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# Isothiocyanate Derivatives of Cocaine: Irreversible Inhibition of Ligand Binding at the Dopamine Transporter

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## SUMMARY

Isothiocyanate derivatives of (−)-cocaine were prepared and tested for inhibitory potency at the cocaine receptor in rat striatal membranes. Coincubation with *m*-isothiocyanatobenzoylecgonine methyl ester (*m*-ISOCOC), *p*-isothiocyanatobenzoylecgonine methyl ester (*p*-ISOCOC), and 3β-(4-isothiocyanatophenyl)tropane-2-carboxylic acid methyl ester (ISOWIN) resulted in inhibition of [$^3$H]WIN 35,428 binding, but the compounds were about 10-fold weaker than (−)-cocaine. However, *p*-ISOCOC was approximately 3-fold more potent than metaphit, an isothiocyanate derivative of phencyclidine. *p*-ISOCOC was equipotent at the serotonin transporter but was much less potent at the norepinephrine transporter and was inactive at the $D_2$ dopamine receptor at 1000 μM concentration. The $IC_{50}$ value for *m*-ISOCOC and *p*-ISOCOC varied with tissue concentration, suggesting irreversible inhibition of binding. Preincubation with *m*-ISOCOC and *p*-ISOCOC resulted in inhibition of [$^3$H]WIN 35,428 binding that could not be removed by washing of the membranes; in contrast, preincubation with (−)-cocaine caused inhibition that was readily removed by washing. Preincubation with 1 μM concentrations of *p*-ISOCOC resulted in a large reduction in $B_{max}$ of the high affinity binding site for [$^3$H]WIN 35,428. Preincubation with 100 μM *p*-ISOCOC eliminated the high affinity site and apparently reduced the affinity at the low affinity site. Coincubation of 10 μM *p*-ISOCOC with 100 μM cocaine prevented the total loss of [$^3$H]WIN 35,428 binding. The uptake of [$^3$H]dopamine was inhibited by *p*-ISOCOC with an $IC_{50}$ comparable to that of cocaine. Additionally, preincubation of rat striatal synaptosomes with 10 μM *p*-ISOCOC reduced the $V_{max}$ of [$^3$H]dopamine uptake after washing. These data suggest that *m*-ISOCOC and *p*-ISOCOC are useful irreversible acylators of (−)-cocaine binding sites at the dopamine transporter.

Dopamine transport is thought to function as the inactivation mechanism for synaptic dopamine, and recently the transporter has been implicated as a cocaine receptor related to drug self-administration (1, 2). The dopamine transporter has been studied in a variety of binding experiments (3–7), including experiments utilizing tritiated cocaine (8–13). Evidence indicating that cocaine binds to the dopamine transporter is that the pharmacological properties of its binding parallel the pharmacological profile of inhibition of the dopamine transporter. Also, the binding is absent or reduced in tissue where there is an absence or reduction of dopaminergic nerve terminals (14–16).

Metaphit, a phencyclidine analogue containing an isothiocyanate group on the aromatic ring, has been shown to bind irreversibly to the dopamine transporter (17–20). However, it is approximately equipotent at the cocaine and phencyclidine receptor sites (17). In an effort to develop more specific irreversible binding ligands to study the dopamine transporter, we have identified compounds that bind to the dopamine transporter more specifically and with higher affinity than does metaphit. Grigoriadis *et al.* (21) showed that $^{125}$I-1-(2-[diphenylmethoxy]ethyl)-4-(2-[4-azido-3-iodiophenyl]ethyl)-piperazine, a derivative of GBR 12909, photolabeled the dopamine transporter. Another GBR derivative, $^{125}$I-1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(2-[4-azido-3-iodophenyl]ethyl)piperazine, is a similar photolabel for the transporter (22). In these experiments, it was shown that the dopamine transporter was a glycoprotein with an approximate molecular weight of 60,000. In the present experiments, we describe the properties of several isothiocyanate derivatives of cocaine, which also may be useful probes for studying the transporter. Acylating agents have been important tools for the characterization of a variety of drug and neurotransmitter receptors (23–28).

## Materials and Methods

**Tissue preparation.** Adult male Sprague-Dawley rats (200–250 g) were sacrificed by decapitation. The striata, midbrain, and frontal

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**ABBREVIATIONS:** *p*-ISOCOC, *p*-isothiocyanatobenzoylecgonine methyl ester; *m*-ISOCOC, *m*-isothiocyanatobenzoylecgonine methyl ester; ISOWIN, 3β-(4-isothiocyanatophenyl)tropane-2-carboxylic acid methyl ester; $IC_{50}$, 50% inhibitory concentration.

cortex of the rats were quickly dissected, frozen on a block of dry ice, and stored at −70° until needed.

**[$^3$H]WIN 35,428 (2β-carbomethoxy-3β-(4-fluorophenyl)tropane) binding.** The striata were weighed and homogenized with a Polytron (setting 5–6) for 15 sec, in 20 volumes of ice-cold 10 mM phosphate buffer (pH 7.4) containing 0.32 M sucrose. The homogenate was centrifuged at 50,000 × *g* (2–4°) for 10 min. The resulting pellet was resuspended in buffer to a tissue concentration of 20 mg of original wet weight/ml, using a Polytron (5–10 sec), and recentrifuged; this procedure was repeated twice. Binding assays were carried out in a total volume of 0.5 ml, containing the above buffer, membranes from 2.0 mg of tissue (original wet weight), and 0.5 nM radioligand. In tissue-dependence experiments, 0.5–6.0 mg (original wet weight) of tissue was added to the tubes. The suspensions were incubated in an ice bath for 2 hr. Incubations were terminated by filtration, with three 5-ml washes of ice-cold buffer, through Whatman GF/B filters that were previously soaked in 0.05% polyethylenimine, using a Brandel M48R filtering manifold (Brandel Instruments, Gaithersburg, MD). Radioactivity was counted in a Beckman LS 3801 liquid scintillation counter with an efficiency of approximately 50%.

Nonspecific binding of [$^3$H]WIN 35,428 was defined in the presence of 50 μM (−)-cocaine. The average value of nonspecific binding with cocaine was less than 5% of total [$^3$H]WIN 35,428 binding. Filter binding in the absence of tissue was negligible.

**Saturation of [$^3$H]WIN 35,428 binding.** Assays were carried out under the conditions described above, with the exception that 0.15 nM [$^3$H]WIN 35,428 was added to each assay tube. Binding was assayed in the presence of increasing concentrations of unlabeled WIN 35,428 (10 pM to 10 μM).

**Tissue preincubation with isothiocyanate derivatives.** Tissue was prepared as described above, but only two washes were performed. Preincubation with the drug, or vehicle as a control, was performed under assay conditions (10 mM phosphate buffer, pH 7.4, containing 0.32 M sucrose, at 4°) at a tissue concentration of 2.0 mg/ml (original wet weight). The drug was added to the tissue suspension and maintained at 4° for the required preincubation period. The reaction was terminated by the addition of 20 volumes of ice-cold buffer and centrifugation for 10 min at 50,000 × *g*. The resulting pellet was resuspended in buffer to a concentration of 20 mg/ml and was maintained at 4° for 5 min before centrifugation. This procedure was usually repeated for a total of three washes. The resulting pellet was resuspended in buffer to a concentration of 2.0 mg/ml. Statistical comparisons of $K_D$ and $B_{max}$ values were by analysis of variance, followed by a Sheffe comparison of the means.

**[$^3$H]Mazindol and [$^3$H]paroxetine binding.** Norepinephrine uptake sites were labeled in the frontal cortex of the rat using [$^3$H]mazindol. Nonspecific binding was defined by the addition of 4 μM desipramine. Approximately 5 mg of original weight wet of homogenized tissue were incubated for 1 hr at 4° in buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4) containing 4 nM ligand and 40 nM GBR 12909. [$^3$H]Paroxetine was used to label serotonin uptake sites in the rat brainstem. Nonspecific binding was defined with 1.5 μM citalopram. Homogenized brainstem (1.5 mg of original wet weight/assay tube) was incubated at room temperature in Tris buffer for 90 min, with a final ligand concentration of 0.2 nM.

**[$^3$H]YM-09151-2 binding.** Dopamine $D_2$ receptors were labeled according to the method of Niznik *et al.* (29). Briefly, rat striata were homogenized in 50 mM Tris, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 1 mM EDTA, pH 7.4, at room temperature. [$^3$H]YM-09151-2 binding assays were conducted in the same buffer as before, except 12 μM nialamide and 0.1% ascorbic acid were added. Homogenized striata (1.5 mg of original wet weight/assay tube) were incubated for 2 hr at room temperature with a final ligand concentration of 100 pM, with or without various concentrations of either *p*-ISOCOC or metaphit. Nonspecific binding was defined with 1.0 μM (+)-butaclamol.

**[$^3$H]Dopamine uptake.** [$^3$H]Dopamine uptake was measured using a modification of the method previously described (30). Briefly, a crude synaptosomal preparation was made from fresh rat striatal tissue (2.0 mg/ml, original wet weight) in 0.32 M sucrose. Inhibition of [$^3$H]dopamine uptake by cocaine or *p*-ISOCOC was determined in Krebs-phosphate buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 16.0 mM $Na_2HPO_4$, 1.4 mM $MgSO_4$, 2 mg/ml dextrose, 0.2 mg/ml ascorbic acid, pH 7.4) containing 10 μM pargyline, using 10–12 concentrations of either inhibitor. Assay blanks were defined using 100 μM mazindol; specific dopamine uptake was defined as the difference between [$^3$H]dopamine taken up by the synaptosomes in the presence and absence of mazindol. Buffer and ligand (0.9 ml, total volume) were preincubated for 10 min at 30°, and then 0.1 ml of tissue was added to the tubes to begin the 3-min incubation. The assay was terminated by the addition of 3 ml of ice-cold 0.32 M sucrose. The tubes were then filtered through GF/B filters, using a Brandel manifold vacuum apparatus. Radioactivity was counted in 5 ml of scintillation cocktail. Protein content was determined according to the method of Lowery *et al.* (31).

In order to determine the effect of *p*-ISOCOC upon the $K_m$ and $V_{max}$ of dopamine uptake, the synaptosomal fraction was incubated with either buffer or *p*-ISOCOC for 20 min. The fraction was pelleted and the $P_2$ preparation was washed twice. [$^3$H]Dopamine uptake was measured in the presence of 10–900 μM concentrations, as described above.

**Materials.** All isothiocyanate derivatives were prepared by treatment of the amino analogs with thiophosgene. *p*-Isothiocyanatococaine (*p*-ISOCOC) (Fig. 1) was prepared by treatment of the known *p*-aminococaine (32) with thiophosgene, resulting in a 50% yield. Similar treatment of *m*-aminococaine and of the *p*-amino WIN 35,065-2 analog gave *m*-ISOCOC (Fig. 1) and ISOWIN (Fig. 1) in 48% and 75% yields, respectively. The two cocaine analogs were isolated as the hydrochloride salts; the WIN 35,065-2 analog was obtained as a fumarate salt. The heretofore unreported *m*-aminococaine and 3β-(4-aminophenyl)tropane-2-carboxylic acid methyl ester were prepared by catalytic reduction of the nitro analogs, using Raney nickel catalyst. The *m*-nitrococaine compound was synthesized as described for the *p*-isomer (32), whereas the WIN analog was prepared by nitration of WIN 35,065-2. The chemical structures of these compounds were

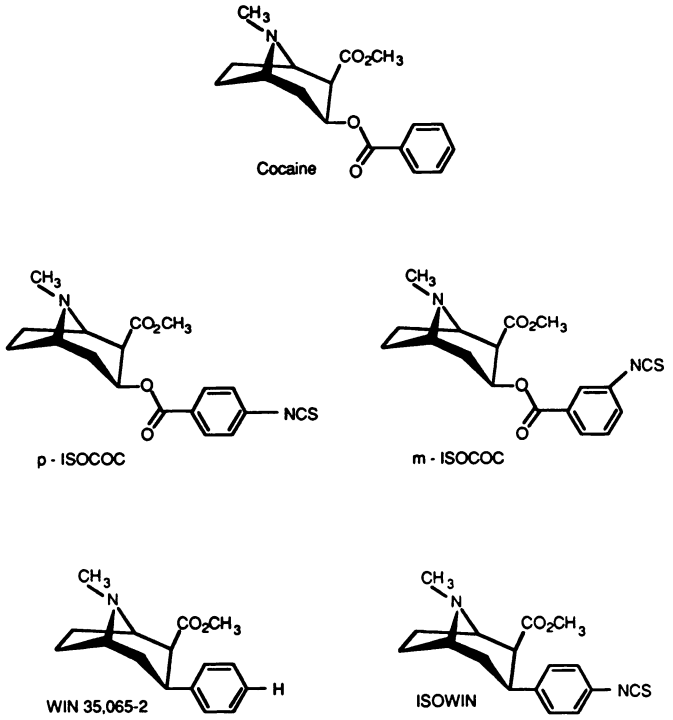


**Fig. 1.** Structures of cocaine, WIN 35,065-2, and isothiocyanate derivatives (*m*-ISOCOC, *p*-ISOCOC, and ISOWIN), as discussed in Materials and Methods.

confirmed by $^1$H NMR analysis, and the chemical purity was ascertained by thin layer chromatography and elemental analysis. The salts were stable when stored as solids at 12°. Experimental details of the synthesis will be described in a subsequent publication. [$^3$H]WIN 35,428 (specific activity, 81.3 Ci/mmol), [$^3$H]mazindol (specific activity, 15.0 Ci/mmol), [$^3$H]paroxetine (specific activity, 22.3 Ci/mmol), [$^3$H]YM-09151-2 (specific activity, 71.8 Ci/mmol), and [$^3$H]dopamine (specific activity, 20.5 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). Unlabeled WIN 35,428, WIN 35,065-2, mazindol, and (−)-cocaine were obtained from the National Institute on Drug Abuse. Metaphit was a generous gift of Warner-Lambert (Ann Arbor, MI), (+)-Butaclamol was purchased from Research Biochemicals (Natick, MA), and all other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

## Results

The analogs of cocaine, *m*-ISOCOC, *p*-ISOCOC, and ISOWIN (Fig. 1), were synthesized as described in Materials and Methods. These compounds, (−)-cocaine, metaphit, and WIN 35,065-2 were tested as inhibitors of [$^3$H]WIN 35,428 binding in rat striatal membranes. The most potent inhibitor was WIN 35,065-2, as previously described (1). Next in potency was (−)-cocaine, whereas the three isothiocyanate derivatives were about 10-fold weaker; metaphit was the weakest (Table 1). The Hill coefficient in these experiments was less than 1 for (−)-cocaine and WIN 35,065-2; this has also been reported by others (33).

Because cocaine and various analogs have been demonstrated to bind to both norepinephrine and serotonin uptake sites (34), the inhibitory properties of *p*-ISOCOC at these sites were also examined. The $IC_{50}$ for cocaine inhibition of [$^3$H]mazindol binding in rat frontal cortex was approximately 150-fold less than that of *p*-ISOCOC (Table 2). In contrast, cocaine and *p*-ISOCOC inhibited [$^3$H]paroxetine binding to rat brainstem with equal potency. Both Hill coefficients for *p*-ISOCOC were close to unity, indicating a single binding site in these regions. There did not appear to be any effect of *p*-ISOCOC upon $D_2$ dopamine receptors, as evidenced by no loss of [$^3$H]YM-09151-2 binding at a *p*-ISOCOC concentration of 1000 μM. In contrast, at this concentration of metaphit there was a significant reduction of [$^3$H]YM-09151-2 binding (data not shown).

Because the $IC_{50}$ of irreversible inhibitors has been shown to depend on tissue concentration (25, 27), we examined the effect of various tissue concentrations on the $IC_{50}$ values of these compounds. The $IC_{50}$ did not demonstrate a dependence on tissue concentration for (−)-cocaine. There was only a slight dependence on tissue concentration for ISOWIN. In contrast, the $IC_{50}$ values for *m*-ISOCOC and *p*-ISOCOC showed a strong dependence on tissue concentration, inasmuch as they varied 10-fold over the 10-fold tissue concentration range examined (Fig. 2).

TABLE 1

**Inhibition of [$^3$H]WIN 35,428 binding by cocaine, WIN 35,065-2, isothiocyanate derivatives, and metaphit**

Membranes (2 mg of tissue/ml) were prepared and radioligand assays were performed as described in Materials and Methods, using at least nine concentrations of inhibitor. The $IC_{50}$ values and Hill coefficients were computed using EBDA. Control values for [$^3$H]WIN 35,428 binding were approximately 23 fmol/mg. Values represent the mean ± standard error of four or five independent experiments, each performed in triplicate.

| Drug | $IC_{50}$ | Hill coefficient |
|---|---|---|
| | μM | |
| (−)-Cocaine | 0.10 ± 0.02 | 0.89 ± 0.05 |
| WIN 35,065-2 | 0.023 ± 0.005 | 0.89 ± 0.02 |
| *m*-ISOCOC | 1.46 ± 0.31 | 1.00 ± 0.09 |
| *p*-ISOCOC | 1.05 ± 0.20 | 1.01 ± 0.05 |
| ISOWIN | 1.65 ± 0.02 | 0.90 ± 0.01 |
| Metaphit | 3.29 ± 0.03 | 1.32 ± 0.03 |

[1] Boja, J. W. and Kuhar, M. J. Unpublished data.

In order to assess the irreversibility of the inhibition by cocaine and the isothiocyanate derivatives, they were preincubated with striatal membranes (2 × $IC_{50}$ concentrations) and subjected to zero, one, two, or three washes, and then the membranes were tested for [$^3$H]WIN 35,428 binding activity. When (−)-cocaine or ISOWIN was used as inhibitor, the inhibiting properties were removed by two washes. In contrast, *m*-ISOCOC and *p*-ISOCOC displayed an inhibition that was not reversed by three washes (Fig. 3).

The time course of inhibition was examined. Preincubation times were varied from 15 sec to 30 min. Maximal irreversible inhibition was found by 1 min (data not shown). The reactivity of isothiocyanate derivatives has been shown to be reduced in an acidic environment (20, 25, 27). In an attempt to examine the effect of reduced pH on ISOCOC blockade, the binding of [$^3$H]WIN 35,428 was measured at pH 5.0. In control experiments, specific [$^3$H]WIN 35,428 binding at pH 5.0 without drug was reduced by 95%, as compared with specific binding at pH 7.4. Because of this, the effect of ISOCOC at pH 5.0 could not be determined.

The effect of irreversible inhibition by *p*-ISOCOC on the $K_D$ and $B_{max}$ of [$^3$H]WIN 35,428 binding in rat striatal membranes was examined. Membranes were preincubated with 1, 10, or 100 μM concentrations of *p*-ISOCOC, 1 μM (−)-cocaine, or buffer alone. After three washes of the membranes, [$^3$H]WIN 35,428 binding was examined. Saturation experiments with membranes that were preincubated with no drug present revealed both a high and a low affinity component of [$^3$H]WIN 35,428 binding (Table 3). There was about a 10-fold difference in $K_D$ and a 3-fold difference in $B_{max}$ between the two components. Two binding sites for [$^3$H]WIN 35,428 have been observed by other investigators (33). Preincubation with (−)-cocaine had no significant effect on the binding constants. However, preincubation with 1 μM *p*-ISOCOC resulted in a large reduction of the $B_{max}$ for the high affinity site. Preincubation with higher concentrations of *p*-ISOCOC resulted in loss of the high affinity site; the $K_D$ of the remaining site appeared to increase after preincubation with 100 μM *p*-ISOCOC. Preincubation of membranes with 10 μM *p*-ISOCOC resulted in a loss of high affinity [$^3$H]WIN 35,428 binding, and the data could be fit to a multiple site model in only two of the eight experiments. Statistical modeling was based on LIGAND, using the *F* test and "goodness of fit" parameters to compare multiple curves simultaneously. Using the same method, Scatchard analysis of [$^3$H]WIN 35,428 binding to membranes that were preincubated with 100 μM *p*-ISOCOC could only be fit to a single-site model and demonstrated a significant difference between single- and multiple-site models at the $p < 0.05$ level of significance in all nine experiments (see Fig. 4).

Cocaine and dopamine were tested for their ability to protect against acylation by *p*-ISOCOC in rat striatal membranes. The striatal membranes were incubated for 10 min with various concentrations of either cocaine or dopamine, alone or in combination with 10 μM *p*-ISOCOC, washed three times, and tested

TABLE 2

**Inhibition of [$^3$H]mazindol binding in rat frontal cortex, [$^3$H]paroxetine binding in rat brainstem, and [$^3$H]YM-09151-2 binding in rat striatum by cocaine and *p*-ISOCOC**

Membranes were prepared and radioligand assays were performed as described in Materials and Methods, using 10–12 concentrations of inhibitor. The $IC_{50}$ values and Hill coefficients were computed using EBDA. Values represent the mean ± standard error of three or four independent experiments, each performed in triplicate.

| Ligand | Site | Drug | $IC_{50}$ | Hill coefficient |
|---|---|---|---|---|
| | | | μM | |
| [$^3$H]Mazindol | Norepinephrine transport | (−)-Cocaine | 0.11 ± 0.02 | 0.83 ± 0.10 |
| | | *p*-ISOCOC | 17.3 ± 4.7 | 1.11 ± 0.13 |
| [$^3$H]Paroxetine | 5-Hydroxytryptamine transport | (−)-Cocaine | 1.10 ± 0.26 | 1.01 ± 0.10 |
| | | *p*-ISOCOC | 1.17 ± 0.09 | 1.43 ± 0.56 |
| [$^3$H]YM-09151-2 | $D_2$ Dopamine | *p*-ISOCOC | >1000 | |

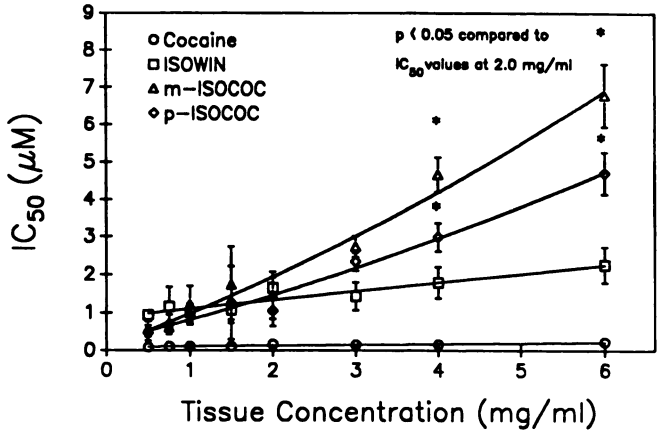


**Fig. 2.** Effect of tissue concentration on $IC_{50}$. The membranes were initially prepared as described in Materials and Methods; however, the tissue was resuspended to 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, or 6.0 mg/ml (original wet weight). Each experiment was conducted by coincubating 0.5 nM [$^3$H]WIN 35,428 and 12 concentrations of inhibitor, to calculate $IC_{50}$; each point is the result of three experiments in triplicate. Data are mean ± standard error. Nonspecific binding was defined with 50 μM (−)-cocaine. $IC_{50}$ values were determined using EBDA (Elsevier Software). Significant differences were determined by analysis of variance and Dunett's comparison of the means.

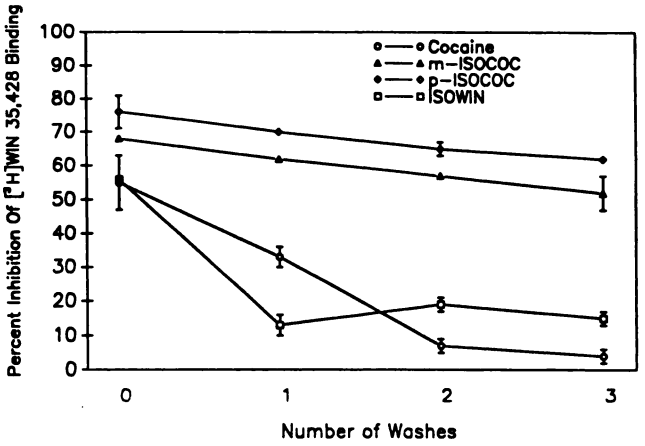


**Fig. 3.** Effect of repeated tissue washes on [$^3$H]WIN 35,428 binding in striatal membranes pretreated with cocaine, *m*-ISOCOC, *p*-ISOCOC, or ISOWIN. Membrane preparations (2.0 mg/ml) were preincubated for 15 min with twice the $IC_{50}$ concentrations (Table 1) of the various drugs, or with buffer for control. The membranes were then centrifuged at 20,000 × *g* for 5 min and resuspended in buffer to 2.0 mg/ml. An aliquot of membrane suspension was removed (0 washes), and [$^3$H]WIN 35,428 binding was assayed as described in Materials and Methods. The remaining membrane preparation was again centrifuged and resuspended, and an aliquot was removed; this was repeated until the membrane preparation had been washed three times. Data are mean ± standard error of three experiments.

for [$^3$H]WIN 35,428 binding inhibition. Preincubation with 100 or 1000 μM cocaine before incubation with 10 μM *p*-ISOCOC resulted in 63% and 100% protection, respectively, against acylation by *p*-ISOCOC (Fig. 5). However, the same concentrations of dopamine provided less protection than that afforded by cocaine. When the concentration of *p*-ISOCOC was increased to 100 μM, neither concentration of either cocaine or dopamine protected against acylation by *p*-ISOCOC (data not shown).

The ability of *p*-ISOCOC to inhibit [$^3$H]dopamine uptake into synaptosomes was compared with that of cocaine. Various concentrations (10 mM to 100 μM) of either *p*-ISOCOC or cocaine were coincubated with [$^3$H]dopamine and a $P_2$ synaptosomal preparation, for 3 min at 30°. The results demonstrated that cocaine (2.75 ± 0.18 μM) and *p*-ISOCOC (2.78 ± 0.07 μM) both inhibit [$^3$H]dopamine uptake with approximately equal potency (Table 4).

Preincubation of the synaptosomal fraction with 10 μM *p*-ISOCOC or cocaine, followed by two washes, resulted in a reduction in the $V_{max}$ of synaptosomal dopamine uptake from 8.39 ± 0.60 pmol/mg of protein/min for cocaine-pretreated synaptosomes to 0.30 ± 0.07 pmol/mg of protein/min for *p*-ISOCOC-treated synaptosomes. The $K_m$ values were relatively unchanged following *p*-ISOCOC pretreatment (122 ± 14 nM, compared with a cocaine-pretreated value of 120 ± 31 nM) (Table 4).

## Discussion

Isothiocyanate derivatives of binding ligands have been useful tools for examining the properties and characteristics of various drug receptors (23, 25, 27, 28). These compounds are thought to interact with bionucleophiles, particularly amino groups on lysine, arginine, and histidine. In studying the cocaine receptor, the analogs examined here may be particularly useful, because they are structurally more similar to cocaine than are any of the other available irreversible ligands for the dopamine transporter (17–22). In addition, *p*-ISOCOC seems to be slightly more potent at the cocaine binding site than metaphit, another isothiocyanate derivative that binds to this site (17, 18, 20, 35).

Our data indicate that the analogs of cocaine, particularly *m*-ISOCOC and *p*-ISOCOC, are irreversible binding inhibitors at the cocaine receptor associated with the dopamine transporter. The compounds are relatively potent inhibitors of [$^3$H]WIN 35,428 binding, and the inhibition cannot be removed from the tissues by washing. The $IC_{50}$ of *p*-ISOCOC was less than that

TABLE 3

**Effect of drug pretreatment on [$^3$H]WIN 35,428 binding parameters**

Membranes were prepared (2 mg/ml) and preincubated for 15 min with the indicated compound, and saturation binding assays were conducted as described in Materials and Methods. Values represent the means of four or more experiments, as indicated in parentheses, with at least 10 concentrations of unlabeled WIN 35,428. $K_D$ and $B_{max}$ values were calculated using LIGAND. Data are mean ± standard error.

| Pretreatment | $K_D$ | | $B_{max}$ | |
|---|---|---|---|---|
| | $K_{Dhigh}$ | $K_{Dlow}$ | $Bmax_{high}$ | $Bmax_{low}$ |
| | nM | | nmol/g | |
| Buffer (4) | 7.1 ± 1.7 | 77.9 ± 21.3 | 1.04 ± 0.35 | 2.76 ± 0.51 |
| 1 μM (−)-Cocaine (5) | 6.1 ± 1.3 | 71.4 ± 26.6 | 0.83 ± 0.18 | 2.52 ± 0.41 |
| 1 μM *p*-ISOCOC (5) | 6.2 ± 2.0 | 66.9 ± 15.4 | 0.12 ± 0.06[a] | 1.56 ± 0.64 |
| 10 μM *p*-ISOCOC (8)[b] | 42.0 ± 4.4 | | 2.68 ± 0.22 | |
| 100 μM *p*-ISOCOC (9)[c] | 173.0 ± 20.0 | | 1.93 ± 0.30 | |

[a] Significantly less than either buffer or cocaine pretreatment ($p < 0.01$).
[b] A two-site model was not statistically preferred in six of eight experiments, using LIGAND.
[c] A two-site model was not statistically preferred in any of the nine experiments, using LIGAND.

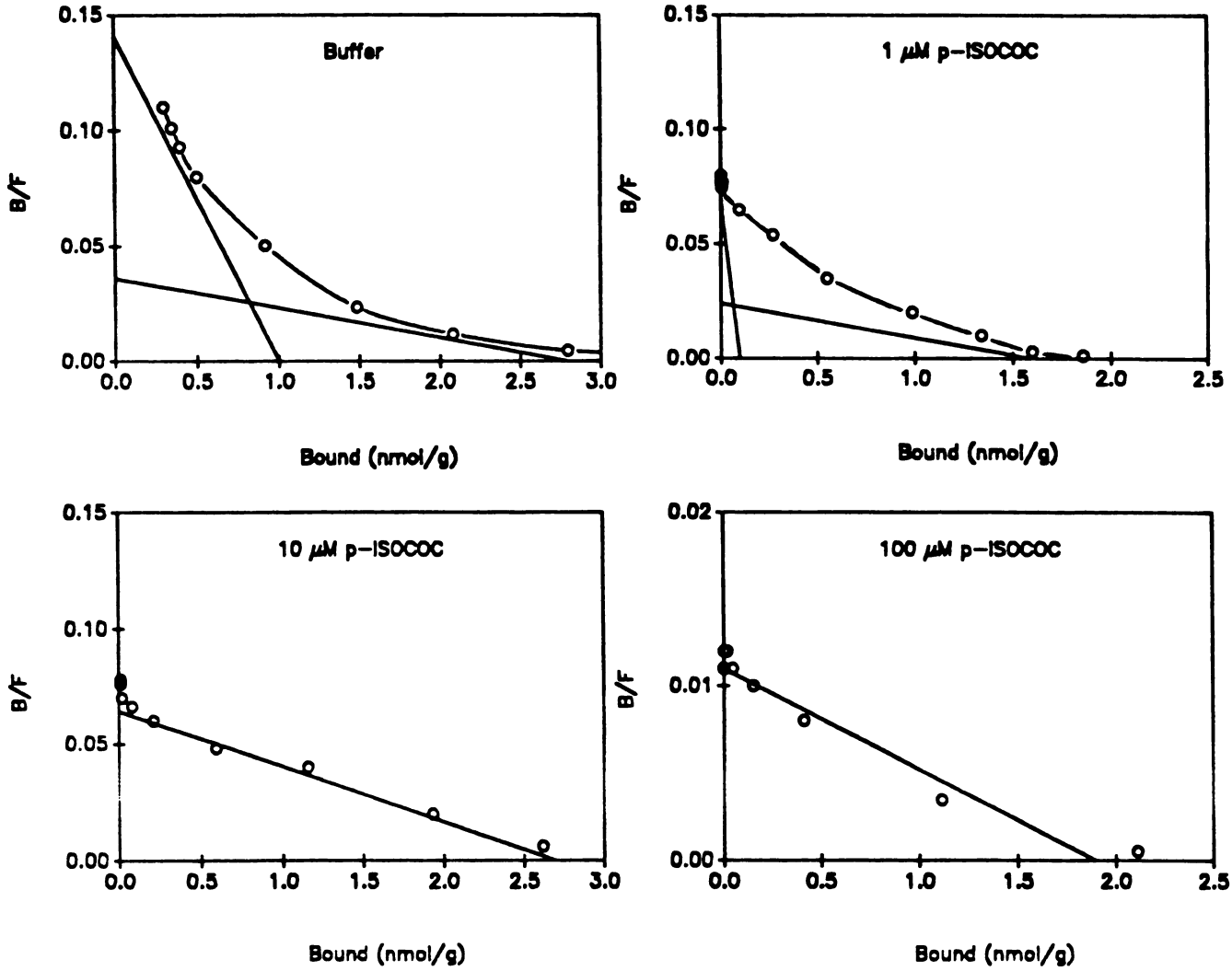


**Fig. 4.** Effect of preincubation with *p*-ISOCOC on [$^3$H]WIN 35,428 binding constants. Membrane preparations were preincubated with 1, 10, or 100 μM *p*-ISOCOC for 15 min at 4° and then washed, and [$^3$H]WIN 35,428 binding was assayed as described in Materials and Methods. The $K_D$ and $B_{max}$ values were calculated using LIGAND. Statistical analyses were performed, using LIGAND and an *F* test, to determine whether those data could be best described by a single- or multiple-state model. A multiple-state model was chosen only if there was a significant increase in the goodness of fit. The Scatchard plots shown are the means of five to nine experiments, as indicated in Table 2. *B/F*, bound/free.

seen for cocaine itself; however, the addition of the isothiocyanate moiety seems to reduce potency, as demonstrated by the reduction of potency shown by metaphit, as compared with the parent phencyclidine compound (18, 35). In addition, the blockade of the [$^3$H]WIN 35,428 binding site indicates that [$^3$H]dopamine transport is blocked by *p*-ISOCOC, because, under the conditions used here, [$^3$H]WIN 35,428 binds to the cocaine receptor at the dopamine transporter (33). Also, the inhibition of these compounds in competition experiments depended strongly on tissue concentration, a finding that is

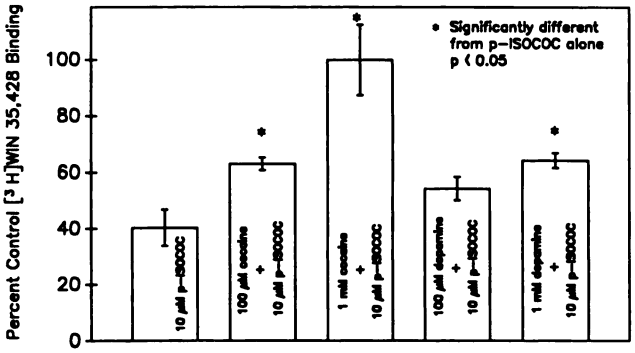


**Fig. 5.** Protection by cocaine or dopamine against acylation by *p*-ISOCOC. The membranes were initially prepared as described in Materials and Methods and then incubated with either buffer or 100 or 1000 μM drug, with 10 μM *p*-ISOCOC, for 10 min. The membrane preparation was centrifuged and washed three times; [$^3$H]WIN 35,428 binding was then assayed as previously described. Statistical analyses were performed using analysis of variance and a Dunett's comparison of the means.

TABLE 4

**Inhibition of [$^3$H]dopamine uptake into rat striatal synaptosomes by coincubation and by preincubation with *p*-ISOCOC and (−)-cocaine**

The ability of *p*-ISOCOC to inhibit [$^3$H]dopamine uptake was compared with that of cocaine, as described in Materials and Methods. Varying concentrations of cocaine and *p*-ISOCOC were coincubated with 100 μM [$^3$H]dopamine, to determine the inhibitory effect of each upon [$^3$H]dopamine uptake. The $IC_{50}$ values were computed using EBDA. Values represent the mean ± standard error of three experiments. The effect of 10 μM *p*-ISOCOC and cocaine on [$^3$H]dopamine transport was determined following a 20-min preincubation of the synaptosomal fraction with the drugs, in a ice bath. The synptosomes were washed twice by centrifugation. $V_{max}$ and $K_m$ values were determined in the presence of 10–900 nM radioligand. Mean ± standard error of $V_{max}$ and $K_m$ values are from three experiments.

| Drug | $IC_{50}$ | Hill coefficient | $V_{max}$ | $K_m$ |
|---|---|---|---|---|
| | μM | | pmol/mg of protein/min | nM |
| Saline | | | 8.43 ± 0.66 | 103 ± 12 |
| Cocaine | 2.75 ± 0.18 | 1.1 ± 0.3 | 8.39 ± 0.60 | 120 ± 31 |
| *p*-ISOCOC | 2.78 ± 0.07 | 1.2 ± 0.5 | 0.30 ± 0.07 | 122 ± 14 |

typical of irreversibly binding ligands (25, 27). Because *p*-ISOCOC appeared to be a more effective irreversible inhibitor of [$^3$H]WIN 35,428 binding than ISOWIN and because these compounds are fairly close structural analogs of each other and exhibit similar affinities for inhibition of [$^3$H]WIN 35,428 binding (Table 1), it is striking that ISOWIN is not involved in site-directed acylation of the cocaine binding site. Presumably, this indicates that ISOWIN lacks one or more of the specific structural requirements associated with irreversible inhibition of cocaine binding. The lack of cocaine binding to the $D_2$ dopamine receptor is reflected by a lack of binding to this receptor by *p*-ISOCOC, although metaphit, on the other hand, did demonstrate inhibition at this site. Although *p*-ISOCOC demonstrated similar potency as cocaine at the serotonin transporter site, it was slightly weaker at the norepinephine uptake site, again suggesting a specificity of action for *p*-ISOCOC.

Scatchard analysis of [$^3$H]WIN 35,428 binding revealed two binding sites, with $K_D$ values in buffer- (7.1 and 77.9 nM) or (−)-cocaine- (6.1 and 71.4 nM) pretreated membranes that are in good agreement with previously published $K_D$ values for [$^3$H]WIN 35,428 (33). Binding experiments with [$^3$H]cocaine or WIN 35,065-2 also revealed two binding sites (11–13, 36). This is in contrast to findings with other ligands, where only one site was found (2–5). The $B_{max}$ values obtained here in rat striatal tissues are approximately 10-fold higher than those obtained by Madras *et al.* (33), using [$^3$H]WIN 35,428 in monkey caudate, but are in good agreement with the values obtained by Calligaro and Eldefrawi (11, 12), using [$^3$H]cocaine. The reasons for this differences are 2-fold. Whereas Calligaro and Eldefrawi (11, 12) determined the number of cocaine binding sites in rat, Madras *et al.* (33) utilized primate tissue. Additionally, Madras *et al.* (33) conducted binding experiments in Tris buffer, whereas Calligaro and Eldefrawi (11, 12) utilized phosphate buffer, which gives higher binding; we utilized phosphate buffer as well. Reith *et al.* (37) determined that Tris buffer can reduce the apparent number of cocaine binding sites, as compared with phosphate buffer. This phenomenon was also apparent in our hands, in that the use of Tris buffer reduced $B_{max}$ values 5–10-fold, while not affecting $K_D$ values.[1]

After pretreatment of membranes with the lowest concentration of *p*-ISOCOC, there was an apparent preferential loss of high affinity sites, as expected. Low concentrations of *p*-ISOCOC (1 μM) significantly reduced the number of high affinity binding sites, whereas higher doses (10 and 100 μM) totally eliminated this site. In contrast, the number of low affinity binding sites did not significantly change with *p*-ISOCOC pretreatment. There was a slight dose-dependent decrease in affinity for this site that was not statistically significant. This finding is similar to that reported by others (17, 20) using metaphit. Low doses of metaphit (10–14 μM) reduced $B_{max}$ approximately 40%, with a slight or no change in affinity; however, those studies (17, 20) were conducted with ligands that recognize only a single binding site. Thus, when a ligand is used that recognizes two binding sites, low doses of *p*-ISOCOC may acylate a nucleophile located at the high affinity binding site, blocking [$^3$H]WIN 35,428 binding; higher doses of *p*-ISOCOC may completely block the high affinity binding site and acylate a second nucleophile located near the low affinity site, partially hindering access of [$^3$H]WIN 35,428, resulting in decreased affinity. These observations suggest that the low and high affinity cocaine binding sites may be structurally or conformationally different. However, additional experiments will be necessary to establish this. In other experiments with both the cocaine (17) and the benzodiazepine (25) receptor, low doses of acylator reduced $B_{max}$ and higher doses affected $B_{max}$ and $K_D$. The decrease in $B_{max}$ values at low doses of irreversible inhibitors, followed by an increase in $K_D$ at higher doses, has been postulated to be due to the presence of a second nucleophile in the vicinity of the receptor (25).

As suggested by blockade of the cocaine binding site, *p*-ISOCOC also inhibits [$^3$H]dopamine uptake, with an $IC_{50}$ similar to that of cocaine itself. This finding is surprising, considering the dissimilar $IC_{50}$ values of cocaine and *p*-ISOCOC for inhibition of [$^3$H]WIN 35,428 binding. This is probably accounted for by the different temperatures at which the two assays were performed (0° and 30°). Thus, although all reaction rates are increased at higher temperatures, this has virtually no effect on cocaine binding, because both the association and the dissociation rates at the receptor are increased. Conversely, because *p*-ISOCOC is an irreversible ligand and, therefore, has a vanishingly small dissociation rate, the result of increasing its association and bonding rates leads to an apparently higher $IC_{50}$. Pretreatment of striatal synptosomes decreased the uptake site $V_{max}$, a finding consistent with the radioligand binding

data. This finding is also consistent with the reduction in $V_{max}$ shown by metaphit (20, 33).

The results of this study suggest that *p*-ISOCOC is a compound that will be useful in the study of the structure and function of the dopamine transporter. *p*-ISOCOC irreversibly blocked both ligand binding to the cocaine recognition site and dopamine uptake by the transporter. Because *p*-ISOCOC blocked the high affinity cocaine binding site in preference to the low affinity sites, it may be valuable tool to study the role of the high affinity site.

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