

Effects of Methylphenidate Analogues on Phenethylamine Substrates for the Striatal Dopamine Transporter: Potential as Amphetamine Antagonists?

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Abstract: Methylphenidate (MPD) was found to inhibit competitively the striatal dopamine transporter (DAT) and bind at sites on the DAT in common with both cocaine (a non-substrate site ligand) and amphetamine (a substrate site ligand). Some methylphenidate analogues modified on the aromatic ring and/or at the nitrogen were tested to determine whether the profile of inhibition could be altered. None was found to stimulate the release of dopamine in the time frame (≤ 60 s) of the experiments conducted, and each of the analogues tested was found to *noncompetitively* inhibit the transport of dopamine. It was found that halogenating the aromatic ring with chlorine (*threo*-3,4-dichloromethylphenidate hydrochloride; compound 1) increased the affinity of MPD to inhibit the transport of dopamine. A derivative of MPD with simultaneous, single methyl group substitutions on the phenyl ring and at the nitrogen (*threo*-*N*-methyl-4-methylphenidate hydrochloride; compound 2) bound at a site in common with MPD. A benzyl group positioned at the nitrogen (*threo*-*N*-benzylmethylphenidate hydrochloride; compound 3) imparted properties to the inhibitor in which binding at substrate and non-substrate sites could be distinguished. This analogue bound at a mutually interacting site with that of methylphenidate and had a K_{int} value of $4.29 \mu M$. Furthermore, the *N*-substituted analogues (compounds 2 and 3), although clearly inhibitors of dopamine transport, were found to attenuate dramatically the inhibition of dopamine transport by amphetamine, suggesting that the development of an antagonist for substrate analogue drugs of abuse may be possible.
Key Words: Amphetamine—Cocaine—Dopamine—Methylphenidate—Methylphenidate analogues—Ritalin—Rotating disk electrode voltammetry—Striatum—Transporter—*m*-Tyramine.

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Methylphenidate (MPD; Ritalin) is currently the most commonly prescribed drug to treat children with attention deficit disorder and is also used to treat narcolepsy (Chiarello and Cole, 1987; Kraus and Burch, 1992).

Originally, amphetamine was used to treat children with attention deficit disorder until its addictive potential was recognized. It was estimated in 1992 that 3% of school-age children were being treated with MPD for some extended interval (McNamara et al., 1993); these numbers have continued to increase. MPD is not thought to stimulate dopamine (DA) synthesis or induce release of DA from nerve terminals (Kolta et al., 1985). The action of MPD is to block the inward transport of DA into the presynaptic terminal, resulting in a prolonged DA stimulus. Although MPD, a psychomotor stimulant agent, has been shown to have abuse potential, it is still the drug of choice for the treatment of children with attention deficit disorder.

MPD has been shown to bind at the DA transporter (DAT) (Héron et al., 1994), and its binding is saturable and specific for the DAT (Schweri et al., 1985), but it is not clear where MPD binds on the transporter relative to DA and other inhibitors of transport. MPD is thought to have behavioral, pharmacological, and binding properties similar to those of amphetamine (Barnett and Kuczenski, 1986) and cocaine (Schweri et al., 1985; Froimowitz et al., 1995; Gatley et al., 1996). Therefore, MPD has been classified as a nontransported inhibitor of DAT (Hurd and Ungerstedt, 1989; Héron et al., 1994), as well as a substrate analogue for DAT when present at high concentrations (Nomikos et al., 1990). These discrepancies have led to the current study, which focuses on

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Abbreviations used: compound 1, *threo*-3,4-dichloromethylphenidate hydrochloride; compound 2, *threo*-*N*-methyl-4-methylphenidate hydrochloride; compound 3, *threo*-*N*-benzylmethylphenidate hydrochloride; $[DA]_o$, extracellular dopamine concentration; DA, dopamine; DAT, dopamine transporter; MPD, methylphenidate; RDE, rotating disk electrode; SER, SE of regression.

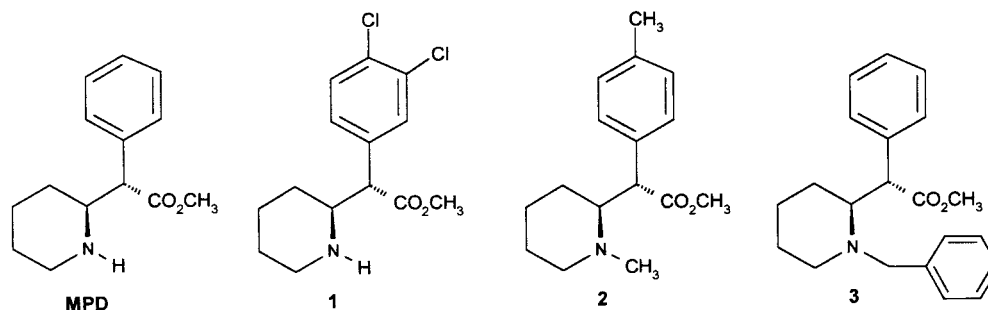


FIG. 1. Structures of the MPD analogues used in the studies herein.

comparing the inhibition properties and binding sites of MPD with those of some MPD structural analogues, amphetamine, and cocaine. The results of previous work by this laboratory in a kinetic model of the actively transporting DAT have shown that amphetamine and *m*-tyramine bind to the same site on the DAT and at a site competitive with DA (Wayment et al., 1998). Thus, it was shown that amphetamine and *m*-tyramine are substrate analogues for the DAT. It was shown that amphetamine binds at a site that is separate but interacting with the inhibitory site of cocaine on the DAT (Wayment et al., 1998).

Various MPD derivatives have been synthesized in an attempt to develop compounds that will block cocaine binding to the DAT but do not interfere with substrate binding or transport (Deutsch et al., 1996; Froimowitz et al., 1997). The test used for their assessment has been to compare the IC₅₀ for inhibition of the transport of DA with the IC₅₀ of the test analogue for the displacement of [³H]WIN 35,428 binding (Deutsch et al., 1996).

In this study, amphetamine, cocaine, and selected structural analogues of MPD (Fig. 1) were used to decipher whether MPD resembles amphetamine or cocaine in its effects on the function of DAT. The MPD derivatives studied in this work were chosen for their particular characteristics. Compound 1, a dichloro-substituted derivative, is one of the more potent MPD derivatives with respect to inhibition of [³H]WIN 35,428 binding. It differs from most other MPD derivatives that have been synthesized in that (a) it is equipotent as an inhibitor of [³H]WIN 35,428 binding and [³H]DA uptake and (b) it has an equilibrium Hill coefficient (*n*_H) determined against [³H]WIN 35,428 binding of ~2. (Most of the other MPD analogues are at least threefold less potent as inhibitors of [³H]DA uptake and have *n*_H values of unity.) Compounds 2 and 3 were included because they are some of the most effective analogues in discriminating between [³H]WIN 35,428 binding and [³H]DA uptake, with a five- to sevenfold separation in potency to inhibit binding versus transport. Also, compound 3 was of interest because the bulky benzyl group at the nitrogen was anticipated to alter markedly the interaction of the core MPD structure with the DAT. Although complete separation of the inhibitory effects of the modified compounds on stimulant binding versus substrate transport

was not achieved, some differentiation between the two functions was obtained (Deutsch et al., 1996; Froimowitz et al., 1997).

MATERIALS AND METHODS

Preparation of striatal tissue suspensions and measurement of DA transport by rotating disk electrode (RDE) voltammetry

Striatal tissue samples were prepared from male Sprague-Dawley rats (weighing 250–400 g), and the transport of DA was measured by RDE voltammetry at 37°C in a glass incubation chamber as previously described by Meiergerd and Schenk (1995), Earles et al. (1998), and Earles and Schenk (1998). All animal protocols were reviewed and approved by the University Laboratory Animal Care and Use Committee. In brief, animals were rapidly decapitated, and the striata were quickly dissected and placed on ice. The striatum was then finely chopped with a razor blade on an ice-cold watch glass and placed in 500 μl of physiological buffer (composition below). The tissue was disrupted by rapidly filling and expelling the tissue/buffer mixture with a 250-μl pipette. This process was repeated 15–20 times and then allowed to settle. The suspension was washed by addition of 250 μl of fresh buffer to the suspension, allowing it to settle, and again removing 250 μl. After eight repeated washes of the suspension a final volume of 500 μl was obtained.

The glassy carbon RDE was placed just slightly below the surface of the suspension and rotated at a rate of 2,000 rpm, at which time a potential of 450 mV relative to an Ag/AgCl reference electrode was applied to the RDE to detect DA. The potential was applied using a Bioanalytical Systems (West Lafayette, IN, U.S.A.) LC4B potentiostat, and the current was monitored on a Nicolet Instruments Corp. (Madison, WI, U.S.A.) model 310 digital storage oscilloscope. Suspensions were incubated until a steady baseline was obtained (~15 min), at which time either an inhibitor or two inhibitors were added, and the effect on the inwardly directed transport of 1.0 μM DA was recorded as extracellular DA concentration ([DA]_o) versus time (*t*). Initial rates (*v*) were determined by the linear slope of a plot of [DA]_o versus time (*t*) where the *y*-intercept is indistinguishable from the initial [DA]_o added to the suspension and is expressed in the units pmol/s and normalized for wet tissue weight. The timing for addition of each inhibitor was based on whether the inhibitor was experimentally found to exhibit a time dependence in its effects on DAT activity (data not shown). An inhibitor whose inhibition decreased over time was added simultaneously with DA, whereas other inhibitors that appear not to have this time dependence were added 30 s before

addition of 1.0 μM DA following the previously used protocols (Meiergerd and Schenk, 1994; Wayment et al., 1998). In the present study MPD and its tested analogues did not exhibit time-dependent inhibition, whereas, as before (Wayment et al., 1998), the substrate analogue inhibitors *m*-tyramine and amphetamine did. Thus, these agents were added simultaneously with DA. Unless otherwise noted, each numerical result reported is from three to seven replicates and each replicate represents a value obtained from a single striatum.

Kinetic analysis

Values of v were also measured over a $[\text{DA}]_o$ range of 0.25–4.0 μM and plotted as a function of $[\text{DA}]_o$. The results were fit to the Michaelis–Menten expression to estimate values of K_m and V_{max} with precision indicated as SE of regression (SER) by nonlinear curve fitting using PRISM version 2.0 (GraphPad, San Diego, CA, U.S.A.). When inhibitions were studied, a K_i was estimated using the Michaelis–Menten expression modified for competitive, noncompetitive, or uncompetitive inhibition (Fersht, 1985; Segel, 1993). The values for K_m and V_{max} were used in the fitting procedures. Starting values for $[I]$ were defined by the IC_{50} of inhibition of a test analogue estimated from Hill analyses modified for use in catalytic (enzymic) experiments (Segel, 1993)

$$\log \frac{v_i}{v_o - v_i} = -n_H \log [I] + \log K \quad (1)$$

where v_i is the velocity of transport in the presence of a given concentration of a test inhibitor, I ; v_o is the control velocity in the absence of inhibitor; n_H is the apparent stoichiometry (Hill coefficient); and K is the apparent dissociation constant for the transporter– I complex. The apparent IC_{50} is taken as the x -intercept, the value for $[I]$ where $v_i = 0.5v_o$.

Fits of any of the tested models to experimental data were evaluated by using the model to calculate a v versus $[\text{DA}]_o$ profile and comparing the result point by point with the experimental data and expressing the goodness of fit as a percent deviation of the model from the data.

Some experiments were conducted to determine the relationship between binding sites for MPD, its analogues, cocaine, *m*-tyramine, and amphetamine. Three binding models were used: a single-site model, a model where the two inhibitors act at separate independent sites, and a model where the two inhibitors act at separate sites that interact. To test whether the inhibitors bind to a single binding site Eq. 2 was used:

$$\left(\frac{v}{v_b} - 1\right) = \left(\frac{v}{v_{\text{test1}}} - 1\right) + \left(\frac{v}{v_{\text{test2}}} - 1\right) \quad (2)$$

where v is the velocity of DA transport in the absence of inhibitor, v_{test1} is the velocity observed at a given concentration of the first test inhibitor, v_{test2} is the velocity observed at a given concentration of the second inhibitor, and v_b is the velocity in the presence of both inhibitors at the same individual concentrations used in the previous two tests. To test whether the inhibitors bind to two different, independent sites, v_b was predicted by the expression

$$\left(\frac{v}{v_b} - 1\right) = \left(\frac{v}{v_{\text{test1}}} - 1\right) + \left(\frac{v}{v_{\text{test2}}} - 1\right) + \left(\frac{v}{v_{\text{test1}}} - 1\right)\left(\frac{v}{v_{\text{test2}}} - 1\right)A \quad (3)$$

where the symbols have the same significance as in Eq. 1 and $A = (1 + f_i/f_{-1})(1 + [\text{DA}]_o/K_m)$ and f_i/f_{-1} is the equilibrium

distribution of carrier between inwardly and outwardly directed conformations. For this work, as in previous work (Meiergerd and Schenk, 1994; Wayment et al., 1998), the value of the ratio used was unity as suggested by Devés and Krupka (1980). To test the case of binding to two separate but mutually interacting sites, v_b was predicted by the expression

$$\left(\frac{v}{v_b} - 1\right) = \left(\frac{v}{v_{\text{test1}}} - 1\right) + \left(\frac{v}{v_{\text{test2}}} - 1\right)\left(1 + \frac{[I]_{\text{test1}}}{K_{\text{int}}}\right) \quad (4)$$

where the symbols have the same significance as in the expressions above except for K_{int} , which is the half-saturation constant for test inhibitor 1 in the presence of saturating concentrations of test inhibitor 2, and K_m , the Michaelis–Menten constant of the inwardly directed transport of DA in the striatum. The predicted v_b was then compared with the experimental v_b . The analyses conducted were based on the work of Déves and Krupka (1980), and the step-by-step procedures followed for the evaluation of the data obtained in studies of the DAT have been presented by this laboratory previously (Meiergerd and Schenk, 1994; Wayment et al., 1998). In brief, when fits to the models were evaluated, if the experimental results were found to be statistically indistinguishable from predictions of the single-site model (Eq. 2) and numerically within 5%, the interaction was deemed as competition at a single site. For comparison, the predicted values of velocity for two independent sites were calculated and shown. However, the values for two interacting sites were not calculated because the last term in that equation would be required to have the value of unity, requiring that the value of $K_{\text{int}} \gg \gg [I]$, a physically unrealistic condition because no inhibition should be observable under this condition.

Ligand binding and studies of [^3H]DA uptake

Some experiments were conducted to evaluate the binding of MPD and its analogues to the DAT. In separate studies the IC_{50} for each analogue to inhibit DAT uptake of [^3H]DA was also estimated. The methods used in these studies were the same as those described by Deutsch et al. (1996), except rats used for the uptake studies were anesthetized before use with CO_2 .

Solutions and chemicals

Solutions were prepared in deionized water and further purified by a Nanopure water purification system (Barnstead, Dubuque, IA, U.S.A.) equipped with ion exchangers, activated carbon cartridges, and an ultra filter. The common buffer salts were obtained from Baker Chemicals (Phillipsburg, NJ, U.S.A.), and DA hydrochloride, (–)-cocaine hydrochloride, *S*(+)-amphetamine sulfate, *m*-tyramine hydrochloride, and MPD hydrochloride were obtained from Research Biochemicals International (Natick, MA, U.S.A.). The structural analogues of MPD (Fig. 1), *threo*-3,4-dichloromethylphenidate hydrochloride (compound 1), *threo*-*N*-methyl-4-methylphenidate hydrochloride (compound 2), and *threo*-*N*-benzylmethylphenidate hydrochloride (compound 3), were synthesized in the laboratory of Prof. H. M. Deutsch (Deutsch et al., 1996; Froimowitz et al., 1997).

The pH 7.4 physiological buffer was composed of 124 mM NaCl, 1.80 mM KCl, 1.24 mM KH_2PO_4 , 2.50 mM CaCl_2 , 1.30 mM MgSO_4 , 26.0 mM NaHCO_3 , and 10.0 mM glucose and saturated with a gas mixture of 95% O_2 and 5% CO_2 .

TABLE 1. Values of IC_{50} and Hill coefficient (n_H) for inhibition of [3H]WIN 35,428 binding and [3H]DA uptake for MPD and some of its analogues

Compound ^a	IC_{50} ^b for		n_H for WIN 35,428 binding	Discrimination ratio ^c
	[3H]WIN 35,428 binding	[3H]DA uptake		
MPD	83.0 ± 7.9 (4)	224 ± 19 (4)	0.90 ± 0.09	2.7 ± 0.34
1	5.30 ± 0.70 (2) ^d	7.00 ± 0.60 (2) ^d	2.08 ± 0.05 ^d	1.3 ± 0.20 ^d
2	140 ± 9.3 (4) ^d	918 ± 150 (4) ^d	1.02 ± 0.03	6.6 ± 1.2 ^d
3	52.9 ± 2.3 (3) ^d	273 ± 28 (3)	1.08 ± 0.02	5.2 ± 0.58 ^d

^a See Fig. 1 for chemical structures. The data for MPD and compound 1 were obtained from the results of a previous study (Deutsch et al., 1996).

^b Values for IC_{50} listed are in nanomolar units with the number of experiments in parentheses.

^c Discrimination ratio = IC_{50} for [3H]DA uptake/ IC_{50} for [3H]WIN 35,428 binding, and the values of precision are propagated errors.

^d $p \leq 0.0002$ relative to the result with MPD in each column.

RESULTS AND DISCUSSION

Some analogues of MPD discriminate between inhibition of tropane inhibitor binding and inhibition of uptake of [3H]DA

Table 1 lists the IC_{50} values for MPD and its tested analogues for inhibition of [3H]WIN 35,428 binding and the uptake of [3H]DA. The rank order, compound 1 > compound 3 \cong MPD > compound 2, of inhibition of binding matched that of the rank order of inhibition of uptake. The values of n_H for inhibition of [3H]WIN 32,428 binding were unity except for compound 1, where the value was found to be 2. Compounds 2 and 3 and to a lesser extent MPD were found to discriminate between inhibition of the binding of the tropane ligand and inhibition of [3H]DA uptake. Compound 1 was found not to discriminate between the two modes of action. Therefore, MPD and compounds 2 and 3 were studied further by RDE methodology (below) to investigate further differences in the kinetic mechanisms of action of these inhibitors in relation to DA transport and their relative binding sites.

MPD competitively inhibits DAT activity, whereas MPD derivatives are noncompetitive inhibitors

Figure 2A illustrates the concentration dependence of the inhibition of the transport of DA by MPD. It fit a first-order expression with an intercept of the curve fit line at 345 ± 30 pmol/s/g wet weight (mean \pm SER), which is indistinguishable from the experimental value of 345 ± 11 pmol/s/g (mean \pm SEM), suggesting that the reaction order for inhibition of DA transport by MPD is unity. MPD and its analogues were not observed to induce release of DA within the incubation times and at the concentrations used in these experiments. Figure 2B shows the results of a Hill plot of the data with the IC_{50} estimated from the x -intercept. The apparent reaction orders and IC_{50} values for MPD and its analogues were estimated in the same fashion and are listed in Table 2. The rank order of the inhibition was compound 1 > compound 3 \cong MPD > compound 2. These results agree with those listed in the experimental results of Table 1. The n_H values for the analogues are close to unity.

Except for the case of compound 1, these results also agree with those listed in Table 1.

Figure 3 illustrates the fit of the velocity of the striatal DAT to the Michaelis–Menten expression (upper curve). The K_m and V_{max} values (mean \pm SER) for controls were found to be $1.18 \pm 0.2 \mu M$ and 829 ± 113 pmol/s/g, respectively. An analysis of the inhibition pattern of MPD by curve-fitting velocity versus $[DA]_o$ in the presence of $2.0 \mu M$ MPD, a concentration close to its IC_{50} , is also shown. The best fit is to that of a competitive inhibition model. Similar analyses were conducted at two other concentrations of MPD, 1.0 and $3.0 \mu M$, and the same result was obtained (raw data not shown). The

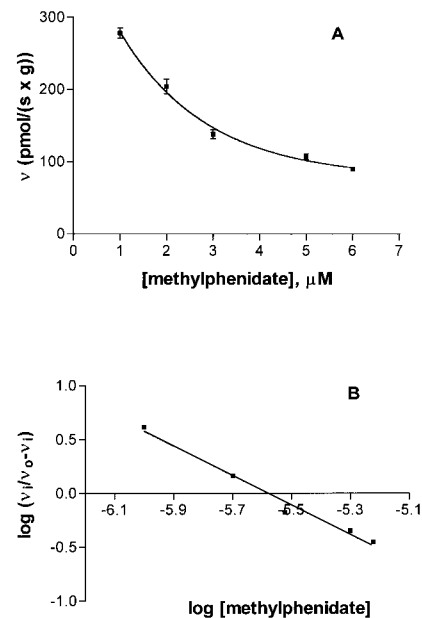


FIG. 2. Concentration-dependent inhibition of the velocity of the striatal transport of DA by MPD and its Hill analysis. **Upper panel:** The solid line is the best fit of a first-order exponential expression to the data. **Lower panel:** Hill plot of the data. Its slope is close to unity (see Table 1), and its correlation coefficient was found to be 0.981 with a post hoc significance of linear regression at the 99.9% confidence level.

TABLE 2. Hill coefficient (n_H), IC_{50} values, mechanism of inhibition, and inhibition constant for inhibition of transport of DA by MPD and its analogues

Analogue	n_H	IC_{50} (μM)	Mechanism of inhibition	K_i (μM)
MPD	1.3 ± 0.1	2.6 ± 0.2	Competitive	1.4 ± 0.4
Compound 3	0.8 ± 0.1	1.9 ± 0.2	Noncompetitive	1.9 ± 0.2
Compound 2	0.7 ± 0.1	6.0 ± 0.4	Noncompetitive	5.9 ± 1.3
Compound 1	0.7 ± 0.1	0.065 ± 0.005	NT	NT

The values for n_H and the IC_{50} were estimated from a fit of the v of the transport observed at $1.0 \mu M$ DA as a function of [analogue] to Eq. 4. The indicators of precision are SER. NT, not tested.

overall fit of all the data to the competitive inhibition model was $\pm 15\%$ with no systematic deviation of estimated values of the competitive inhibition constant (K_{ic} ; average propagated SER, $1.4 \pm 0.4 \mu M$; range, 1.4 – $1.5 \mu M$), whereas the fits to the other models varied systematically by $>50\%$, and the values of the inhibition constants also varied systematically with the tested [MPD]. Table 2 lists the results of similar studies conducted with the MPD analogues, compounds 2 and 3. Both analogues were found to exhibit a noncompetitive mechanism of inhibition.

MPD binds to phenethylamine substrate and cocaine binding sites at the kinetically active DAT

The results of previous work from this laboratory suggest that amphetamine and *m*-tyramine bind at the same site as DA as well as with each other, indicating that these inhibitors are substrate analogues (Wayment et al., 1998). Also, at the incubation times and concentrations used, these agents were not observed to induce release of DA and were deemed to be acting only as substrate analogues at the DAT (Wayment et al., 1998). In contrast, cocaine was not found to possess properties of a substrate analogue, and its binding can be distinguished from that of the substrate analogues (Meiergerd and Schenk, 1994; Wayment et al., 1998). Thus, MPD was tested against *m*-tyramine and amphetamine (Fig. 4). The results are that MPD, amphetamine, and *m*-tyramine bind at a single site on the

DAT. MPD and cocaine were found also to bind to the same site (Fig. 5). Thus, MPD appears to have a binding site that overlaps with cocaine and amphetamine, two inhibitors whose binding to the DAT can be differentiated in this kinetic model.

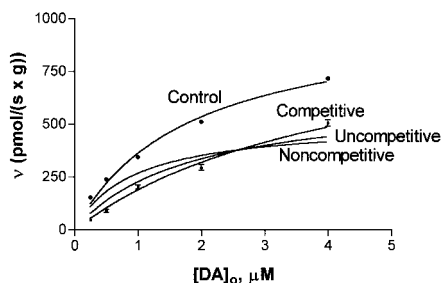


FIG. 3. Velocity (v) versus $[DA]_o$ profile and its fit (upper line) to the Michaelis–Menten expression in controls and to Eqs. 1–3 in the presence (lower lines) of $2.0 \mu M$ MPD. The inhibition pattern fits an apparent competitive mechanism. Data are mean \pm SEM (bars) values, and SEM values not visible are within the dimensions of the symbol.

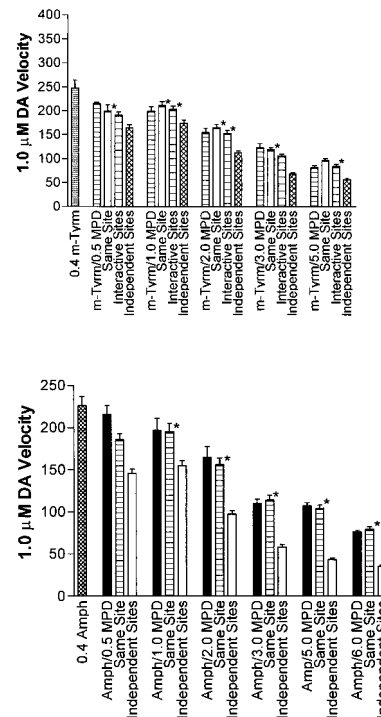


FIG. 4. Comparisons of inhibitions of DAT activity by MPD with those observed with the substrate analogue inhibitors *m*-tyramine (*m*-Tyrm) and amphetamine (Amph). The indicators of precision are SEM of values of velocity obtained by linear regression analyses, and the errors indicated in the values predicted for a given model (Eqs. 1–3) are the experimental SEM propagated through the mathematics of the individual model used. The asterisks indicate predicted results not statistically different from experimental data. The bars without asterisks represent differences of predicted results from the experimental data at $p \leq 0.001$ via a z test. The transport velocity for all experiments shown was measured at $1.0 \mu M$ DA, which in the absence of an inhibitor has a velocity of 345 ± 11 pmol/s/g wet weight. The inhibited control velocities were 248 ± 14 , 278 ± 7 , 204 ± 10 , 138 ± 4 , 107 ± 4 , and 90 ± 1 in the presence of 0.5 , 1.0 , 2.0 , 3.0 , 5.0 , and $6.0 \mu M$ MPD, respectively.

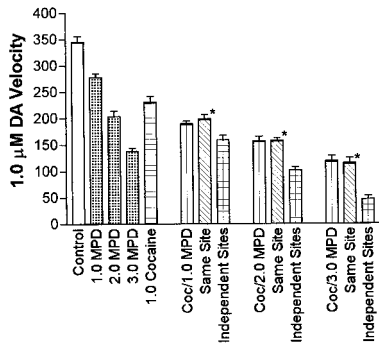


FIG. 5. Comparison of inhibition of DAT activity by MPD with that observed with the non-substrate analogue inhibitor cocaine (Coc). The indicators of precision and the asterisks have the same significance as in Fig. 4.

MPD modified by a benzyl group at the nitrogen binds at a separate but interacting site with MPD

When MPD was tested against its analogues, it was found that compound 2 and compound 1 inhibited the transport of DA at a site in common with MPD (Fig. 6). It was also found that the dichloro analogue (compound 1) was ~ 20 -fold more potent than compound 2 in its ability to inhibit DA transport. Figure 6 also shows that the MPD analogue, modified by the introduction of a large bulky group (a benzyl group at the nitrogen), compound 3, possesses properties different from those of the other two analogues in that it binds to a different but interacting site relative to MPD with a K_{int} value of $4.29 \pm 0.79 \mu M$.

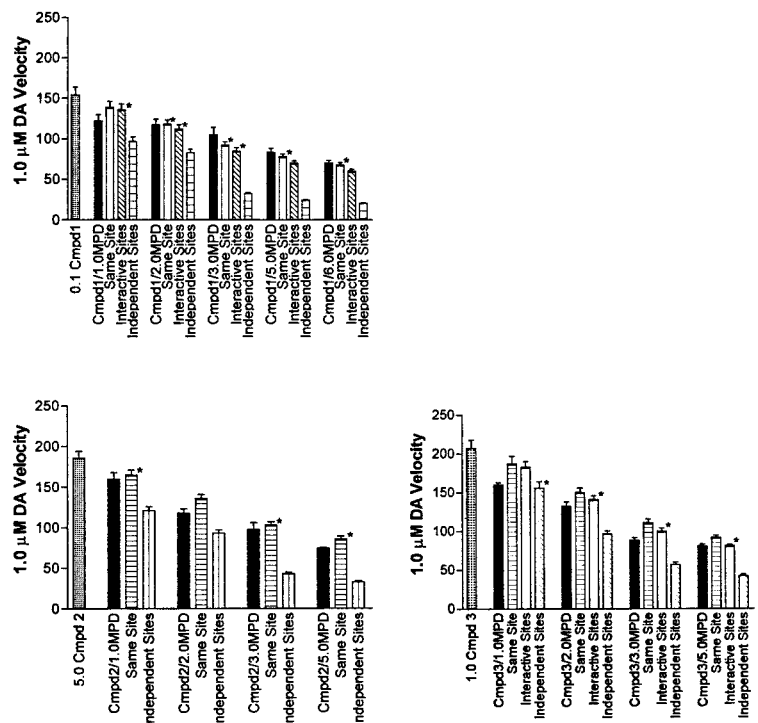


FIG. 6. Comparison of inhibitions of DAT activity by MPD with that observed with the MPD analogues, compounds (Cmpds) 1, 2, and 3. The indicators of precision and the asterisks have the same significance as in Fig. 4. The transport velocity for all experiments shown was measured at $1.0 \mu M$ DA. The control velocity (in units of pmol/s/g wet weight) was 324 ± 10 , and the values in the presence of MPD are the same as those in Fig. 4.

MPD modified at the nitrogen antagonizes the effects of amphetamine on DAT activity and binds to a site in common with cocaine

The analogues, compounds 2 and 3, were tested further against cocaine (Fig. 7) and amphetamine (Figs. 8 and 9). These two analogues were chosen because they have similar IC_{50} values for inhibition of DA transport and because one (compound 2) binds at an apparent site in common with MPD, whereas the other (compound 3) binds at a separate but interacting site with MPD. Like MPD these analogues were found to bind to a site in common with cocaine. In contrast, the MPD analogues did not fit one of the three binding models when tested against amphetamine (Figs. 8 and 9). It was found that the inhibition of DA transport for compound 2 paired with amphetamine (Fig. 8, upper panel) was *less* than that predicted by the same site model given by Eq. 1, and the two lines were found to be statistically different. The same site model predicts the smallest inhibitions of the three models. However, the experimental and predicted lines for compound 2 converged at larger concentrations of amphetamine. When the concentration of compound 2 was varied at a constant concentration of amphetamine (Fig. 8, lower panel), the two lines were not found to be different and are deemed to fit the same site model. These results suggest that compound 2 may “antagonize” the action of amphetamine and that amphetamine overcomes this antagonism only at larger concentrations.

A more dramatic demonstration of the possibility of “antagonizing” the action of one inhibitor of DA transport by another is shown in Figs. 9 and 10. Figure 9

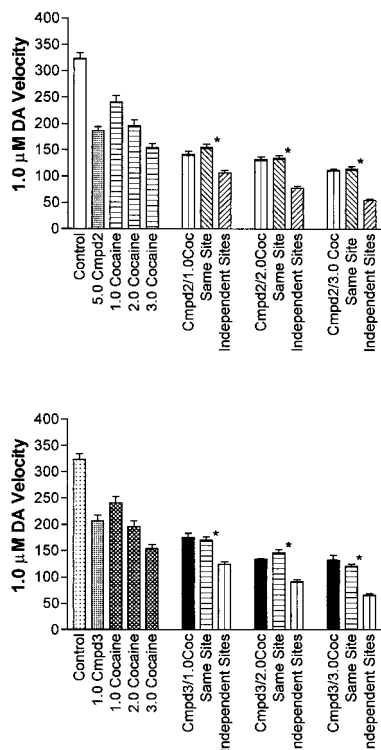


FIG. 7. Comparison of inhibitions of DAT activity by the MPD analogues, compounds (Cmpds) 2 and 3, with that by cocaine (Coc). The transport velocity for all experiments shown was measured at 1.0 μM DA. The indicators of precision and the asterisks have the same significance as in Fig. 4.

shows clearly that the MPD analogue, compound 3, antagonizes the action of amphetamine in the upper panel, and the converse is shown in the lower panel. In Fig. 10, compounds 2 and 3 were tested against each other. When compound 2 was present at a constant concentration (5.0 μM) and the concentration of compound 3 varied, no increase in inhibition was observed as a function of the concentration of compound 3, and the observed degree of inhibition was less than that predicted by a single binding site model (Fig. 10, upper panel). Clearly, compound 2 “antagonized” the action of compound 3. When the experiment was reversed such that compound 3 was present at a constant concentration (1.0 μM) and the concentration of compound 2 was varied, at small concentrations significantly less inhibition was observed than would have been expected in a model predicting binding at a site in common, and this effect was overcome only at higher concentrations of compound 2 (Fig. 10, lower panel).

Summary of the overall findings

MPD was found to inhibit the striatal transport of DA by a mechanism (competitive) distinguishable from that of cocaine [*uncompetitive* (see McElvain and Schenk, 1992)] but not from that of amphetamine [*competitive* (see Wayment et al., 1998)]. However, MPD was not found to induce release of DA, suggesting that it is not a

substrate analogue at the DAT, a finding consistent with those of others (Sonders et al., 1997). When the inhibition sites for these three inhibitors were evaluated in various paired combinations with MPD, all three appeared to bind at a common inhibitory binding site on the striatal DAT. When MPD was modified by halogenating the aromatic ring with chlorine, the affinity for DAT was greatly increased. Placing a methyl group on the nitrogen and at position 4 on the aromatic ring or a benzyl substituent on the nitrogen of MPD altered the inhibitory profile of the parent MPD by converting the inhibition pattern from a competitive mechanism to a *noncompetitive* mechanism with respect to DA and providing an interaction with the DAT that strongly antagonizes the action of amphetamine.

Comments on “antagonism” at a transporter binding site and suggestions for further study

The finding here that MPD analogues may interfere with the effects of amphetamine at the DAT requires further examination experimentally as well as conceptually. Amphetamine has been shown by us in the RDE-monitored model as well as by others using other systems of transport monitoring to be a substrate analogue (see Wayment et al., 1998, and references therein) at the DAT. The analogues of MPD do not exhibit properties suggesting that they too are substrate analogues; rather,

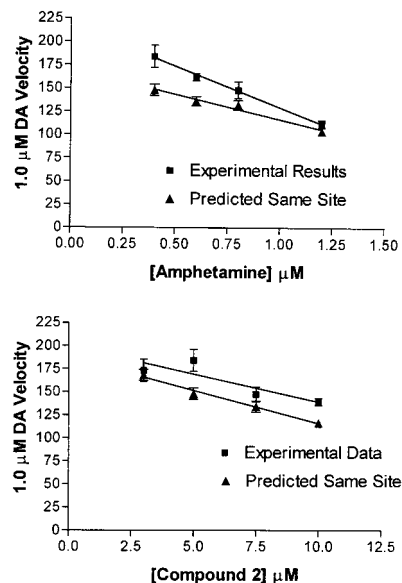


FIG. 8. Concentration dependence of inhibition of the DAT by amphetamine in the presence of a constant 5.0 μM concentration of compound 2 (upper panel) and compound 2 at a constant concentration of 0.4 μM amphetamine (lower panel). Each panel also shows a line from predictions made from the same-site model, Eq. 1. In the upper panel the two lines are statistically different at the 99.99% confidence level, whereas the two lines in the lower panel are not different from each other. The transport velocity for all experiments shown was measured at 1.0 μM DA. The values of velocity at 5.0 μM compound 2 and 0.4 μM amphetamine were 186 ± 8 and 226 ± 11 pmol/s/g wet weight, respectively.

these compounds exhibit characteristics of allosteric modulators of DAT activity. The findings here suggest that an allosteric modulator of the activity of DAT can modify the effects of a transporter substrate analogue (amphetamine) without further affecting the activity of the natural substrate (DA) at the transporter. This result suggests several challenges to the understanding of these findings. First, the term *antagonist*, usually reserved for describing a pharmacological effect at a receptor whereby the activity of the natural ligand is reduced by the presence of the antagonist, is not met here. What is observed here is that an inhibitor of transport activity is antagonized by an agent that spares some activity of the natural ligand; however, the inhibitor appears (via kinetic analysis) to act at the same site as the natural ligand. This places the development of a model to account for these findings in a particularly challenging position of describing how one substrate analogue can act differently from another substrate analogue. It would have to be believed that the catalysis of DA transport requires that DA and the DAT achieve a transition state whereby a path for movement of DA through the protein matrix can occur. By analogy, this requirement must also exist for amphetamine to act as a substrate analogue. Perhaps the allosteric (relative to the substrate site) binding of the MPD analogues serves to make the formation of the transition state for amphetamine more difficult than for DA; thus,

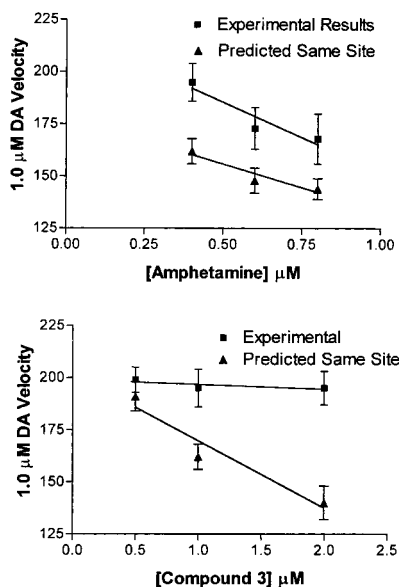


FIG. 9. Concentration dependence of inhibition of DAT by amphetamine in the presence of a constant 1.0 μM concentration of compound 3 (**upper panel**) and compound 3 at a constant concentration of 0.4 μM amphetamine (**lower panel**). Each panel also shows a line from predictions made from the same-site model, Eq. 1. Analyses by linear regression indicated that the two lines in the upper panel are statistically indistinguishable, whereas the two lines in the lower panel are statistically different. The transport velocity for all experiments shown was measured at 1.0 μM DA. The values of velocity at 1.0 μM compound 3 and 0.4 μM amphetamine were 207 ± 11 and 226 ± 11 pmol/s/g wet weight, respectively.

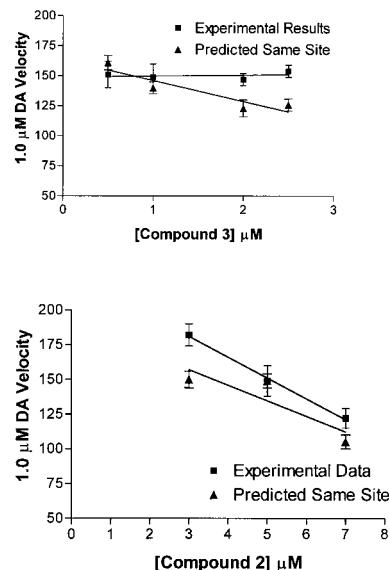


FIG. 10. Concentration dependence of inhibition of DAT by compound 3 in the presence of a constant 5.0 μM concentration of compound 2 (**upper panel**) and compound 2 at a constant concentration of 1.0 μM compound 3 (**lower panel**). Each panel also shows a line from predictions made from the same-site model, Eq. 1. The two lines in the upper panel were found to be statistically different in both the slopes and intercepts, whereas the two lines in the lower panel were found to be statistically indistinguishable. The transport velocity for all experiments shown was measured at 1.0 μM DA, and the values of velocity for the inhibitions observed for compound 2 at 5.0 μM and compound 3 at 1.0 μM are given in the legends of Figs. 8 and 9.

the catalysis of the movement of DA can proceed where that for amphetamine is blocked.

Comments on the potential use of MPD analogues as antagonists of the action of amphetamine

It is intriguing to note that a *noncompetitive* (allosteric) inhibitor of the DAT can block the action of a substrate analogue inhibitor (amphetamine) without significantly reducing further the velocity of transport of the natural substrate, DA. Although the exact mechanisms defining these observations are unknown at present (see above), these results offer the hope of developing agents to antagonize the action of the amphetamine class of drugs of abuse while sparing functional transport of DA by the DAT. These agents may be derived from the MPD class of inhibitors of the DAT. It appears from the studies here that at least micromolar concentrations of MPD analogues will be required in the brain to produce the pharmacological effect on amphetamine. The results of recent pharmacokinetic and pharmacodynamic studies of MPD suggest that this may be possible given that a systemic dose of MPD results in $\sim 1,000$ ng/g of whole tissue levels and concentrations of MPD in in vivo microdialysates of ~ 1 μM (Aoyama et al., 1996).

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