

# Relationship between Conformational Changes in the Dopamine Transporter and Cocaine-Like Subjective Effects of Uptake Inhibitors

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## ABSTRACT

Cocaine exerts its stimulatory effect by inhibiting the dopamine transporter (DAT). However, novel benztropine- and rimcazole-based inhibitors show reduced stimulant effects compared with cocaine, despite higher affinity and selectivity for DAT. To investigate possible mechanisms, we compared the subjective effects of different inhibitors with their molecular mode of interaction at the DAT. We determined how different inhibitors affected accessibility of the sulfhydryl-reactive reagent [2-(trimethylammonium)ethyl]-methanethiosulfonate to an inserted cysteine (I159C), which is accessible when the extracellular transporter gate is open but inaccessible when it is closed. The data indicated that cocaine analogs bind an open conforma-

tion, whereas benztropine and rimcazole analogs bind a closed conformation. Next, we investigated the changes in inhibition potency of [<sup>3</sup>H]dopamine uptake of the compounds at a mutant DAT (Y335A) characterized by a global change in the conformational equilibrium. We observed a close relationship between the decrease in potencies of inhibitors at this mutant and cocaine-like responding in rats trained to discriminate cocaine from saline injections. Our data suggest that chemically different DAT inhibitors stabilize distinct transporter conformations and that this in turn affects the cocaine-like subjective effects of these compounds in vivo.

Abuse of cocaine represents an increasing worldwide problem, with up to 3 million cocaine abusers in the United States alone. The personal consequences of cocaine abuse are severe, sometimes leading to persistent psychiatric disease (Nnadi et al., 2005). Cocaine abuse also represents a major socioeconomic burden with increased medical expenses, lost

earnings, and increased crime. It is accordingly a long-sought wish to develop a medication that can aid in the treatment of cocaine addiction, but attempts to develop such a drug have thus far been unsuccessful.

Cocaine exerts its stimulatory effect by competitively inhibiting the function of the dopamine transporter (DAT) (Giros et al., 1996; Chen et al., 2006). This transporter, which belongs to the solute carrier 6 gene family of Na<sup>+</sup>/Cl<sup>-</sup>-coupled transporters, tightly controls the termination of dopaminergic signaling by mediating rapid reuptake of dopamine from the synaptic cleft (Giros et al., 1996). Inhibition of the DAT by cocaine increases extracellular dopamine levels that in turn produce the psychomotor stimulant and reinforcing effect that underlies cocaine abuse (Volkow et al., 1997).

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**ABBREVIATIONS:** DAT, dopamine transporter; BZT, benztropine; TM, transmembrane domain; WIN 35,428, 2 $\beta$ -carbomethoxy-3 $\beta$ -(4-fluorophenyl)tropane; RTI-55, 2 $\beta$ -carbomethoxy-3 $\beta$ -(4-iodophenyl)tropane; AHN 1-055, 3 $\alpha$ -[bis-(4-fluorophenyl)methoxy]tropane; AHN 2-005, *N*-allyl-3 $\alpha$ -[bis-(4-fluorophenyl)methoxy]tropane; RT, room temperature; MTSET, [2-(trimethylammonium)ethyl]-methanethiosulfonate; FR, fixed ratio; ANOVA, analysis of variance; LeuT, leucine transporter; WT, wild type; NET, norepinephrine transporter; SERT, serotonin transporter; Ro 41-0960, 2'-fluoro-3,4-dihydroxy-5-nitrobenzophenone.

Recently, there has been increasing focus on novel dopamine uptake inhibitors as potential medications for cocaine addiction (Dutta et al., 2003). Among these compounds are analogs of bupropion (BZT) or rimcazone that have similar or higher affinity and selectivity for the DAT than cocaine (Newman and Kulkarni, 2002). The compounds tested so far readily cross the blood-brain barrier (Raje et al., 2003), and they produce increases in extracellular levels of dopamine for longer durations than cocaine (Tanda et al., 2005). Nonetheless, several of these DAT inhibitors are less effective than cocaine as behavioral stimulants (Newman et al., 1995; Katz et al., 2003, 2004; Desai et al., 2005). Furthermore, one BZT analog, JHW 007, was found to potently antagonize the behavioral effects of cocaine (Desai et al., 2005). Assuming a correlation between behavioral effects of cocaine in laboratory animals and abuse potential in humans, these findings suggest JHW 007 as a potential lead for development of cocaine abuse pharmacotherapeutics (Desai et al., 2005).

If the differential behavioral effects of DAT inhibitors are not related to distinct pharmacokinetic properties, it is tempting to suggest that they are linked to different modes of interaction at the DAT. In support of this hypothesis, photoaffinity labeling and site-directed mutagenesis studies have indicated that the binding domain for cocaine in the DAT is at least partially different from that of other structurally divergent dopamine uptake inhibitors (Vaughan et al., 1999; Chen et al., 2004). Moreover, BZT and cocaine have been shown to differentially affect the reaction of sulfhydryl-reactive reagents with endogenous cysteines, indicating that BZT and cocaine stabilize distinct conformational states of the DAT (Reith et al., 2001).

Here, we analyze in detail the relationship between behavioral effects of a series of BZT and rimcazone analogs and their molecular mode of interaction with the DAT. The subjective effects of these compounds are assessed in rats by the cocaine discrimination test. The molecular mode of interaction is assessed 1) by determining conformational changes promoted by the different inhibitors based on the chemical reactivity of a cysteine inserted into transmembrane domain (TM)3 (I159C), and 2) by analyzing how the different compounds are affected by a mutation (Y335A) changing the conformational equilibrium of the transporter (Loland et al., 2002). Taken together, the data demonstrate that different classes of transporter inhibitors promote distinct conformations of the DAT. Moreover, they suggest a relationship between the conformations induced and the different behavioral effects produced in vivo by the distinct transport inhibitors.

## Materials and Methods

**DAT Inhibitors.** The drugs tested are displayed in Fig. 1. Cocaine HCl (Sigma-Aldrich, St. Louis, MO); WIN 35,428 (Sigma-Aldrich); RTI-55 and RTI-31 (generous gifts from Dr. F. Ivy Carroll, Research Triangle Institute, Research Triangle Park, NC); and BZT (Sigma-Aldrich) were obtained from the designated sources; all other inhibitors were synthesized in the Medicinal Chemistry Section (Intramural Research Program, National Institute on Drug Abuse, Baltimore, MD) according to published procedures: diCl-BZT, AHN 1-055 (Newman et al., 1995); AHN 2-003, AHN 2-005, JHW 007, GA 103 (Agoston et al., 1997); GA 2-99 (Robarge et al., 2000); PG01053 (Grundt et al., 2004); MFZ 2-71 (Zou et al., 2003); MFZ 4-86 (Zou et al., 2006); JJC 1-059 (Cao et al., 2001); and JJC 2-010, JJC 2-006

(Cao et al., 2003). Cocaine, BZT, JJC 2-006, AHN 2-005, and GA 103 were dissolved in water, for the in vitro assays; the rest were initially dissolved in dimethyl sulfoxide and subsequently diluted so the concentration of dimethyl sulfoxide never exceeded 0.1% in the experiments. This concentration had no measurable effect on the cells for the duration of the assays (~10 min).

**Site-Directed Mutagenesis.** All mutants were generated in the synthetic human dopamine transporter by two-step polymerase chain reaction mutagenesis as described previously (Loland et al., 2004). E2C DAT in which two external endogenous cysteines were mutated to alanines (C90A-C306A) was kindly provided by Dr. Jonathan A. Javitch (Columbia University, New York, NY).

**Cell Culture and Transfection.** COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.01 mg/ml gentamicin at 37°C in 10% CO<sub>2</sub>. Wild-type and mutant constructs in pcDNA3 were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as described previously (Loland et al., 2004).

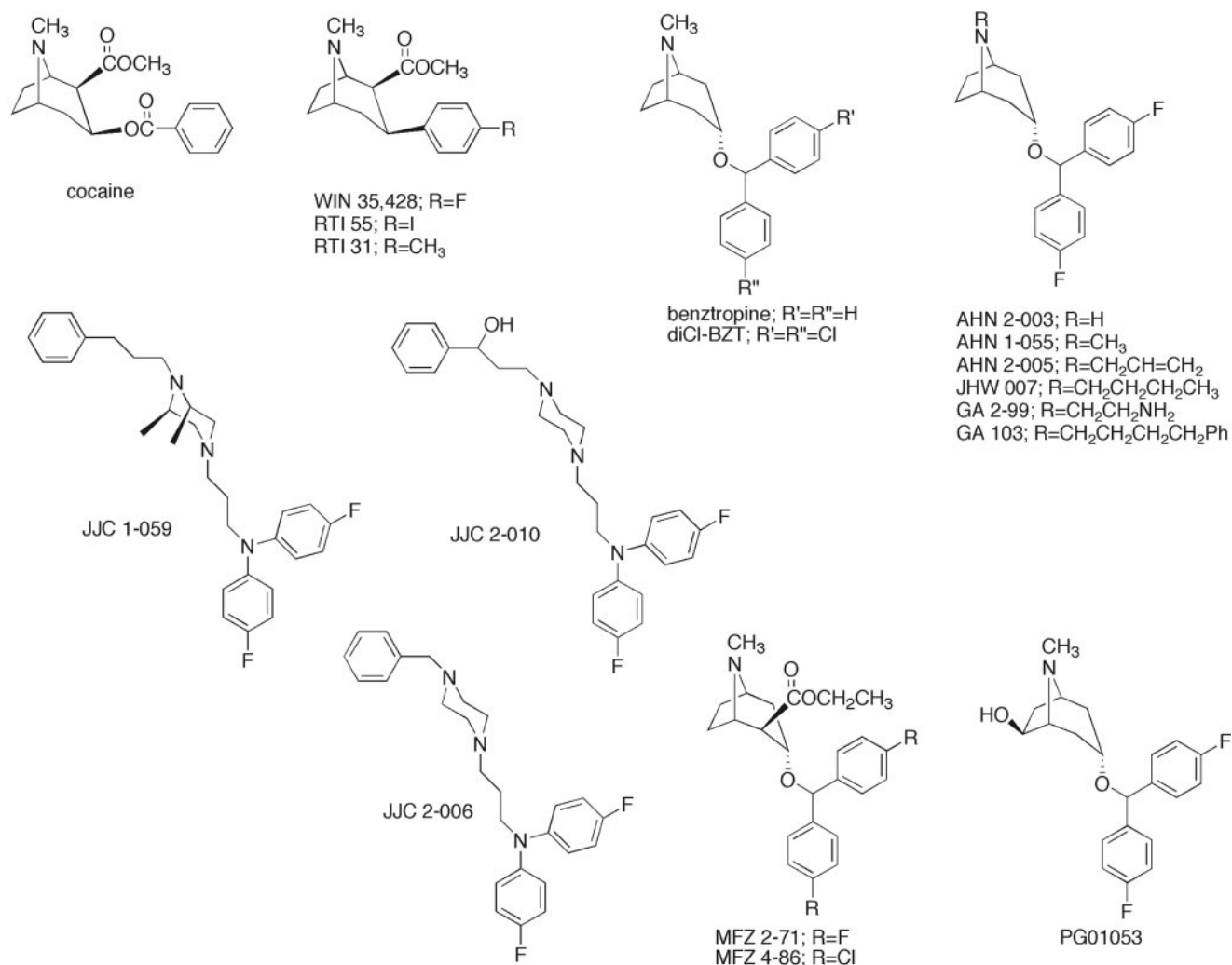
**[<sup>3</sup>H]Dopamine Uptake Experiments.** Uptake assays were essentially performed as described previously (Loland et al., 2004) using 2,5,6-[<sup>3</sup>H]dopamine (8–10 Ci/mmol) (GE Healthcare, Uppsala, Sweden). Transfected COS-7 cells were plated in either 24-well dishes (10<sup>5</sup> cells/well) or 12-well dishes (3 × 10<sup>5</sup> cells/well) coated with poly-D-lysine to achieve an uptake level of no more than 10% of total added [<sup>3</sup>H]dopamine. The uptake assays were carried out 2 days after transfection. Before the experiment, the cells were washed once in 500 μl of uptake buffer (25 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1 mM L-ascorbic acid, 5 mM D-glucose, and 1 μM of the catechol-O-methyltransferase inhibitor Ro 41-0960 (Sigma-Aldrich), pH 7.4, at room temperature (RT). The compound to be tested was added to the cells, and uptake was initiated by addition of 40 nM [<sup>3</sup>H]dopamine in a final volume of 500 μl. After 5 min of incubation at 37°C, the cells were washed twice with 500 μl of ice-cold uptake buffer, lysed in 250 μl (24-well) or 300 μl (12-well) of 1% SDS, and left for 30 min at 37°C. All samples were transferred to 24-well counting plates (PerkinElmer Life and Analytical Sciences, Waltham, MA), 500 μl of Optiphase HiSafe 3 scintillation fluid (PerkinElmer Life and Analytical Sciences) was added, and then the plates were counted in a Wallac Tri-Lux beta-scintillation counter (PerkinElmer Life and Analytical Sciences). Nonspecific uptake was determined in the presence of 1 mM nonlabeled dopamine (Sigma/RBI, Natick, MA). All determinations were performed in triplicate.

**MTSET Labeling Experiments.** Two days after transfection, COS-7 cells expressing either DAT E2C or DAT E2C I159C and seeded in 12- or 24-well plates, were washed once with 500 μl of uptake buffer (same as described above). Subsequently, 400 μl of uptake buffer and 50 μl of either buffer or inhibitor were added in the following concentrations: dopamine, 100 μM; cocaine, 10 μM; WIN 35,428, 0.1 μM; RTI-55, 0.05 μM; RTI-31, 0.05 μM; AHN 1-055, 0.1 μM; GA 103, 0.1 μM; MFZ 2-71, 0.1 μM; MFZ 4-86, 0.5 μM; 4',4"-diCl-BZT, 0.5 μM; BZT, 1 μM; JJC 1-059, 0.5 μM, PG 01053, 0.5 μM, and JHW 007, 5 μM, or as indicated for the dose-dependence analysis. Note that the concentration of inhibitor was chosen as the highest possible concentration that could be washed away to allow subsequent proper [<sup>3</sup>H]dopamine analysis. [2-(Trimethylammonium)-ethyl]-methanethiosulfonate (MTSET; Toronto Research Chemicals, Toronto, ON, Canada) was added to a final concentration of 0.5 mM (or 1 mM for the dose-dependence analysis), and the cells were incubated at RT for 10 min. The stock MTSET solution was freshly prepared in H<sub>2</sub>O, and it was immediately diluted 10-fold by application to the transfected cells into a final volume of 500 μl uptake buffer. After incubation, the cells were washed three times in 500 μl of uptake buffer at RT before initiation of [<sup>3</sup>H]dopamine uptake, performed as described above, but here only the maximal uptake (without unlabeled dopamine) and the nonspecific uptake (in the presence of 1 mM unlabeled dopamine) were determined, both in triplicate. The reaction was stopped and uptake was counted as described above. The effects of the added

compound on MTSET reactivity were determined by calculating the effect of preincubation with the compound alone and with MTSET all performed in parallel on the same plate using triplicate determinations.

**[<sup>3</sup>H]WIN 35,428 Binding Experiments.** Binding assays were carried out on whole cells using [<sup>3</sup>H]WIN 35,428 (87 Ci/mmol; PerkinElmer Life and Analytical Sciences) as radioligand. Cells transfected with synthetic human dopamine transporter E2C I159C were treated as described above. Before the binding experiment, cells were washed once in uptake buffer (as described above at RT), and they were preincubated with 10 μM cocaine and either buffer or 1 mM MTSET for 10 min at RT to achieve maximal labeling of Cys159. Competition binding assays were performed in a final volume of 500 μl of uptake buffer containing 2 to 4 nM [<sup>3</sup>H]WIN 35,428 and indicated concentrations of compound to be tested. Binding was terminated after 2 h at 4°C by washing the cells twice in 500 μl of uptake buffer (4°C) before lysis in 500 μl 1% SDS for 30 min at 37°C. All samples were transferred to 24-well counting plates (PerkinElmer Life and Analytical Sciences), 500 μl of Optiphase HiSafe 3 β-scintillation fluid (PerkinElmer Life and Analytical Sciences) was added, followed by counting of the plates in a Wallac Tri-Lux beta-scintillation counter (PerkinElmer Life and Analytical Sciences). Nonspecific binding was determined in the presence of 10 mM WIN 35,428 (RBI/Sigma). All determinations were made in triplicate.

**Cocaine Discrimination Tests.** Experimentally naive male Sprague-Dawley rats (Taconic Farms; Hudson, NY) weighing 300 to 360 g were individually housed (12-h light/dark cycle; 7:00 AM/7:00 PM) and maintained at 85% of their unrestricted feeding weights. The rats had free access to water, and they were fed 10 to 15 g of food daily, 1 h after testing. Experiments were conducted between 9:00 AM and noon, with subjects placed in a 29.2 × 24.2 × 21-cm operant-conditioning chamber (modified ENV-001; MED Associates, St. Albans, VT) containing two response keys (levers requiring an activation force of 0.4 N) and a centrally located dispenser for delivery of 45-mg food pellets (BioServ, Frenchtown, NJ). A pair of green and a pair of yellow light-emitting diodes were situated above each lever. The chamber was enclosed in a ventilated enclosure that provided light and sound attenuation. White noise was delivered to the chamber at all times to mask extraneous noise. Rats were initially trained with food reinforcement to press both levers, and they were eventually trained to press one after cocaine (10 mg/kg i.p.) and the other after saline (i.p.) injection. Each response produced an audible click. The ratio of responses to food pellets (fixed ratio or FR) was gradually increased until, under the final conditions, the completion of 20 consecutive responses on the cocaine- or saline-appropriate lever produced food. Incorrect responses reset the FR response requirement. The right versus left assignments of cocaine- and saline-appropriate levers were counterbalanced among subjects. Subjects



**Fig. 1.** Chemical structures of the investigated compounds. These compounds include cocaine and cocaine analogs (WIN 35,428, RTI-55, and RTI-31), BZT and BZT analogs (diCl-BZT, AHN 2-003, AHN 1-055, AHN 2-005, JHW 007, GA 2-99, GA 103, MFZ 2-71, MFZ 4-86, and PG 01053), and rimcazole analogs (JJC 1-059, JJC 2-010, and JJC 2-006).



were injected, and then they were placed in chambers. Sessions started with a 5-min time-out period during which lights were off and responses had no consequences, other than producing a click. Following the time-out, the house light was turned on until the completion of the FR20 response requirement and the presentation of food. Sessions ended after 20 food presentations or 15 min, whichever occurred first, and they were conducted 5 days per week, with cocaine or saline sessions scheduled in a mixed sequence. Training continued until subjects met the criteria on four consecutive sessions of at least 85% cocaine- or saline appropriate responding over the entire session, and the first FR. Once these criteria were met, testing began. Test sessions were conducted with the administration of different doses of cocaine, or other test drugs rather than cocaine before sessions. Test sessions were identical to training sessions with the exception that responses on either lever were reinforced with the completion of the FR requirement.

**Locomotor Activity.** Experimentally naive mice were placed singly in clear acrylic chambers (40 cm<sup>3</sup>) contained within monitors (Accuscan Instruments, Inc., Columbus, OH) that were equipped with light-sensitive detectors. The detectors were spaced 2.5 cm apart along two perpendicular walls with infrared light sources mounted on the opposing walls and directed at the detectors. Activity counts were registered for each interruption of a light beam. Mice were injected (i.p. in volumes of 1 ml/100 g), and then they were immediately placed in the apparatus for 1 h, with activity counts totaled each 10 min. Each drug dose was studied in six to eight mice, and mice were used only once.

**Data Calculations.** All data from uptake and binding experiments were analyzed by nonlinear regression analysis using Prism 4.0 (GraphPad Software Inc., San Diego, CA). The IC<sub>50</sub> values were calculated from means of pIC<sub>50</sub> values and the SE interval from the pIC<sub>50</sub> ± S.E. Note that uptake inhibition assays are done under nonequilibrium conditions; therefore, they cannot allow calculation of accurate binding constants. The IC<sub>50</sub> values were accordingly used

as an estimate of the binding potency of the tested compounds. A one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used for identifying values significantly different from cocaine in the MTSET assay.

Locomotor activity in mice was assessed with counts collected and analyzed separately during the first and second 30 min, and data are reported for the time at which maximal effects were observed. Effects of individual doses were determined significant by ANOVA. The maximal locomotor activity induced is expressed as a percentage of the maximum obtained with cocaine. Values for several compounds were taken from the references indicated in Table 1. The values for compounds studied by Cline et al. (1992) were obtained from a 40-min time; therefore, they were expressed as 75% of the total counts obtained.

For each of the rats studied in the cocaine discrimination procedure, the overall rate of response over time and the percentage of responses occurring on the cocaine-appropriate lever (lever selection) were calculated. The mean values were calculated for each measure at each drug dose tested. If less than half of the rats responded at a particular dose, a mean value for lever selection was considered unreliable and it was not calculated. At least 15% cocaine-appropriate responding was adopted as a conservative criterion at which to assume a significant difference from saline; 85% or higher cocaine-appropriate responding was taken as similar to the training dose of cocaine, and intermediate levels of cocaine-appropriate responding were considered partial substitution.

A Fisher's exact test was used to assess the association between whether the drug had a Y335A:WT IC<sub>50</sub> ratio greater than 73, and whether the drug effect met the criterion for full cocaine substitution (>85% cocaine-appropriate responding) in the discrimination procedure or stimulation of locomotor activity significantly greater than values obtained with vehicle injections. The value of 73 was chosen as midway between a gap in the distribution of Y335A:WT IC<sub>50</sub> ratios, between the ratio of 58 obtained with AHN 2-005 and 88 obtained with MFZ 2-71.

TABLE 1

Potencies of inhibitors for DAT WT and Y335A compared with the maximal substitution for cocaine in the cocaine discrimination test and the maximal basal locomotor activity induced by the compounds

IC<sub>50</sub> values were calculated by nonlinear regression analysis of [<sup>3</sup>H]dopamine uptake competition assays in COS-7 cells transiently expressing DAT WT or Y335A. The S.E. interval for each IC<sub>50</sub> value is indicated, and it was calculated from the pIC<sub>50</sub> ± S.E. The maximal substitution for cocaine ± S.E. was determined by the cocaine discrimination test shown in Fig. 5 (bold), or it is listed from previously published data as indicated. The maximal locomotor activity induced was determined for the compounds ± S.E. shown in bold as described under *Materials and Methods* or it was taken from the references indicated.

| Compound         | WT IC <sub>50</sub><br>(S.E. Interval) | <i>n</i> | Y335A IC <sub>50</sub><br>(S.E. Interval) | Maximum Cocaine<br>Substitution ± S.E. | Maximum Locomotor<br>Activity ± S.E. |
|------------------|--|----------|---|--|--------------------------------------|
|                  | <i>nM</i>                              |          | <i>nM</i>                                 | %                                      | counts/30 min                        |
| Cocaine          | 230 [190, 260]                         | 12       | 24,500 [19,900, 30,200]                   | 99.7 ± 0.3 <sup>a</sup>                | 16,880 ± 690 <sup>a,b,c</sup>        |
| WIN 35,428       | 30 [27, 34]                            | 6        | 3100 [2600, 3600]                         | 99.9 ± 0.1 <sup>a</sup>                | 16,900 ± 1300 <sup>b,d</sup>         |
| RTI-31           | 4.3 [3.6, 5.2]                         | 4        | 420 [330, 550]                            | 100 ± 0 <sup>a</sup>                   | 18,900 ± 3200 <sup>b,d</sup>         |
| RTI-55           | 5.0 [4.1, 6.2]                         | 3        | 450 [350, 580]                            | 99.5 ± 0.5 <sup>a</sup>                | 20,800 ± 4600 <sup>d</sup>           |
| <b>MFZ 2-71</b>  | 140 [130, 150]                         | 5        | 12,000 [9100, 16,100]                     | <b>97.3 ± 1.35</b>                     | <b>15,500 ± 3200</b>                 |
| AHN 2-003        | 15 [13, 17]                            | 4        | 880 [660, 1200]                           | 54 ± 20 <sup>a</sup>                   | 6000 ± 8600 <sup>c</sup>             |
| <b>GA 2-99</b>   | 25 [21, 30]                            | 4        | 1300 [1100, 1600]                         | <b>40 ± 17</b>                         | <b>7460 ± 550</b>                    |
| <b>PG 01053</b>  | 24 [20, 29]                            | 4        | 1100 [770, 1600]                          | <b>18 ± 9</b>                          | <b>10,100 ± 2300</b>                 |
| diCl-BZT         | 220 [180, 270]                         | 6        | 9200 [4400, 1900]                         | 18 ± 17 <sup>b</sup>                   | 5500 ± 470 <sup>a</sup>              |
| BZT              | 66 [49, 91]                            | 5        | 2500 [2100, 3100]                         | 49 ± 18 <sup>b</sup>                   | 9500 ± 1600 <sup>a</sup>             |
| AHN 1-055        | 40 [36, 46]                            | 4        | 1400 [1200, 1700]                         | 64 ± 33 <sup>b</sup>                   | 12,450 ± 970 <sup>a</sup>            |
| AHN 2-005        | 120 [89, 170]                          | 4        | 4100 [3300, 5200]                         | 42 ± 17 <sup>c</sup>                   | 6370 ± 610 <sup>c</sup>              |
| <b>JJC 1-059</b> | 120 [100, 130]                         | 3        | 3500 [2700, 4400]                         | <b>19 ± 17</b>                         | <b>8050 ± 510</b>                    |
| <b>JJC 2-010</b> | 66 [44, 99]                            | 3        | 2000 [1400, 2700]                         | <b>65 ± 32</b>                         | N.D.                                 |
| JHW 007          | 130 [95, 170]                          | 4        | 3100 [2400, 4000]                         | 8 ± 5 <sup>c</sup>                     | 6800 ± 320 <sup>c</sup>              |
| <b>MFZ 4-86</b>  | 730 [630, 850]                         | 6        | 9800 [7400, 13,000]                       | <b>35 ± 23</b>                         | <b>11,900 ± 2600</b>                 |
| GA 103           | 310 [280, 340]                         | 4        | 3600 [3100, 4300]                         | 10 ± 7 <sup>c</sup>                    | 8480 ± 480 <sup>c</sup>              |
| JJC 2-006        | 370 [330, 430]                         | 6        | 2700 [2100, 3500]                         | N.D.                                   | N.D.                                 |
| Dopamine         | 850 [760, 940]                         | 9        | 250 [200, 310]                            | N.D.                                   | N.D.                                 |

N.D., not determined.

<sup>a</sup> Data taken from Katz et al. (1999).

<sup>b</sup> Data taken from Izenwasser et al. (1994).

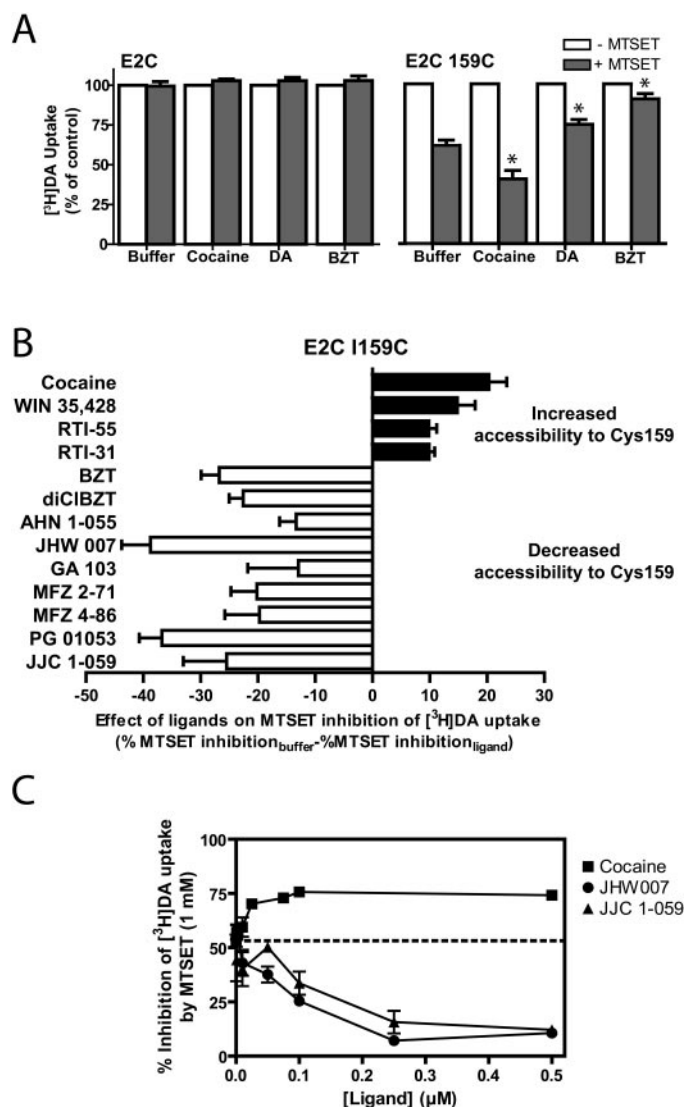
<sup>c</sup> Data taken from Katz et al. (2004).

<sup>d</sup> Data taken from Cline et al. (1992) for comparison.

## Results

**BZT and Rimcazole Analogs Promote Distinct Conformational Changes in the DAT Compared with Cocaine.** To explore possible conformational changes in DAT in response to binding of BZT and rimcazole analogs versus cocaine and cocaine analogs (Fig. 1), we used an assay that allowed us to assess the conformational state of the transporter upon binding of the different ligands. The assay was based on the reactivity of cysteine inserted into position 159 in TM3 of the DAT. Previous observations in DAT, and in the transporters for norepinephrine (NET) and serotonin (SERT), have suggested that the accessibility of a cysteine in this position is dependent on whether the extracellular gate is open or closed: Cys159 is accessible to the extracellular environment when the extracellular gate is open, but it is inaccessible when the gate is closed (Chen and Rudnick, 2000; Loland et al., 2004). In agreement with this notion, the position is inaccessible in the crystallized conformation of the LeuT (Ile111), a bacterial homolog of the DAT, which is characterized by a closed extracellular gate (Yamashita et al., 2005). Importantly, reaction of the cysteine with the sulfhydryl reactive, cell-impermeable, and positively charged methanethiosulfonate MTSET results in inactivation of the transporter, allowing the use of dopamine uptake as a functional readout for I159C reactivity (Chen and Rudnick, 2000; Loland et al., 2004). The I159C mutant was generated in a DAT background (E2C) in which the two external endogenous cysteines were mutated to alanines (C90A-C306A), resulting in DAT E2C I159C (Loland et al., 2004).

As shown in Fig. 2A, preincubation with 0.5 mM MTSET results in a marked inhibition of [ $^3$ H]dopamine uptake in COS-7 cells expressing E2C I159C ( $39 \pm 4\%$  inhibition; mean  $\pm$  S.E.;  $n = 5$ ), whereas no inhibition is observed in COS-7 cells expressing E2C (Fig. 2A). Preincubation with 10  $\mu$ M cocaine together with MTSET potentiated the inhibition ( $60 \pm 5\%$  inhibition; mean  $\pm$  S.E.;  $n = 5$ ), consistent with stabilization of the transporter in a conformation open to the extracellular environment (Fig. 2A). Similarly, preincubation with 100  $\mu$ M dopamine protects against MTSET inactivation ( $25 \pm 3\%$  inhibition; mean  $\pm$  S.E.;  $n = 5$ ) consistent with substrate-promoted closure of the external gate (Fig. 2A). Interestingly, preincubation with BZT did not potentiate MTSET inactivation, as observed for cocaine, but protected against MTSET inactivation, as observed for dopamine ( $9.5 \pm 3\%$  inhibition; mean  $\pm$  S.E.;  $n = 5$ ) (Fig. 2A). The difference between cocaine and BZT was further substantiated by testing several different inhibitors (Fig. 2B). Similar to cocaine, the cocaine analogs WIN 35,428, RTI-31, and RTI-55 all potentiated the effect of MTSET on uptake in E2C I159C by  $14 \pm 3$ ,  $10 \pm 1$ , and  $10 \pm 1\%$ , respectively, relative to the effect of MTSET alone (Fig. 2B). In contrast, we observed protection of E2C I159C by all tested BZT and rimcazole analogs. The highest degree of protection was observed for JHW 007 and PG 01053 ( $-39 \pm 5$  and  $-37 \pm 4\%$ , respectively; Fig. 2B). Note that the concentration of inhibitor was chosen as the highest possible concentration that subsequent to the MTSET incubation could be washed away to allow proper [ $^3$ H]dopamine uptake analysis. Thus, the data in Fig. 2B reflect a qualitative rather than quantitative measurement of whether a compound protects or exposes I159C. To also obtain a more quantitative measure, we determined the concentration dependence for protection or potentiation of



**Fig. 2.** Effect of dopamine transporter inhibitors on MTSET inhibition of [ $^3$ H]dopamine uptake in DAT E2C I159C. A, left, effect of MTSET with and without coinubation ligands on DAT E2C (a DAT mutant in which two endogenous cysteines, Cys90 and Cys306, have been changed to alanines, rendering it insensitive to MTSET). Right, effect of MTSET with and without coinubation ligands on DAT E2C I159C in which Ile159 in TM3 has been substituted with cysteine in the E2C background. DA, dopamine. Data are means  $\pm$  S.E. of five experiments performed in triplicate. Asterisk (\*) indicates significant change ( $p < 0.02$ ) compared with buffer only (unpaired  $t$  test). B, effect of cocaine analogs, BZT analogs, and rimcazole analogs on the MTSET inactivation of E2C I159C compared with MTSET alone. COS-7 cells transiently expressing E2C I159C were preincubated with each compound (see *Materials and Methods* for concentrations) and 0.5 mM MTSET for 10 min followed by thorough washing of the cells and subsequent measurement of [ $^3$ H]dopamine uptake activity. The effects of each compound on MTSET inhibition are expressed as their effect in percentage with the effect of MTSET alone subtracted (% MTSET inhibition<sub>buffer</sub> - % MTSET inhibition<sub>ligand</sub>). Open bars indicate values significantly different from the value observed for cocaine ( $p < 0.01$ , one-way ANOVA with Dunnett's post hoc test). Closed bars indicate values that are not significantly different from cocaine. Data are means  $\pm$  S.E. of three to four experiments performed in triplicate. C, effect of MTSET reactivity as a function of inhibitor concentration. MTSET (1 mM) was added together with different concentrations of either cocaine (■), the BZT JHW 007 (●), or the rimcazole analog JJC 1-059 (▲). A dose-dependent increase in [ $^3$ H]dopamine inhibition was observed for cocaine, whereas JHW 007 and JJC 1-059 showed a dose-dependent protection of the MTSET induced inhibition of [ $^3$ H]dopamine uptake. Data are means  $\pm$  S.E. of three to four experiments performed in triplicate.

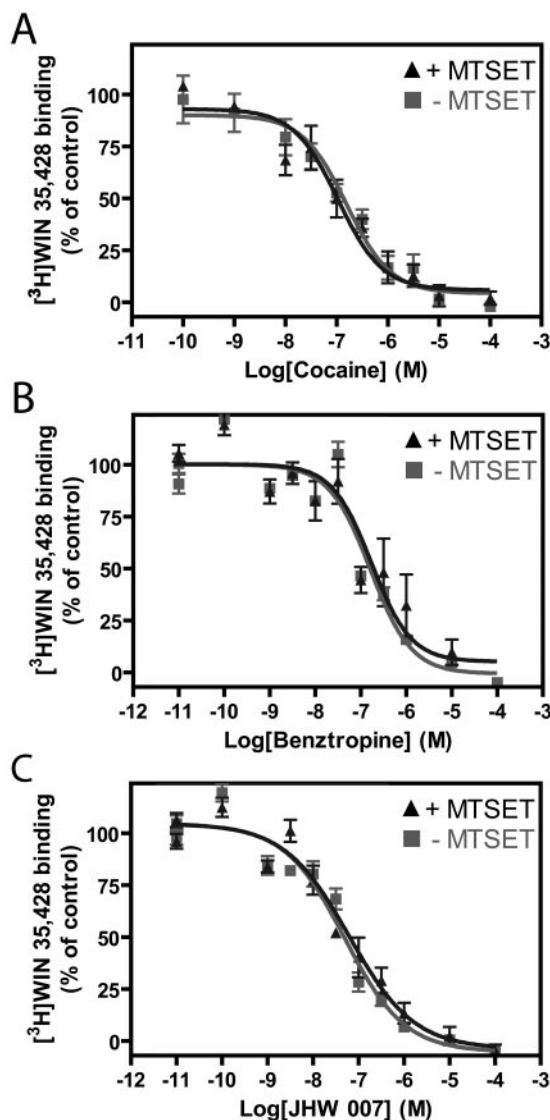
representative compounds including cocaine, the benzotropane analog JHW 007, and the rimcazole analog JJC 1-059. As shown in Fig. 2C, cocaine dose-dependently enhanced the reactivity of I159C, reaching maximum potentiation at a concentration of 0.1 to 0.5  $\mu$ M, whereas both JHW 007 and JJC 1-059 dose-dependently protected against MTSET inactivation, reaching maximum protection at 0.25 to 0.5  $\mu$ M (Fig. 2C).

The protection against MTSET afforded by the BZT and rimcazole analogs could either be due to a direct shielding of the cysteine residue by these compounds because they bind at Cys159 or the result of a conformational change that causes shielding of Cys159. However, if the BZT and rimcazole analogs bind at Cys159, we would expect a decrease in their binding affinities when E2C I159C is premodified with MTSET. To exclude this possibility, we performed a binding assay in which we used the cocaine analog [ $^3$ H]WIN 35,428 as radioligand, and we assessed the binding affinities of selected BZT analogs in competition assays with and without MTSET prelabeling. Prelabeling was obtained with 1 mM MTSET together with 10  $\mu$ M cocaine. This treatment only marginally impaired [ $^3$ H]WIN 35,428 binding (data not shown). As seen in Fig. 3, there were no substantial differences in the affinities for either cocaine ( $EC_{50}$  buffer = 150 [110, 210] nM;  $EC_{50}$  MTSET = 110 [78, 150] nM), BZT ( $EC_{50}$  buffer = 160 [120, 210] nM;  $EC_{50}$  MTSET = 170 [116, 255] nM), or JHW 007 ( $EC_{50}$  buffer = 51 [41, 63] nM;  $EC_{50}$  MTSET = 50 [37, 67] nM). These results suggest that the protection against MTSET by all tested analogs is unlikely the result of a direct shielding of Cys159, but rather it is the result of distinct conformational changes in the DAT protein resulting in closure of the predicted external gate.

**Differential Effects by Mutation of Tyr335 in DAT on the Apparent Binding Affinities of Cocaine, BZT, and Rimcazole Analogs.** In previous studies, we provided evidence that Tyr335 in DAT is critical for regulating conformational isomerization in the transport cycle. Tyr335 is located in the third intracellular loop, and it is 100% conserved throughout the entire family of neurotransmitter/sodium symporter proteins (<http://www.tcd.org/tcd/index.php?tc=2.A.21>). Our data suggested that mutation of this residue changes the conformational equilibrium of the DAT, resulting in a transporter residing preferentially in an inward facing conformation (Loland et al., 2002, 2004) [i.e., the conformational state in which the substrate binding site is accessible to intracellular environment according to an alternating access model for transporter function (Loland et al., 2003)]. In agreement with these predictions, the recent high-resolution crystal structure of LeuT indicates that Tyr335 (Tyr268 in LeuT) is stabilizing a salt bridge between the cytoplasmic ends of TM1 and TM8 that controls access to the substrate binding site from the intracellular side (Yamashita et al., 2005).

Mutation of Tyr335 in DAT to alanine (Y335A) not only produced impaired transport capacity but also the potency of cocaine to inhibit [ $^3$ H]dopamine uptake was markedly impaired (~100-fold, Table 1; Loland et al., 2002). This was unlikely to be due to disruption of direct interaction between cocaine and Tyr335 but rather the result of a changed conformational equilibrium of the DAT during the translocation cycle. Thus, if cocaine binds to an outward facing conformation (in which the substrate binding site is exposed to the

extracellular environment), a major alteration in the conformational equilibrium toward the inward facing conformation would decrease the time during which the cocaine binding site is exposed and thus result in a large decrease in binding potency. Conversely, if a compound recognizes a conformation that is more likely to occur when mutating Tyr335A compared with WT, or the binding site for the compound does not alter significantly during the protein movement-associated translocation, the potency of the compound is likely less, or not affected. Therefore, it should be possible to obtain insight into the binding modes at the DAT for different chemical classes of transport inhibitors by determining their potencies at Y335A, and by comparing them with those obtained at the wild-type DAT. We tested a series of 14 analogs



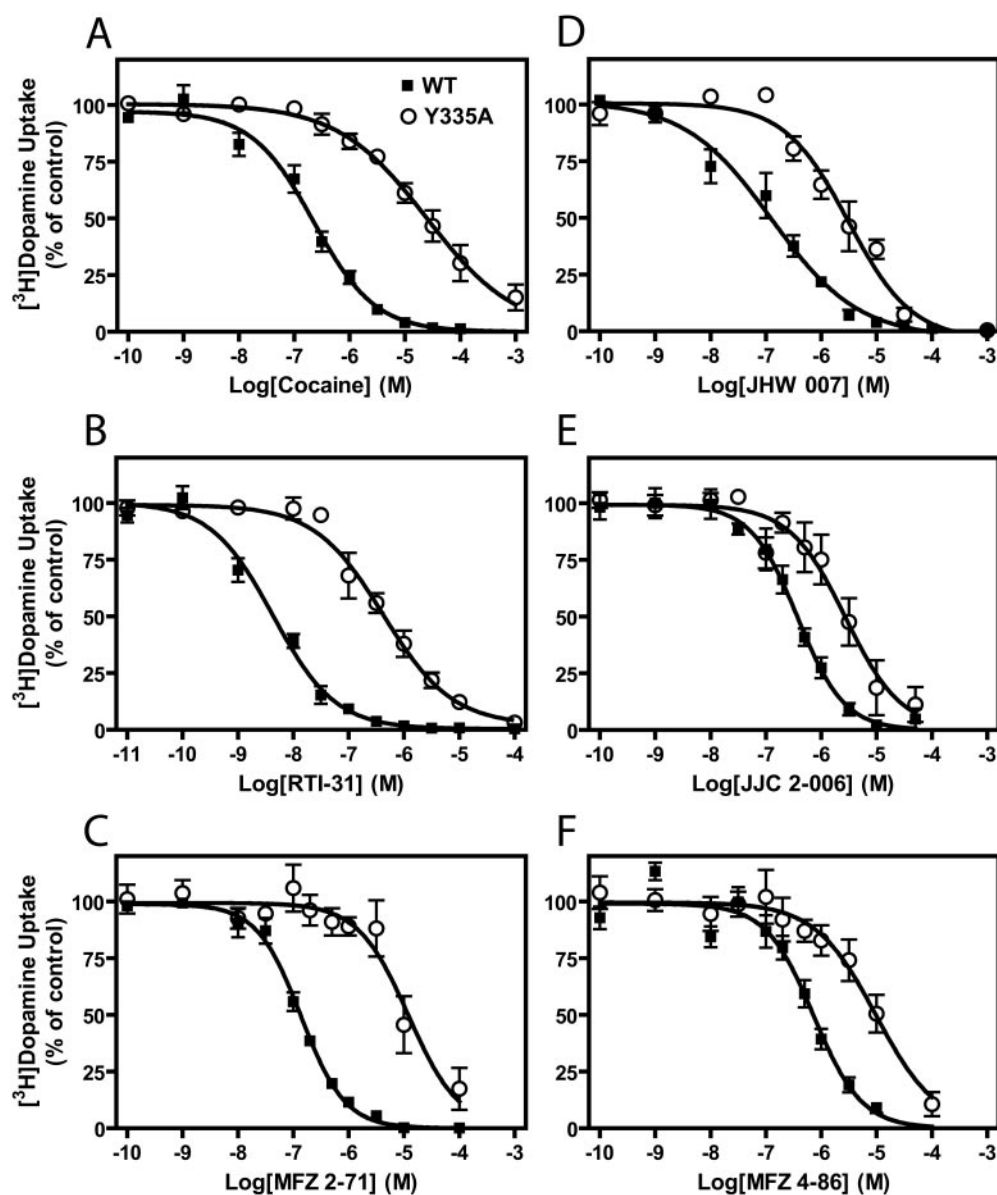
**Fig. 3.** MTSET modification of Cys159 does not change the binding affinity of selected BZT analogs for DAT E2C I159C. Radioligand binding was performed on whole cells (COS-7 cells transiently expressing DAT E2C I159C) using [ $^3$ H]WIN 35,428 as radioligand with and without MTSET prelabeling (1 mM MTSET together with 10  $\mu$ M cocaine). This treatment did not impair [ $^3$ H]WIN 35,428 binding (data not shown). Binding affinities were determined for cocaine (A), BZT (B), and JHW 007 (C). Data are means  $\pm$  S.E. of three experiments performed in triplicate. No effect was observed of the MTSET treatment, suggesting that the protection by BZT analogs against MTSET inactivation (see Fig. 2) is the result of a distinct conformational change induced by the ligands.



of BZT and rimcazole and we compared them with cocaine and three of its analogs (Fig. 1) in [ $^3\text{H}$ ]dopamine uptake inhibition assays performed in COS-7 cells transiently expressing Y335A or wild-type DAT (Table 1; Fig. 4). Interestingly, the potencies of the compounds tested were affected very differently by the Y335A mutation. Whereas cocaine and cocaine analogs, such as WIN 35,428 and RTI-31, displayed around a 100-fold loss in their potency for Y335A compared with wild type, most of the BZT or rimcazole analogs, such as JJC 2-006, JHW 007, MFZ 4-86, and BZT itself, displayed only 7- to 38-fold loss in potency. However, one of the compounds from the BZT class, MFZ 2-71, had an Y335A:WT  $\text{IC}_{50}$  ratio of 88, which was comparable with the cocaine analogs tested and considerably higher than the highest change observed for the remaining BZT and rimcazole analogs (<60-fold) (Fig. 1; Table 1). Notably, this suggests that the I159C reactivity assay and the Y335A assay are unlikely to provide the same conformational readouts since MFZ 2-71 behaved like the other BZTs and not like

cocaine in the I159C reactivity assay (see below for further discussion).

**MFZ 2-71, but Not MFZ 4-86, Is Recognized as Cocaine-Like by Rats Trained to Discriminate Cocaine from Saline.** Previously, we tested several of the BZT and rimcazole analogs in the cocaine discrimination procedure (Cline et al., 1992; Katz et al., 1999, 2004; Table 1); however, MFZ 2-71, MFZ 4-86, JJC 1-059, JJC 2-010, PG 01053, and GA 2-99 had not been tested previously. The results of the experiments with these compounds are shown in Fig. 5, top, as the percentages of responding on the cocaine-appropriate lever. The rimcazole analogs (JJC 1-059 and JJC 2-010) and the BZT analogs (PG 01053, GA 2-99, and MFZ 4-86) each produced either saline-like (<15%) or an intermediate level of cocaine-appropriate responding considered partial substitution. Remarkably, the rats responded to MFZ 2-71 as they did to cocaine, with  $97.3 \pm 1.35\%$  cocaine-appropriate response for the dose that gave maximal substitution (5.6 mg/kg) (Fig. 5A). This was unexpected as most of the BZT and



**Fig. 4.** Inhibition of [ $^3\text{H}$ ]dopamine uptake in COS-7 cells transiently expressing the DAT WT (■) or Y335A (○) by cocaine (A), RTI-31 (B), MFZ 2-71 (C), JHW 007 (D), JJC 2-006 (E), and MFZ 4-86 (F). The calculated difference in  $\text{IC}_{50}$  values between WT and Y335A for a compound is the basis for the data shown in Fig. 6A and Table 1. Data are means  $\pm$  S.E. of 4 to 11 experiments performed in triplicate.

rimcazole analogs that have been tested previously were not considered cocaine like (<85% cocaine-appropriate responding; Cline et al., 1992; Katz et al., 1999, 2003, 2004). However, the full substitution of MFZ 2-71 corresponds with its measured Y335A:WT IC<sub>50</sub> ratio; hence, MFZ 2-71 was the only BZT analog studied with a change in potency comparable with that of cocaine (88- and 101-fold change for MFZ 2-71 and cocaine, respectively; Table 1). In contrast, the structurally similar compound MFZ 4-86 (Fig. 1) did not produce a full substitution for cocaine, but rather it produced a maximal response similar to that seen with the other tested compounds (Table 1; Fig. 5).

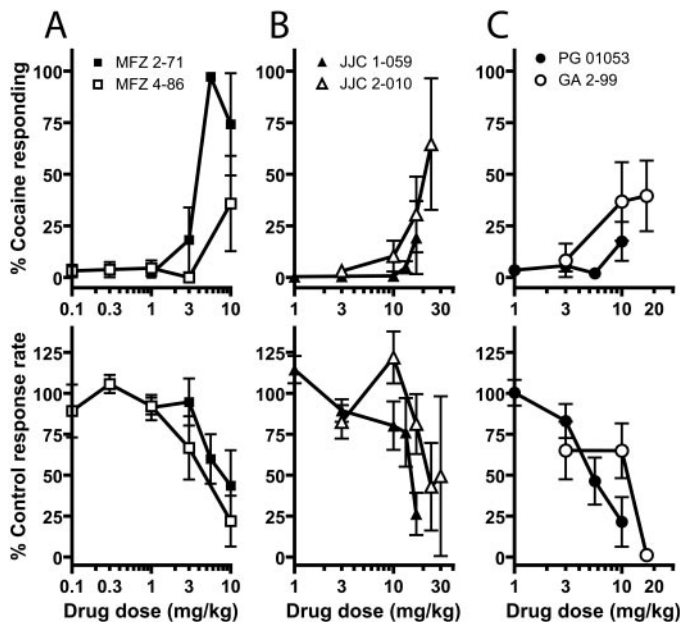
Each of the drugs was tested over a range of doses to ensure that appropriate behaviorally active doses were examined. Each was tested from doses having little or no effect on the rate of response over time to those that virtually eliminated responding (Fig. 5, bottom). In all cases, higher doses could not be tested due to their pronounced behavioral effects that virtually eliminated all responding, or acute toxic effects. Only with MFZ 2-71 was a dose reached that produced greater than 85% cocaine-appropriate responding before producing excessive behavioral disruption or toxicity.

**Effects of BZT Analogs on Locomotor Activity in Mice.** Because the compounds also decreased response rates of rats discriminating cocaine injections, and those effects may have been mediated by another mechanism, it remains possible that the decreases in response rates interfered with the expression of full cocaine-like effects. Therefore, we also

examined the effects of these drugs for stimulation of locomotor activity, a benchmark psychomotor stimulant effect. We previously tested several of the BZT analogs for stimulation of locomotor activity (Cline et al., 1992; Izenwasser et al., 1994; Katz et al., 1999, 2004; Table 1). Tests with MFZ 2-71, MFZ 4-86, JJC 1-059, PG 01053, and GA 2-99 had not been conducted. Among these compounds, MFZ 2-71, MFZ 4-86, and JJC 1-059 produced a statistically significant stimulation of locomotor activity, although the effects of all of these compounds were less than those of cocaine, with the order being MFZ 2-71 > MFZ 4-86 >> JJC 1-059. The maximal effects of these compounds along with those reported previously are shown in Table 1 (fifth column).

**Correlation between Cocaine Discrimination and Change in Measured IC<sub>50</sub> Values at Y335A.** To more fully assess the relationship between cocaine-like subjective effects as determined from the cocaine discrimination procedure and the potency change induced by the Y335A mutation, we compared the maximal cocaine-appropriate responding for all compounds tested in the cocaine discrimination test (Table 1) to the measured Y335A:WT IC<sub>50</sub> ratio (Fig. 6A). The change in potency for each compound was also plotted against its maximal substitution for cocaine. As shown in Fig. 6B, we observed a significant correlation between the two data sets, with  $r^2 = 0.74$  ( $p < 0.0001$ ) in support of a relationship between the behavioral effects of the tested compounds and their mode of interaction with the DAT. As mentioned above, the decreases in response rates (Fig. 5, bottom) may have interfered with the expression of cocaine-like discriminative-stimulus effects. Therefore, we also examined the correlation between maximal stimulant effects (Table 1) and the Y335A:WT IC<sub>50</sub> ratio (Fig. 6A). As shown in Fig. 6C, these effects were also significantly correlated ( $r^2 = 0.590$ ;  $p = 0.0005$ ), further supporting a relationship between the behavioral effects of the tested compounds and their mode of interaction with the DAT.

Because the data as shown in Fig. 6, B and C, had the appearance of drugs with distinct groupings, we further examined the relationships among Y335A:WT IC<sub>50</sub> ratios and behavioral effects by separating the compounds into four groups based on whether they met the criterion for full cocaine substitution (>85% cocaine-appropriate responding) and whether they had an IC<sub>50</sub> ratio greater than 73. (This value was chosen as the midpoint between what seemed to be a natural demarcation between the groups of drugs.) Drugs that both fully substituted for cocaine and had an IC<sub>50</sub> ratio greater than 73 were cocaine, WIN 35,428, RTI-31, RTI-55, and MFZ 2-71. Drugs that did not substitute and had an IC<sub>50</sub> ratio less than 73 were AHN 2-003, GA 2-99, PG 01053, diCl-BZT, BZT, AHN 1-055, AHN 2-005, JJC 1-059, JJC 2-010, JHW 007, MFZ 4-86, GA 103, and JJC 2-006. None of the compounds met the criteria of substituting for cocaine, with an IC<sub>50</sub> ratio less than 73, or not substituting, with an IC<sub>50</sub> ratio greater than 73. The same grouping of drugs was achieved by segregating based on their Y335A:WT IC<sub>50</sub> ratio and whether their stimulant effects were greater than 75% of those produced by cocaine. A Fisher's exact test on these groupings of drugs resulted in a significant association of the IC<sub>50</sub> ratio and cocaine-like subjective effects or in stimulation of locomotor activity to 75% of the response to cocaine (two-sided;  $p = 0.0002$ ).



**Fig. 5.** Effects of the novel compounds MFZ 2-71 (■) and MFZ 4-86 (□) (A), JJC 1-059 (▲) and JJC 2-010 (△) (B), and PG 01053 (●) and GA 2-99 (○) (C) in rats trained to discriminate injections of cocaine from saline. Top, ordinates, percentage of responses on the cocaine-appropriate lever. The percentage of responses emitted on the cocaine-appropriate lever was considered unreliable and not plotted, if less than half the subjects responded at that dose. Bottom, ordinates, rates of response over time (as a percentage of response rates after saline administration). Abscissae, drug dose in milligrams per kilogram (log scale). Each point represents the effect in 4 to 16 rats. Note that only the BZT, MFZ 2-71, fully substituted, and JJC 2-010 approached the effects of cocaine. The remaining BZT analogs tested failed to substitute for cocaine.



## Discussion

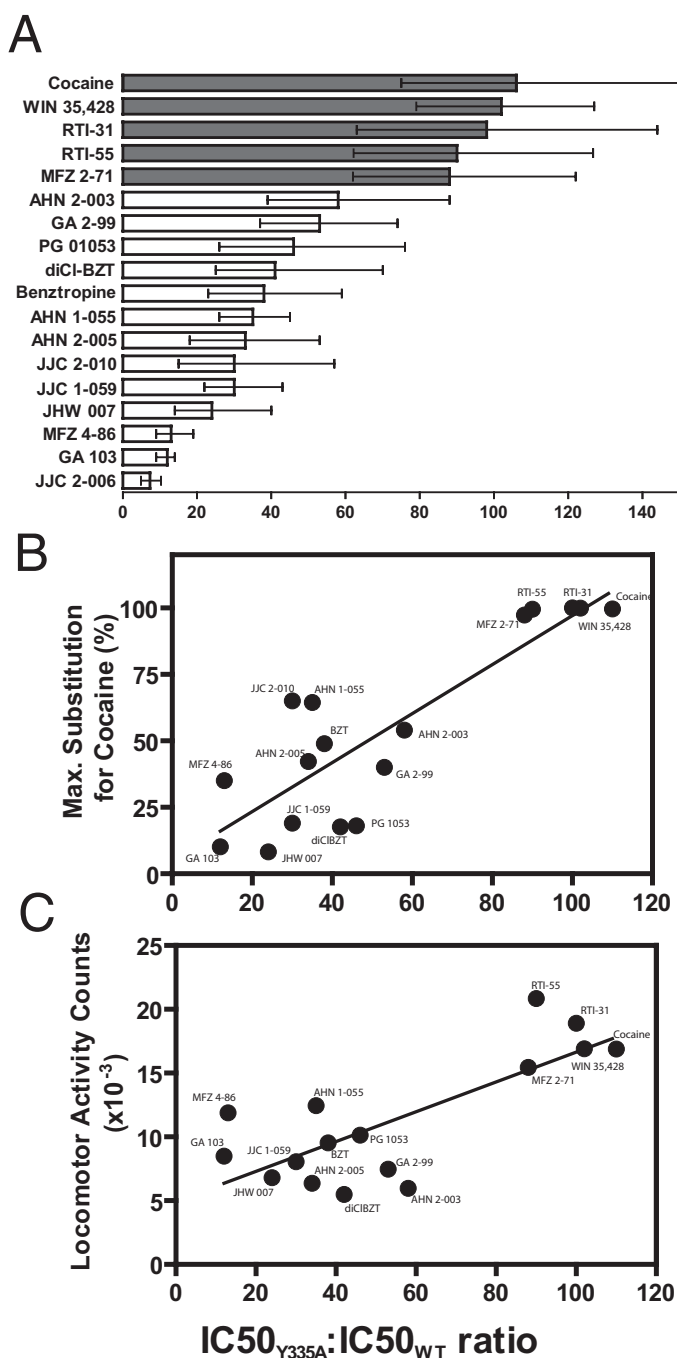
There are currently no medications clinically proven effective for the treatment of cocaine addiction. Therefore, it is highly revealing that several of the DAT inhibitors studied here do not share the same subjective and reinforcing effects as observed for cocaine in drug discrimination (Katz et al., 1999, 2004) and self-administration (Woolverton et al., 2000, 2001) animal models. The compounds might accordingly represent useful leads toward development of medications for cocaine abuse. The mechanisms underlying the reduced behavioral response to BZT and rimcazone analogs have nonetheless remained obscure.

One possible explanation for the reduced behavioral response could be differences in their pharmacokinetic properties. There is, for example, increasing evidence that the rate of DAT occupancy might be an important determinant for the reinforcing and addictive effects of cocaine (Volkow et al., 1997; Desai et al., 2005). Also, the rate by which cocaine is delivered into the brain could be important (Samaha and Robinson, 2005). However, BZT and rimcazone analogs readily cross the blood-brain barrier (Raje et al., 2003), and they cause increases in the extracellular levels of dopamine for longer durations than cocaine (Tanda et al., 2005). Therefore, it is tempting to speculate that the reduced reinforcing effect of these DAT inhibitors are not, at least solely, determined by their pharmacokinetic differences, but rather by their pharmacodynamic properties.

In the present study, we have accordingly explored the mode of interaction of 14 BZT and rimcazone analogs with the DAT, and we directly compared the results of these experiments with the effects of the same compounds in behavioral models. In a first approach we used a cysteine reactivity assay and we obtained evidence that BZT, rimcazone, and cocaine analogs promote distinct conformational changes in the DAT. Cocaine and cocaine analogs increased the reactivity of I159C in TM3, whereas all the tested BZT and rimcazone analogs decreased the reactivity. In a second approach, we compared BZT and rimcazone analogs to cocaine and several of its analogs in DAT Y335A, a mutant characterized as causing a shift in the conformational equilibrium (Loland et al., 2002, 2004). By quantifying the behavioral response to the different compounds according to the cocaine discrimination test, we obtained a correlation between the Y335A:WT  $IC_{50}$  ratio for the tested compounds and cocaine-appropriate responding (Fig. 6). Thus, it seemed that the behavioral response to a DAT inhibitor might be predictable from its interaction with DAT Y335A, and thereby by its mode of interaction with the transporter.

The Y335A:WT  $IC_{50}$  ratio correlation was observed in both the locomotor activity test and the cocaine discrimination test. This limits the chances that the reduced and differential effects of the BZT and rimcazone analogs are the result of unknown "side effects" at other sites than DAT. In that case, such an action would not only have to be shared within the group of tested drugs of various structures that were less efficacious than cocaine but also that action would have to decrease rates of learned operant behavior in the discrimination procedure and interfere with the stimulation of locomotor activity.

$IC_{50}$  Y335A: $IC_{50}$  WT ratio for each compound and its ability to induce an increase in basal locomotor activity. The  $r^2$  value was 0.59 ( $p < 0.0005$ ). Note that the error bars in this figure have been excluded for clarification. The S.E. values for all data are listed in Table 1.



**Fig. 6.**  $IC_{50}$  Y335A: $IC_{50}$  WT ratio for the tested dopamine uptake inhibitors and the respective correlation to the degree to which they can substitute for cocaine in a cocaine discrimination test and a basal locomotor activity test. A, effect of the Y335A mutation on  $IC_{50}$  values for inhibitors compared with DAT WT displayed as an  $IC_{50}$  Y335A: $IC_{50}$  WT ratio between the two data sets shown in Table 1. Closed bars, compounds that completely substituted for cocaine in the cocaine discrimination test (Table 1). Open bars, compounds that failed completely or only partially substituted for cocaine in the cocaine discrimination test. The change in  $IC_{50}$  ratio is calculated from the inhibition of [ $^3$ H]dopamine uptake by the compound in COS-7 cells transiently expressing either DAT WT or Y335A as shown in Fig. 4. The error bars are calculated by the division of the higher S.E. value for Y335A with the lower S.E. value for WT listed in Table 1. Similarly, the range of the lower error bar is calculated by dividing the lowest S.E. value for Y335A with the highest S.E. value for the WT. This calculation will naturally produce large error bars. B, correlation between the  $IC_{50}$  ratio for each compound and its ability to substitute for cocaine in a cocaine discrimination test. The  $r^2$  value was 0.74 ( $p < 0.0001$ ). Data are mean from the maximal cocaine substitution plotted against the mean  $IC_{50}$  Y335A: $IC_{50}$  WT ratio (A). C, correlation between the mean

From a molecular perspective, it is interesting that in contrast to the Y335A:WT IC<sub>50</sub> ratio assay, the I159C cysteine reactivity assay did not show the same correlation between behavioral response and transporter conformation. Specifically, MFZ 2-71, which displayed a cocaine-like behavioral response, protected reactivity of I159C like the other BZT and rimcazole analogs (Fig. 2), whereas its Y335A:WT IC<sub>50</sub> ratio resembled that of the cocaine analogs. One implication of this result is that the cysteine reactivity assay is not providing the same conformational readout as the Y335A assay. This is not entirely surprising; the Y335A assay probably reports more global changes by providing a measure of how a given compound recognizes the most prevalent conformations of the mutated transporter compared with the wild-type transporter. Thus, we hypothesize that mutation of Tyr335 alters the time constants of the different conformations in the translocation cycle. This means that a prevalent conformation in the wild type, e.g., the cocaine binding conformation, is likely to be less prevalent in Y335A. When performing [<sup>3</sup>H]dopamine uptake inhibition experiments, both WT and Y335A are “forced” to assume the conformations necessary for a translocation cycle, but the equilibrium between the different states will differ between WT and Y335A at any given time. From this perspective, the IC<sub>50</sub> ratio from the uptake inhibition experiments should reflect the difference in the probability of the inhibitor binding to the WT or Y335A conformations. In contrast, the cysteine reactivity assay may involve a more specific site in the transporter molecule and thereby depend on a more “local” conformational change, for example at the extracellular gate. It is noteworthy that all compounds were affected to some degree by the Y335A mutation. In the simplest case, it would be predicted that if the benztropine and rimcazole analogs induce an inward facing conformation they would bind more avidly to Y335A compared with the wild type. The most conceivable explanation for this discrepancy is that none of the compounds stabilize a conformation identical to the most dominating conformation of Y335A. However, the benztropine and rimcazole analogs are likely to stabilize conformations more similar to Y335A than those promoted by the cocaine analogs. We should also emphasize that although all the BZT and rimcazole analogs seem to protect the reactivity of I159C, they are not necessarily promoting identical conformations of the transporter.

An intriguing question is how different modes of interaction as reflected in the Y335A assay might translate into an altered effect in vivo compared with cocaine. In this context, it is interesting that JHW 007, which substitutes very poorly for cocaine in the discrimination paradigm, occupies DAT in vivo much more slowly than cocaine (Desai et al., 2005) despite its rapid delivery to the brain (Raje et al., 2003). The slow rate of DAT occupancy by JHW 007 might be correlated to its distinct mode of interaction with DAT. The combined low sensitivity to the Y335A mutation and protection of I159C fit well into this scenario. Thus, the predicted more “closed” conformation stabilized by JHW 007 and other novel dopamine uptake inhibitors tested here is conceivably on average a markedly less probable conformation in the wild-type transporter than the “open” cocaine binding conformation. Accordingly, a compound that binds the closed conformation is more likely to display a markedly slower on-rate compared with a compound that binds the open and likely

more prevalent conformation. Indeed, recent studies with [<sup>3</sup>H]JHW 007 on striatal membrane have shown that its on-rate is 4.5-fold slower than the one observed for [<sup>3</sup>H]WIN 35,428 (Kopajtic et al., 2006). It follows that such a compound, despite its rapid delivery to the brain, will cause a much slower rise in the extracellular dopamine concentration as compared with cocaine (Tanda et al., 2005). It should also be considered that the distinct conformational states stabilized by the different inhibitors might alter the interaction of the transporter with associated proteins. A growing number of cellular proteins have been shown to interact with the DAT (for review, see Torres, 2006), and alterations in these protein-protein interactions could easily be envisioned to affect the in vivo effects of an inhibitor.

BZT itself and some of its analogs have activity not only at the DAT but also at other sites that may influence the in vivo effects of these drugs. These include histamine H<sub>1</sub> and muscarinic M<sub>1</sub> antagonist effects. However, previous studies suggest that at least the H<sub>1</sub> and M<sub>1</sub> antagonist actions are contributing little if at all to the decreased cocaine-like effects of BZT analogs (Katz et al., 1999, 2004; Campbell et al., 2005). Major activity at other nontransporter sites is also quite unlikely as determined by testing selected BZT/rimcazole compounds at any of 31 mammalian receptors (results with some of the compounds have been published; Katz et al., 2004). Additionally, it is unlikely that activity at the homologous norepinephrine (NET) and serotonin (SERT) transporters interferes with our observations. Previous studies showed that DAT knockout mice self-administer cocaine and that only the dual DAT/SERT knockout mice are insensitive to cocaine (Sora et al., 2001), suggesting a role for SERT in the abuse-related effects of cocaine. However, the present compounds are relatively selective for the DAT (Agoston et al., 1997). Furthermore, there is a wide array of compounds with affinity for the monoamine transporters SERT, NET, and DAT, and many of these have differing ratios of affinities for the DAT compared with SERT or NET. Nonetheless, despite these varying selectivities, most if not all of the standard selective dopamine uptake inhibitors (e.g., methylphenidate, bupropion, and mazindol) fully substitute for cocaine in the cocaine-discrimination test, whereas serotonin and norepinephrine uptake inhibitors do not (e.g., Baker et al., 1993). Finally, the recent generation of a knockin mouse expressing a DAT mutant incapable of binding cocaine has provided strong evidence that the stimulatory effects of cocaine are indeed primarily mediated via its action at the DAT despite its significant affinity at NET and SERT (Giros et al., 1996; Chen et al., 2006). Therefore, it is the activity at the DAT that seems to solely determine the potency of the various monoamine uptake inhibitors in generalizing to cocaine.

In summary, the present study represents the first comprehensive investigation aimed at characterizing the relationship between molecular mode of interaction with the DAT and behavioral response for a broad spectrum of different DAT inhibitors. The correlation between the cocaine-like subjective effects of compounds and their response in the Y335A mutation in principle allows an a priori prediction from in vitro to in vivo effects. In addition to potential medical treatments for cocaine abuse, this could be relevant for high-throughput evaluation of potential leads in drug discovery processes in which inhibitor activity at DAT is wanted

but without subjective effects like those of cocaine, including potential "triple action" antidepressants with activity at NET, SERT, and DAT (Demitrack, 2002). Together with previous findings, the present results suggest a scenario in which the behavioral response to a DAT inhibitor is the result of a complex interplay between the rate of delivery to the brain and the mode of interaction with the transporter molecule. Whereas the former is better established conceptually, the latter represents a novel aspect for consideration as a factor in how the inhibition of the DAT by a drug is transduced into in vivo effects.

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