | 20 | — |
| Chloripramine | 70 | 25 | 1,400 |
| Amitriptyline | 47 | 25 | — |
| Nortriptyline | 120 | — | — |
| Iprindole | 28 | 5,500 | 17,000 |
| Doxepin | 95 | 300 | 1,000 |
| Orphenadrine | 160 | — | — |
| Opipramol | 7 | — | — |
| Maprotiline | 100 | — | — |
| Mianserin | 2,000 | 20,000 | 2,250 |
| Trazodone | >10,000 | — | — |
| Cocaine | 5,000 | 900 | — |
| Fluoxetine | 135 | 200 | — |

[a,b] $K_i$ values from U'Prichard *et al.* (4) for inhibition of [$^3$H]WB-4101 and [$^3$H]clonidine binding, respectively.
[c] $IC_{50}$ values from Bylund and Snyder (5) for inhibition of [$^3$H]dihydroalprenolol binding.
[d] $IC_{50}$ values from Romano and Goldstein (6) for inhibition of [$^3$H]nicotine binding.
[e] $IC_{50}$ values from Snyder and Yamamura (7) for inhibition of [$^3$H]QNB binding.
[f,g] $K_i$ values from Burt *et al.* (8) for inhibition of [$^3$H]dopamine and [$^3$H]haloperidol binding, respectively.
[h,i] $K_i$ values from Peroutka and Snyder (9) for inhibition of [$^3$H]5-HT and [$^3$H]spiroperidol binding, respectively.
[j] $K_i$ values from Tran *et al.* (10) for inhibition of [$^3$H]mepyramine binding.
[k] $IC_{50}$ values from Raisman *et al.* (11) for inhibition of [$^3$H]imipramine binding.
[l] $IC_{50}$ values from Lee and Snyder (12) for inhibition of [$^3$H]desipramine binding.

sites with $IC_{50}$ values in the nanomolar range. Several phenothiazine neuroleptics (perphenazine, fluphenazine, trifluoperazine, prochlorperazine, thioridazine, and chlorpromazine) were very potent inhibitors of [$^3$H]DM binding, having $IC_{50}$ values considerably less than or equal to the $IC_{50}$ value for DM. Various typical and atypical antidepressants (imipramine, protriptyline, chloripramine, amitriptyline, nortriptyline, doxepin, iprindole, opipramol, fluoxetine, and maprotiline) also exhibited significant affinity for DM binding sites. Other drugs acting as potent displacers of [$^3$H]DM binding are *d*- and *l*-propranolol, benztropine, α-flupenthixol, haloperidol, cinanserin, and trimeprazine. The displacement curves for most of these drugs were parallel to the displacement curve for DM, and Scatchard analysis of DM binding in the presence of several of these drugs indicated that the interaction with DM binding sites was competitive in nature (data not shown).

Table 4 lists additional drugs which, when used in competition studies, were found to be poor displacers of [$^3$H]DM binding. Included are the putative neurotransmitters GABA, glycine, and glutamate as well as other compounds for which specific central nervous system binding sites have been identified (for example, diazepam and phencyclidine).

*Effect of DPH on [$^3$H]DM binding.* During the competition studies we found that the anticonvulsant DPH, in the concentration range of 10–100 μM, enhanced DM binding (Fig. 6), producing an increase of 50–80% at 100 μM. The effect was specific for DPH and was not produced by the 5-*p*-hydroxy metabolite of DPH or by other anticonvulsants such as clonazepam, diazepam, carbamazepine, and sodium phenobarbital. The enhancement of DM binding produced by DPH resembled that produced by noscapine: DPH increased the affinity of DM by significantly decreasing the DM dissociation rate (Fig. 7); the number of DM binding sites was not changed. The Hill coefficients ($n_H$) for [$^3$H]DM binding in the presence and absence of DPH were 0.96 and 1.03, respectively. Also like those of noscapine, the effects of DPH were completely reversible and, in subcellular fractionation studies, were observed almost exclusively in the microsomal fraction. Monovalent and divalent ions generally did not affect the magnitude of the enhancement of DM binding by DPH. However, on several occasions we have observed antagonism by calcium, but these results have not been consistently obtained. DPH did not alter nonspecific binding or binding of [$^3$H]DM to the glass-fiber filters. It also did not increase the concentration of free [$^3$H]DM.

When [$^3$H]DM binding was measured in the presence

TABLE 4

*Miscellaneous compounds which do not significantly alter specific [$^3$H]DM binding to brain homogenate*

The following compounds have $IC_{50}$ values greater than 1 μM or else produce no effect on specific [$^3$H]DM binding at concentrations as high as 100 μM.

| | | |
|---|---|---|
| D-Amphetamine | GABA | Prostaglandins $E_1$ and $E_2$ |
| Bicuculline | Hemicholinium-3 | Phencyclidine |
| Carbamazepine | Hexamethonium | Pargyline |
| Choline | Histidine | Phenobarbital |
| Clonazepam | Iproniazid | Phenylethylamine |
| Dexoxadrol | Ketamine | Procaine |
| Diazepam | Levoxadrol | Quinidine |
| Dipyridamole | Mecamylamine | Ro 20-1724 |
| Doxapram | Melatonin | Taurine |
| Eserine | Neostigmine | Tetraethylammonium chloride |
| Glutamate | Octopamine | Theophylline |
| Glycine | Ouabain | |

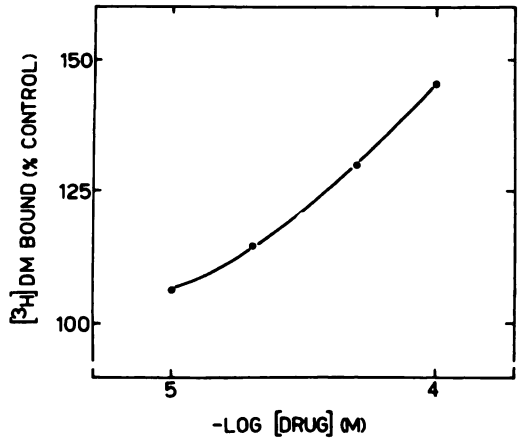



FIG. 6. *Enhancement of specific [$^3$H]DM binding to guinea pig brain stem homogenate by DPH*

Increasing concentrations of DPH were incubated with brain homogenate and 4 nM [$^3$H]DM under standard assay conditions. The results shown are representative of two experiments performed in duplicate.

of both DPH and noscapine, the enhanced binding equaled that produced by DPH alone, indicating that the effects of both drugs were not additive. This result would be expected since noscapine and DPH alter the same kinetic parameter of DM binding.

*[$^3$H]DM binding in rat and mouse brain.* [$^3$H]DM binds with high affinity to brain homogenate of rat and mouse. The $K_d$ calculated for specific [$^3$H]DM binding in rat and mouse were 7 nM and 8 nM, respectively, values similar to that described for guinea pig brain. In addition, [$^3$H]DM binding was stereoselective and could be displaced by drugs which are potent inhibitors of DM binding in guinea pig brain. However, considerable species differences were observed for the $IC_{50}$ values of several drugs. As the results in Table 5 demonstrate, caramiphen had the same relative inhibitory potency as DM in rat and guinea pig but was 15 times more potent than DM in mouse, having an $IC_{50}$ value of 0.8 nM. In mouse and guinea pig, carbetapentane had a greater inhibitory potency than DM but was less potent than DM in rat. Dextrorphan was a rather poor competitor for DM binding sites in rat and guinea pig but was considerably more potent in mouse, since the $IC_{50}$ value was only 10-fold greater than that of DM as compared with 100-fold and 400-fold in guinea pig and rat, respectively. Unlike the effects seen in guinea pig brain, binding of [$^3$H]DM in rat and mouse was only minimally enhanced by DPH (5–20%), whereas noscapine neither enhanced nor inhibited specific DM binding.

*Search for an endogenous ligand for DM binding sites.* Since we did not find a correlation of DM binding with any of the known or putative neurotransmitters, we tried to detect an endogenous substance from brain which might interact with DM binding sites. As described under Materials and Methods, an acid extract of guinea pig brain was fractionated on Sephadex G-50 and aliquots of each fraction were tested for inhibitory activity in the DM binding assay. The results from these experiments demonstrated that there was no discrete peak of inhibitory activity eluting from the column. Instead, we found

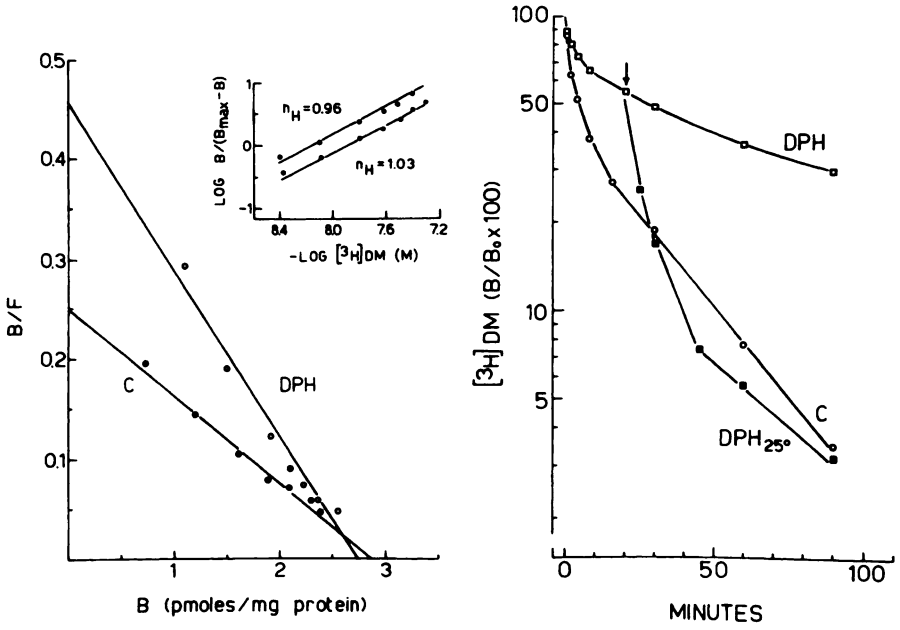



FIG. 7. *Effect of DPH on [$^3$H]DM binding*

Left, Scatchard analysis of [$^3$H]DM binding in the presence and absence of DPH. Brain stem homogenate was incubated with increasing concentrations of [$^3$H]DM (4–50 nM) in the absence (●) and presence (○) of 0.1 mM DPH. Values were obtained from duplicate determinations and are representative of two experiments. Control (no drug): $K_d$ = 11.74 nM; $B_{max}$ = 2.91 pmoles/mg of protein. In the presence of DPH: $K_d$ = 5.92 nM; $B_{max}$ = 2.70 pmoles/mg of protein. Inset, Hill plot of the same data. Right, Dissociation of specific [$^3$H]DM binding in the presence and absence of DPH. 10 μM unlabeled DM was added to samples incubated to equilibrium and the dissociation of [$^3$H]DM was determined at 0° in the presence (□) and absence (○) of 0.1 mM DPH. At the *arrow*, the incubation temperature was increased to 25°. The data shown are representative of two experiments.

TABLE 5

*Inhibition of [$^3$H]DM binding in guinea pig, rat, and mouse brain by various drugs*

The inhibition of specific binding of [$^3$H]DM (4 nM) was determined with five or six concentrations of the competing drugs. All samples were assayed in duplicate. The mean inhibitory concentration ($IC_{50}$) values were determined by log-probit analysis and are the mean of two separate experiments.

| Drug | $IC_{50}$ | | |
|---|---|---|---|
| | Guinea pig | Rat | Mouse |
| | | *n*M | |
| DM | 25 | 12 | 12 |
| Levomethorphan | 500 | 200 | 400 |
| Dextrorphan | 2500 | 5000 | 140 |
| Caramiphen | 25 | 12 | 0.8 |
| Carbetapentane | 9 | 19 | 3 |
| Dimethoxanate | 41 | 60 | 45 |
| Perphenazine | 3 | 3 | 5 |
| Iprindole | 28 | 70 | 50 |

that all fractions inhibited DM binding by 30–40% and that the magnitude of this inhibition was the same whether tissue extract equivalent to 4 or 11 mg of brain (wet weight) was assayed.

*Competition studies of [$^3$H]DM binding in liver homogenates.* Binding of [$^3$H]DM displaceable by 10 μM unlabeled DM has been detected in homogenates of peripheral organs of guinea pig such as liver, kidney, and spleen. We have investigated DM binding sites in liver and have found that they are saturable, stereoselective, and display high affinity for DM ($K_d$ = 17 nM). However, competition studies indicated that the DM binding sites in liver are different from those in brain (Table 6). Although the phenothiazine antitussive dimethoxanate had the same inhibitory potency for liver and brain DM binding sites, the phenylcyclopentylalkylamine antitussives caramiphen and carbetapentane, potent inhibitors of DM binding in brain, were poor inhibitors of DM binding in liver. We also found that noscapine and DPH, drugs which enhance DM binding in brain, did not increase DM binding in liver. In fact, noscapine inhibited [$^3$H]DM binding to liver homogenates at the same concentration in which it maximally increased binding of [$^3$H]DM to brain homogenates.

TABLE 6

*Effect of various drugs on [$^3$H]DM binding in guinea pig brain and liver homogenates*

Specific [$^3$H]DM binding was determined in brain and liver homogenates in the presence and absence of the various drugs indicated. The results were obtained from duplicate determinations and are typical of at least two experiments.

| Drug | Concentration | [$^3$H]DM binding | |
|---|---|---|---|
| | | Brain | Liver |
| | μM | *% of control* | |
| DM | 0.01 | 59 | 60 |
| | 0.10 | 24 | 14 |
| Carbetapentane | 0.10 | 20 | 90 |
| Caramiphen | 0.10 | 30 | 81 |
| Dimethoxanate | 0.10 | 28 | 33 |
| Noscapine | 5.00 | 133 | 75 |
| DPH | 100.00 | 154 | 100 |

A subcellular fractionation study of [$^3$H]DM binding in liver indicated that the high-affinity DM binding component was present in the microsomal fraction. We have also observed that incubation of [$^3$H]DM with liver microsomes in the presence of NADPH results in almost complete degradation of the radioligand (1). These findings may indicate that DM binding sites in liver represent sites of drug metabolism.

## DISCUSSION

One of the most important conclusions of this study is that DM binding sites in brain do not comprise a nonstereospecific subclass of opiate receptors. The results of the competition experiments have clearly demonstrated that opiate agonists and antagonists do not potently displace [$^3$H]DM binding. Thus, our findings are consistent with the observation that the antitussive effects of DM are not naloxone-reversible (13) and are probably elicited at sites other than opiate receptors. We have ruled out a naloxone-insensitive, nonspecific interaction of DM at opiate receptors since we did not detect a population of [$^3$H]dihydromorphine binding sites in guinea pig lower brain stem membranes that was inhibited by low concentrations of DM or its *O*-demethylated metabolite dextrorphan.[4]

Concerning the specificity of [$^3$H]DM binding in relation to antitussive activity, we have demonstrated that two classes of non-narcotic antitussives, namely the phenylcyclopentylalkylamines (caramiphen and carbetapentane) and the phenothiazines (dimethoxanate and pipazethate), are competitive inhibitors of binding and display high affinity for DM sites. Moreover, we have found that, in the guinea pig, the centrally acting antitussive noscapine enhances binding. The fact that the effects of caramiphen, carbetapentane, and noscapine on DM binding in liver homogenates are not the same as those in brain indicates that the peripheral and central nervous system sites are different. Since DM is rapidly degraded by liver microsomes, it is possible that high-affinity DM binding in liver may represent sites for drug-metabolizing enzymes.

It is difficult to establish a correlation between antitussive potency and affinity for DM binding sites. In humans, cough is a highly variable response which does not lend itself easily to the controlled conditions necessary for accurately evaluating antitussive agents. This applies not only to cough of pathological origin but also to cough that is artificially induced in healthy volunteers. As a result, the data derived from clinical studies does not provide a reliable index of cough-suppressant activity for the various non-narcotic agents. An additional problem is the lack of information concerning the absorption, distribution, and metabolism of the different antitussive drugs. Since cough suppressants are administered orally, these factors are of major importance if antitussive activity is to be correlated with a direct biochemical measurement such as binding affinity. Regarding animal studies, there is no ideal testing model which serves as a bioassay for measuring antitussive potency (14). Moreover, despite the multitude of studies reporting the cough-suppressant efficacy of DM and other centrally acting, non-narcotic

[4] G. L. Craviso and J. M. Musacchio, unpublished observation.

antitussives, differences in testing methods and experimental conditions have not allowed meaningful quantitative comparisons to be made. These differences and how they influence the evaluation of antitussive activity have been reviewed (14, 15) and include such factors as the choice of species, the route of administration of the antitussive, the state of the animal (conscious or unconscious), the choice of stimulus to initiate the cough response, and the method by which cough production is measured.

The results of the competition studies using the optical isomers of opiate analgesics indicate that DM binding sites display some structural specificity. Replacement of the methoxy group in DM with a hydroxyl group, as seen in dextrorphan, results in a 100-fold decrease in affinity for DM sites. (+)-Codeine has a negligible effect at DM sites, probably due to the presence of the alcoholic group on ring C or the oxygen bridge linking rings A and C; (+)-cyclazocine has only a micromolar affinity for DM sites. These findings are of interest since antitussive activity has been reported for each of these compounds (ref. 16 and literature citations in ref. 1). However, the fact that such structurally similar compounds have poor affinity for DM sites makes it difficult to understand why caramiphen and carbetapentane, compounds which appear to bear no resemblance to the rigid morphinan structure of DM, should act as potent competitive inhibitors of DM binding. The interaction of the phenylcyclopentylalkylamines and phenothiazines with DM sites could perhaps be better understood on the basis of their 3-dimensional configurations and not their 2-dimensional structural formulae.

Although our results have shown that all centrally acting antitussives do not interact with DM binding sites, this finding might be expected. First, the cough center itself is not confined to a highly localized area or discrete structure within the lower brain stem. Rather, it is an intricate neuronal network (17) parts of which have been mapped in several regions of the pons and medulla (18). Because of the complexity of the neuronal network comprising the cough center, Bucher (17) has suggested that there are many points where the cough reflex can be interrupted. Therefore, one cannot assume that there is only one mechanism to suppress cough and that all centrally acting antitussives have the same qualitative effects. Second, if one considers the chemical diversity of the compounds reported to suppress cough by a central mechanism, it seems unlikely that such a variety of drugs acts at only one site and through a common mechanism. Although some drugs may produce their antitussive effects by the same mode of action as DM, others may not. The antitussive action of codeine and other opiate analgesics are reversed by narcotic antagonists (13, 19), indicating that these drugs mediate their effects via opiate receptors. Fominoben displaces [$^3$H]flunitrazepam binding in brain, and it is thought that the cough-suppressant activity of this drug is due to an interaction with central benzodiazepine receptors (20).

Noscapine is a non-narcotic benzylisoquinoline alkaloid of opium which, apart from its antitussive properties, has no other known pharmacological actions in man. It is structurally related to bicuculline but lacks affinity for GABA receptors since it does not have the proper stereoconfiguration (21). The effect of noscapine on DM binding demonstrates a different type of interaction by a non-narcotic antitussive, namely, an increase in affinity of DM for its central binding sites. Kinetic studies have shown that the enhanced affinity is the result of a decrease in the dissociation rate of DM and that both the slowly and rapidly dissociating components of DM binding are affected. In all probability, such an effect could be attributed to an allosteric mechanism which induces a conformational change at or near the DM binding site. It could be speculated that noscapine replaces an endogenous modulator of binding that was lost during the homogenization procedure or else that it dissociates an endogenous factor which is inhibitory to DM binding. However, the latter possibility seems unlikely since the effects of noscapine are reversed after membranes are washed free of the drug. Of the noscapine-related compounds that were studied (including bicuculline), only hydrastine enhanced DM binding, thus demonstrating that there is some structural specificity with respect to this effect on DM binding. The finding that noscapine enhances DM binding in guinea pig while having no effect in mouse and rat is another example of the species differences that have been observed for DM sites. It might be recalled that the affinities of caramiphen and carbetapentane are significantly different in each species.

While it is clear that [$^3$H]DM binding sites are not a subclass of opiate receptors, it is also clear that the binding characteristics of [$^3$H]DM do not correspond to the putative neurotransmitters that we have tested. Because of the nanomolar affinity of caramiphen and carbetapentane for DM sites and the fact that these drugs have significant atropine-like activity (3), we considered whether DM sites may be associated with central muscarinic receptors. This is most likely not the case since, with the exception of benztropine, binding of [$^3$H]DM is not potently inhibited by other muscarinic agents such as atropine, scopolamine, trihexiphenidyl, and oxotremorine.

Even though most of the neuroleptic phenothiazines we examined are potent competitors for DM binding sites, binding of [$^3$H]DM does not appear to be related to dopamine receptor sites. Therefore, it can be assumed that the interaction of these phenothiazine drugs with DM sites is the result of their similarity in structure to the antitussives pipazethate and dimethoxanate. It is noteworthy that cough-suppressant activity has been reported in animals and humans for several phenothiazines, including the neuroleptics chlorpromazine, perphenazine, prochlorperazine, and thioridazine as well as the antihistamines trimeprazine and promethazine (3, 22). Moreover, chlorpromazine is the drug of choice for intractable hiccough. After studying the antitussive effects of various phenothiazines in guinea pigs, Bossier and Pagny (23) concluded that the ability of these drugs to suppress cough was due to their central nervous system-depressant effects rather than to a specific antitussive effect. However, the phenothiazines dimethoxanate and pipazethate are antitussive but do not have central nervous system-depressant effects (3). In addition, Kasé *et al.* (24) have found that by introducing certain chem-

ical groups, such as a piperidino moiety, into the side chain of phenothiazines, it is possible to enhance antitussive activity but not general central nervous system depression. These findings favor the suggestion that there is some structural basis for the cough-suppressant activity of phenothiazines, rather than a strictly nonspecific central nervous system-depressant effect. Our findings demonstrate the importance of the phenothiazine nucleus at DM sites, since the sulfoxide derivative of chlorpromazine is 1000 times less effective than chlorpromazine as an inhibitor of binding. It is more likely that general central nervous system depression is responsible for the antitussive effects of sedatives and hypnotics, such as hexobarbital and phenobarbital (25, 26).

Several other drugs, including most of the antidepressants investigated, are almost as potent as the phenothiazines at DM binding sites. To our knowledge none of these compounds has ever been tested for antitussive activity, and therefore we cannot readily interpret our binding data regarding these drugs.

The enhancement of DM binding by DPH is similar in many of its characteristics to that described for noscapine and is probably due to a conformational change which results in an increased affinity of [$^3$H]DM for DM sites. Tallman and Gallager (27) have reported that DPH increases the affinity of [$^3$H]diazepam for central benzodiazepine receptors, but the lack of any correlation between DM sites and benzodiazepine receptors makes it unlikely that the effects we have observed are related to those described for benzodiazepine binding. In trying to gain some insight into the nature or significance of the effect of DPH at DM sites, it should be noted that the concentration range in which DPH increases binding (10–100 $\mu$M) falls within that found for plasma and brain levels of the drug during anticonvulsant therapy (28). Thus, it might prove useful to determine whether or not DM is anticonvulsant; anticonvulsant activity has been reported for caramiphen (3). Moreover, in view of the fact that the actions of DPH at DM sites resemble so closely those of noscapine, it would be interesting to test whether DPH has antitussive properties.

As we have indicated in the preceding paper (1), DM has central nervous system effects in addition to antitussive activity. Of particular interest are the subjective effects in man, which resemble certain aspects of the sensory disturbances produced by the mixed narcotic agonist-antagonist cyclazocine. Drug discrimination experiments in animals have demonstrated that DM shares some of the features of the discriminative properties of cyclazocine as well as the dissociative anesthetic phencyclidine. For example, in squirrel monkeys trained to discriminate cyclazocine from saline, DM substitutes for cyclazocine and produces drug-appropriate responding (29). Likewise, pigeons trained to discriminate phencyclidine from saline generalize to DM (30). One could interpret these findings to mean that there is a common site of action for the discriminative effects of these drugs. However, our binding data do not support this suggestion, since cyclazocine and phencyclidine are not potent displacers of [$^3$H]DM binding.

In summary, the results reported here have shown that DM binding sites in brain are distinct from opiate receptors and are also not associated with receptor binding sites for several putative central neurotransmitters. The fact that several classes of non-narcotic antitussives interact with DM binding sites suggests that [$^3$H]DM may be labeling sites which are relevant to its pharmacological activity. However, since the mode of action of DM and other centrally acting antitussives is virtually unknown, further studies are necessary before this can be established with greater certainty. A more detailed investigation of the inhibition of binding by neuroleptic phenothiazines and various antidepressants as well as the interactions with DPH may provide the necessary clues to identify the nature of DM sites.

## REFERENCES


1. Craviso, G. L., and J. M. Musacchio. High-affinity dextromethorphan binding sites in guinea pig brain. I. Initial characterization. *Mol. Pharmacol.* **23**:619–628 (1983).
2. Snady, H., and J. M. Musacchio. Quinonoid dihydropterin reductase. II. Regional and subcellular distribution of rat brain enzyme. *Biochem. Pharmacol.* **27**:1947–1953 (1978).
3. Eddy, N. B., H. Friebel, K.-J. Hahn, and H. Halbach. Codeine and its alternatives for pain and cough relief. 4. Potential alternatives for cough relief. *Bull. WHO* **40**:639–719 (1969).
4. U'Prichard, D. C., D. A. Greenberg, and S. H. Snyder. Binding characteristics of a radiolabeled agonist and antagonist at central nervous system alpha-noradrenergic receptors. *Mol. Pharmacol.* **13**:454–473 (1977).
5. Bylund, D. B., and S. H. Snyder. *Beta* adrenergic receptor binding in membrane preparations from mammalian brain. *Mol. Pharmacol.* **12**:568–580 (1976).
6. Romano, C., and A. Goldstein. Stereospecific nicotine receptors on rat brain membranes. *Science (Wash. D. C.)* **210**:647–650 (1980).
7. Snyder, S. H., and H. I. Yamamura. Antidepressants and the muscarinic acetylcholine receptor. *Arch. Gen. Psychiatry* **34**:236–239 (1977).
8. Burt, D. R., I. Creese, and S. H. Snyder. Properties of [$^3$H]haloperidol and [$^3$H]dopamine binding associated with dopamine receptors in calf brain membranes. *Mol. Pharmacol.* **12**:800–812 (1976).
9. Peroutka, S. J., and S. H. Snyder. Multiple serotonin receptors: differential binding of [$^3$H]5-hydroxytryptamine, [$^3$H]lysergic acid diethylamide, and [$^3$H] spiroperidol. *Mol. Pharmacol.* **16**:687–699 (1979).
10. Tran, V. T., R. S. L. Chang, and S. H. Snyder. Histamine $H_1$ receptors identified in mammalian brain membranes with [$^3$H]mepyramine. *Proc. Natl. Acad. Sci. U. S. A.* **75**:6290–6294 (1978).
11. Raisman, R., M. Briley, and S. Z. Langer. Specific tricyclic antidepressant binding sites in rat brain. *Nature (Lond.)* **281**:148–150 (1979).
12. Lee, C.-M., and S. H. Snyder. Norepinephrine neuronal uptake binding sites in rat brain membranes labeled with [$^3$H]desipramine. *Proc. Natl. Acad. Sci. U. S. A.* **78**:5250–5254 (1981).
13. Cavanagh, R. L., J. A. Gylys, and M. E. Bierwagen. Antitussive properties of butorphanol. *Arch. Int. Pharmacodyn. Ther.* **220**:258–268 (1976).
14. Kasé, Y. Evaluation of antitussive agents, in *Selected Pharmacological Testing Methods*, Vol. 3 (A. Burger, ed.). Marcel Dekker, New York, 363–419, (1968).
15. Eddy, N. B., H. Friebel, K.-J. Hahn, and H. Halbach. Codeine and its alternatives for pain and cough relief. 3. The antitussive action of codeine—mechanism, methodology and evaluation. *Bull. WHO* **40**:425–454 (1969).
16. Chau, T. T., F. E. Carter, and L. S. Harris. Antitussive effects of ketocyclazocine, (−)cyclazocine and (+)cyclazocine in the cat. *Pharmacologist* **24**:230 (1982).
17. Bucher, K. Antitussive drugs. *Physiol. Pharmacol.* **11**:175–200 (1965).
18. Kasé, Y. Antitussive agents and their sites of action. *Trends Pharmacol. Sci.* **1**:237–239 (1980).
19. Kito, G., Y. Kasé, and T. Miyata. A cough-like respiratory response induced by electrical stimulation of the amygdaloid complex in the cat. *Arch. Int. Pharmacodyn. Ther.* **227**:82–92 (1977).
20. Antoniadis, A., W. E. Muller, and U. Wollert. Central nervous system stimulating and depressing drugs as possible ligands of the benzodiazepine receptor. *Neuropharmacology* **19**:121–124 (1980).
21. Enna, S. J., J. F. Collins, and S. H. Snyder. Stereospecificity and structure-activity requirements of GABA receptor binding in rat brain. *Brain Res.* **124**:185–190 (1977).
22. Salem, H., and D. M. Aviado. Phenothiazines and nervous system depressants, in *International Encyclopedia of Pharmacology and Therapeutics*, Section 27: Antitussive agents, Vol. 2 (H. Salem and D. M. Aviado, eds.). Pergamon Press, Oxford, 535–582 (1970).
23. Bossier, J. R., and J. Pagny. Action antitussive de quelques phenothiazines. *Therapie* **15**:97–102 (1960).

24. Kasé, Y., T. Yuizono, and M. Muto. Piperidino groups in antitussive activity. *J. Med. Chem.* **6**:118–122 (1963).
25. Tedeschi, R. E., D. H. Tedeschi, J. T. Hitchens, L. Cook, P. A. Mattis, and E. J. Fellows. A new antitussive method involving mechanical stimulation in unanesthetized dogs. *J. Pharmacol. Exp. Ther.* **126**:338–344 (1959).
26. Hahn, K. J., and H. Friebel. Wirkungen hustenhemmender Pharmaka im zentralen Anteil der Hustenreflexbahn. *Med. Pharmacol. Exp.* **14**:87–97 (1966).
27. Tallman, J. F., and D. W. Gallager. Modulation of benzodiazepine binding site sensitivity. *Pharmacol. Biochem. Behav.* **10**:809–813 (1979).
28. Houghton, G. W., A. Richens, P. A. Toseland, S. Davidson, and M. A. Falconer. Brain concentrations of phenytoin, phenobarbitone and primidone in epileptic patients. *Eur. J. Clin. Pharmacol.* **9**:73–78 (1975).
29. Teal, J. J., and S. G. Holtzman. Stereoselectivity of the stimulus effects of morphine and cyclazocine in the squirrel monkey. *J. Pharmacol. Exp. Ther.* **215**:369–376 (1980).
30. Herling, S., G. D. Winger, E. H. Coale, D. W. Hein, and J. H. Woods. Similarity of the discriminative stimulus effects of dextrorphan, ketamine and cyclazocine in the pigeon. *Fed. Proc.* **39**:994 (1980).

**Send reprint requests to:** Dr. José M. Musacchio, Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, N. Y. 10016.