R-Modafinil (Armodafinil): A Unique Dopamine Uptake Inhibitor and Potential Medication for Psychostimulant Abuse

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Background: (±)-Modafinil has piqued interest as a treatment for attention-deficit/hyperactivity disorder and stimulant dependence. The R-enantiomer of modafinil might have unique pharmacological properties that should be further investigated.

Methods: (±)-Modafinil and its R-(−)- and S-(+)-enantiomers were synthesized and tested for inhibition of [^{3}H] dopamine (DA) uptake and [^{3}H]WIN 35428 binding in human dopamine transporter (DAT) wild-type and mutants with altered conformational equilibria. Data were compared with cocaine and the atypical DA uptake inhibitor, JHW 007. R- and S-modafinil were also evaluated in microdialysis studies in the mouse nucleus accumbens shell and in a cocaine discrimination procedure.

Results: (±)-, R-, and S-modafinil bind to the DAT and inhibit DA uptake less potently than cocaine, with R-modafinil having approximately threefold higher affinity than its S-enantiomer. Molecular docking studies revealed subtle differences in binding modes for the enantiomers. R-modafinil was significantly less potent in the DAT Y156F mutant compared with wild-type DAT, whereas S-modafinil was affected less. Studies with the Y335A DAT mutant showed that the R- and S-enantiomers tolerated the inward-facing conformation better than cocaine, which was further supported by [2-(trimethylammonium)ethyl]-methanethiosulfonate reactivity on the DAT E2C I159C. Microdialysis studies demonstrated that both R- and S-modafinil produced increases in extracellular DA concentrations in the nucleus accumbens shell less efficaciously than cocaine and with a longer duration of action. Both enantiomers fully substituted in mice trained to discriminate cocaine from saline.

Conclusions: R-modafinil displays an in vitro profile different from cocaine. Future trials with R-modafinil as a substitute therapy with the potential benefit of cognitive enhancement for psychostimulant addiction are warranted.

Key Words: Abuse liability, addiction, cocaine, dopamine transporter, methamphetamine, microdialysis

The development of medications to treat stimulant abuse disorders remains an unmet medical need, despite decades of research (1–3). One approach to this challenge is using "reverse translation" of clinically available medications that might have mechanisms of action that are related to those associated with addictive drugs, such as inhibition of dopamine (DA) reuptake via the dopamine transporter (DAT). By further investigating these agents at the molecular level and relating these observations to behavior, a rationale for testing these agents in humans addicted to psychostimulants might be provided. Herein we compare cocaine with the clinically available (±)-modafinil, its R-enantiomer (armodafinil), and S-modafinil at the DAT.

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Modafinil (Figure 1) is used as a wake-promoting agent for the treatment of narcolepsy and other sleep disorders (4). Modafinil has been described as a psychostimulant but is not amphetamine-like in chemical structure, pharmacological profile, or mechanism of action. As such, it has sparked interest for the treatment of cognitive dysfunction in disorders such as attention-deficit/hyperactivity disorder (4,5). Modafinil has also attracted attention for the treatment of cocaine (6) and methamphetamine dependence (7,8). In addition, the emerging emphasis on cognitive impairment in neuropsychiatric disorders, including addiction (9), has stimulated investigations into the potential pro-cognitive effects of modafinil in this population (10–12).

The mechanisms of action underlying the therapeutic actions of modafinil have been debated. Modafinil modulates the activity of hypocretin, histamine, α-adrenergic, γ-aminobutyric acid, and/or glutamate receptors (4,13). However, its ability to bind to the DAT and block DA reuptake, although with low affinity compared with cocaine, has received the most attention (14–17). Positron emission tomography studies in human subjects have demonstrated that modafinil binds to the DAT at therapeutic doses leading to alerts with regard to its abuse potential (18). Although, preclinical data have suggested that modafinil is like cocaine or might reinstate cocaine taking (19–23), the preponderance of clinical literature indicates a low abuse liability (24–26).

Modafinil comprises R-(−)- and S-(+)-enantiomers (Figure 1) and was originally prescribed as the racemate Provigil (Cephalon, Frazer, Pennsylvania) (27). However, more recent human studies suggest that R-(–)-modafinil is the more metabolically stable and longer-acting enantiomer (28–31). We recently prepared the R- and S-modafinil enantiomers, along with a series of analogues, and showed that the R-enantiomer had approximately threefold-higher affinity for the DAT than the S-enantiomer in rat brain tissue (17). Furthermore, although the (±)-, R-, and S-enantiomers all stimu-

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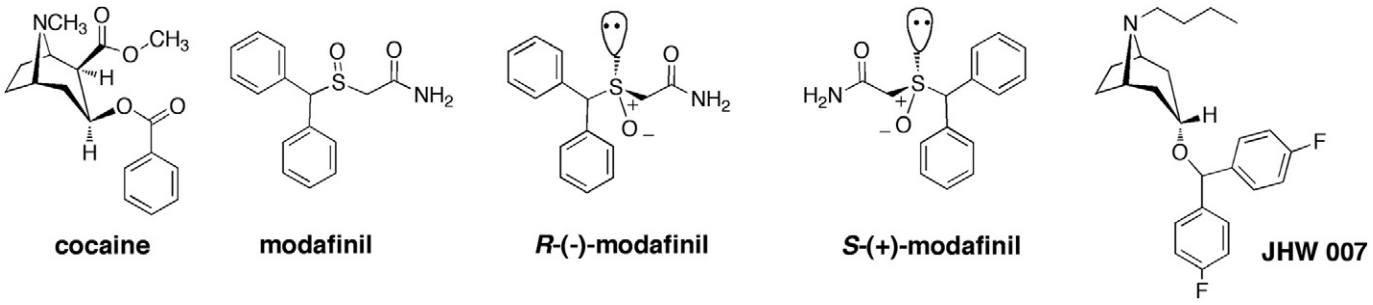

Figure 1. Chemical structures of drugs used in the study.

lated locomotor activity in mice, they were less effective and less potent than cocaine. The reduced efficacy and unusual structure of modafinil suggested that it might bind to the DAT in a different mode than cocaine. Indeed, the biphenyl ring system resembles that of the benztropine class of DAT inhibitors, exemplified by JHW 007 (Figure 1), which has been extensively characterized as being "atypical" with potential for development as a medication to treat cocaine addiction (32,33).

We previously compared the binding and DA uptake inhibition of a series of tropane-based DAT inhibitors in several DAT mutants that were designed to shift the conformational equilibrium toward either an outward- or inward-facing state (34,35). In that study we discovered that the cocaine-like compounds (e.g., WIN 35428) preferred an outward-facing conformation of the DAT, whereas the benztropines (e.g., JHW 007) preferred a more occluded conformation. Remarkably these data correlated with effectiveness in producing cocaine-like effects in rats (36). In subsequent studies, these differences in binding modes were supported and further highlighted different DAT binding interactions between these structurally distinct classes of DAT inhibitors (37,38).

We hypothesized, on the basis of those studies, that R- and S-modafinil might also bind the DAT differently from cocaine, contributing to their in vivo pharmacological profiles. Hence in the present study, we compare the binding of the enantiomers and their potency for inhibition of DA uptake in human DAT transfected COS-7 cells with those of cocaine. We then tested the enantiomers in DAT mutants biased toward inward- or outward-facing conformations to investigate the induction of specific conformations of the DAT by modafinil binding. In addition DA concentrations in the mouse nucleus accumbens (NAc) shell were assessed in vivo with microdialysis procedures. This brain area has been suggested to play a significant role in mediating the reinforcing effects of abused drugs (39–41). Finally, we tested modafinil and its enantiomers in a cocaine-discrimination procedure to determine whether the enantiomers substituted for the discriminative-stimulus effects of cocaine as (±)-modafinil has been reported to do in other species (19,22,23).

Methods and Materials

In Vitro Studies

The [^{3}H]DA uptake and [^{3}H]WIN 35428 binding experiments were carried out with standard methods on transiently transfected COS-7 cells expressing the human DAT wild-type (WT) or mutants as described previously (35) and in detail in Supplement 1. The [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET) labeling experiments were performed essentially as before (36) and described in detail in Supplement 1. In short, the ligands dissolved in uptake buffer were added to the intact cells expressing either DAT E2C or DAT E2C I159C in the following concentrations: (±)-modafinil: 100 μmol/L, R-(−)-modafinil: 100 μmol/L, S-(+)-modafinil: 100 μmol/L, DA: 100 μmol/L, cocaine: 30 μmol/L, and JHW 007: 5 μmol/L. The concentration of inhibitor was chosen as the highest possible concentration that could be washed away to allow subsequent [^{3}H]DA analysis. The MTSET was added at a final

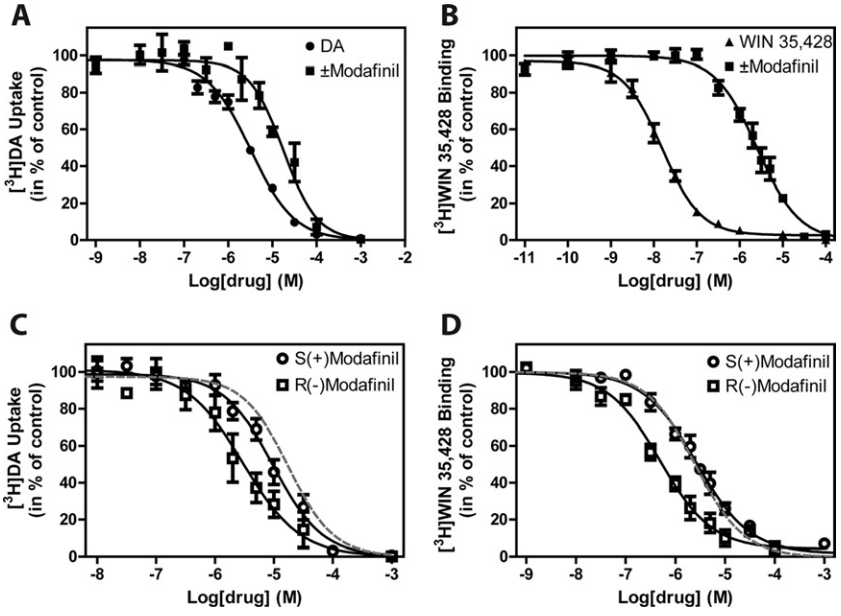

Figure 2. Characterization of (±)-, R-, and S-modafinil binding to the dopamine transporter (DAT). Inhibition of **(A, C)** [^{3}H] dopamine (DA) uptake and **(B, D)** [^{3}H]WIN 35428 binding in COS-7 cells transiently expressing DAT wild-type by (●) DA, (▲) WIN 35428, (■) (±)-modafinil, (○) S-modafinil, and (□) R-modafinil. The grey punctured lines in **(C)** and **(D)** are the observed IC_{50} values for (±)-modafinil from **(A)** and **(B)**, respectively, shown for comparison. The observed IC_{50} value for a compound is the basis for the calculated inhibition potencies and K_i values in Table 1. Data are means ± SEM of 6–10 experiments performed in triplicate.

concentration of .5 mmol/L, and the cells were incubated at room temperature for 10 min. The preincubation was stopped, and [^{3}H]DA uptake was initiated to determine the degree of transport inactivation by MTSET.

Modeling of DAT/Ligand Complexes

The complexes between DAT and modafinil enantiomers were modeled similarly to that described previously, (38) with a well-established induced-fit docking protocol (42), and are described in detail in Supplement 1.

Microdialysis

Methods have been described in detail elsewhere (43) and provided in Supplement 1. Briefly, approximately 45 hours after the surgical procedures and starting at 9:00 AM microdialysis sessions were initiated with probes connected via swivels and perfused with Ringer's solution at a constant flow rate of 1 μL/min. Dialysate sampling (10 μL/10 min) started after approximately 30 min. Mice received cocaine, (±)-, S-, or R-modafinil or vehicle injections only when stable DA values were obtained. Sample collection continued for 360 min but after 2 hours occurred every 20 min. Dialysate samples were immediately injected without purification into a high-performance liquid chromatography coupled with an ESA (Bedford, Massachusetts) 5200 coulochem detector to quantify DA. Assay sensitivity for DA was 2 fmoles/sample.

Cocaine Discrimination

Experimental details are essentially identical to those described previously (44) and provided in Supplement 1. In brief, subjects were placed in operant-conditioning chambers with overall illumination, two response levers, and pairs of green and yellow lights above each lever. Mice were trained with food reinforcement to press both levers and eventually trained to press one after cocaine (10 mg/kg) and the other after saline IP injections, on a double-alternation daily schedule. The ratio of responses to food pellets was ultimately 10 (fixed-ratio 10). Experimental sessions started after a 5-min period in darkness during which responses had no consequences. After this period lights were turned on until the completion of the fixed-ratio requirement and the presentation of food. Sessions ended after 20 food presentations or 15 min, whichever occurred first, and were conducted 5 days/week. Testing with different doses of cocaine or modafinil was initiated after subjects met the training criteria (Supplement 1). Test sessions were identical to training sessions with the exception that 10 responses on either lever were reinforced.

Results

Assessment of the Affinity for (±)-Modafinil and its R- and S-Enantiomers to the DAT

(±)-Modafinil and its enantiomers were tested for inhibition of [^{3}H]DA uptake and displacement of [^{3}H]WIN 35428 (Figure 2). (±)-Modafinil inhibited [^{3}H]DA uptake with a potency that was more than sevenfold lower than observed for DA (inhibition potency for DA and (±)-modafinil for the DAT was 1.7 and 13 μmol/L, respectively) (Figure 2A; Table 1). Inhibition of [^{3}H]WIN 35428 binding by (±)-modafinil revealed lower K_i values as compared with [^{3}H]DA uptake inhibition (K_i = 2.3 μmol/L) (Figure 2B and Table 1) and comparable to our previously published values in rat brain tissue (17). Interestingly, (±)-modafinil was less potent in inhibiting [^{3}H]DA uptake than both the R- and S- enantiomers (Figure 2C and Table 1). However, for the inhibition of [^{3}H]WIN 35428 binding, the observed affinity for the S-enantiomer was almost indistinguishable from the affinity for the racemate, whereas the R-enantiomer had a higher affinity (Table 1). The discrepancy between inhibition potencies of DA

Table 1. [^{3}H]DA Uptake Inhibition and [^{3}H]WIN 35428 Binding Data in DAT WT and Mutants

hDAT Mutants	[^{3}H]DA Uptake (*n*)	IC_{50} (μmol/L) (SE interval)	[^{3}H]WIN 35428 Binding (*n*)	K_d (or K_i; μmol/L) (SE interval)
hDAT				
DA	6	1.7 (1.5–2.1)		
Cocaine	12	.23 (.19–.26)[a]	3	.45 (.34–.59)[b]
WIN 35428			10	.013 (.012–.014)
(±)-modafinil	6	13 (10–16)	7	2.3 (1.9–2.6)
R-modafinil	9	4.0 (2.6–6.4)	7	.78 (.67–.90)
S-modafinil	9	8.7 (7.5–10)	8	2.5 (2.2–2.9)
hDAT Y156F				
Cocaine			5	.35 (.28–.45)[b]
WIN 35428			6	.013 (.0096–.017)
(±)-modafinil			5	8.0 (6.4–9.9)
R-modafinil			3	11 (7.5–15)
S-modafinil			5	5.5 (4.7–6.4)
hDAT Y335A				
DA	3	.99 (.60–1.7)		
Cocaine	12	24 (20–30)[a]		
(±)-modafinil	4	83 (48–143)		
R-modafinil	3	43 (26–71)		
S-modafinil	3	129 (97–170)		

The inhibition potency for [^{3}H]dopamine (DA) uptake, and the K_d or K_i for [^{3}H]WIN 35428 binding were calculated from nonlinear regression analysis of uptake and binding data, respectively, performed on COS7 cells transiently transfected with human dopamine transporter (hDAT) wild-type (WT) or the indicated mutant. The IC_{50} values used in the estimation of K_i and K_d values were calculated from means of pIC_{50} values, and the SE interval was calculated from the pIC_{50} ± SE (see Materials and Methods in Supplement 1).

nd, not determined.

[a]Data from Loland *et al.* (36).

[b]Data from Beuming *et al.* (37).

transport and K_i values in the binding assay could be because [^{3}H]DA uptake inhibition is not performed under equilibrium conditions, as the binding experiments are.

We were not able to measure any significant binding or transport inhibition of the R- and S-enantiomers to either of the homologous transporters for norepinephrine (NET) or serotonin (SERT), assessed by their ability to inhibit [^{3}H]DA uptake or [^{3}H]nisoxetine binding at the NET and [^{3}H]5-HT at the SERT ($IC_{50} > 100$ μmol/L for all experiments, $n = 3$, data not shown). These observations are also in accord with binding experiments performed in native rat brain tissue (17).

The Modafinil Binding Site in DAT Overlaps with the Central Binding Site for Substrate

To characterize the modafinil binding site in the DAT, we carried out a docking study of the two enantiomers with DAT models described previously (37,38) on the basis of the crystal structure of the bacterial homologue LeuT (Figures 3A, B). The modafinil enantiomers were docked in the primary binding pocket (S1) in the center of the protein that is also the binding site for DA and cocaine (37) as well as for the atypical DAT inhibitor JHW 007 and its analogues (38). The top-ranked binding poses revealed a significant similarity between the binding modes of the enantiomers, however, with unique interactions. One distinctive structural feature of modafinil, compared with either cocaine or JHW 007, is that it lacks a charged pyramidal nitrogen. Thus, although the orientations of the biphenyl ring systems in the binding modes of both modafinil enantiomers might be similar to those of the benztropine derivatives (38), there is no direct interaction with Asp79. In contrast, the terminal amide moiety of modafinil tends to stack with the phenyl ring of Phe76 and H-bond to the backbone of Ser321. These restraints result in different positioning of the chiral S = O in the R- and S-enantiomers. Although both enantiomers are in close vicinity to Tyr156, the S = O of R-modafinil interacts with the -OH group of the Tyr156 residue, whereas this interaction does not occur with S-modafinil.

Thus, we sought to experimentally validate the docking results and hypothesized that the removal of the OH-group of Tyr156 by changing it to a phenylalanine (Y156F) could disrupt the interaction with R-modafinil but have little effect on the S-modafinil binding interaction. Indeed R-modafinil showed a marked decrease in affinity for the DAT Y156F mutant as compared with DAT WT protein (Figure 3C) with a 14-fold change (Table 1, compare WT and Y156F DAT binding), whereas only a twofold difference in affinity was observed for S-modafinil by the Y156F mutation (Figure 3D; Table 1). These data are in agreement with the docking models and suggest that only R-modafinil interacts with Tyr156 in the primary binding pocket.

R- and S-Modafinil Preferentially Bind to a Different DAT Conformation Than Cocaine

To identify the preferred DAT conformational state induced by R- and S-modafinil, two previously established methods (36) that assess the conformational state of the DAT upon binding of different ligands were employed: 1) the shift in IC_{50} for [^{3}H]DA uptake inhibition by the modafinil enantiomers between the WT and a Tyr335 to alanine mutation, and 2) the accessibility of a cysteine inserted in TM3 to the cysteine-reactive compound MTSET. We have previously shown that Tyr335 is critical for

Figure 3. Localization of the binding sites for R- and S-modafinil in the dopamine transporter (DAT). **(A, B)** The predicted binding modes of R-modafinil and S-modafinil in the central binding site of DAT, respectively. Note the amide bond of both modafinils are stacked with the phenyl ring of Phe76 and carboxyl group of Asp79, whereas only the sulfinyl group of R-modafinil interacts with the hydroxyl group of Tyr156. The modafinil enantiomers are in thicker stick presentation with the carbon colored in cyan. **(C)** Removal of the hydroxyl group on Tyr156 in DAT (Y156F. open symbols) results in a >14-fold decrease in IC_{50} for R-modafinil as compared with the DAT wild-type (WT) (solid symbols). **(D)** For S-modafinil, the Y156F mutation caused a twofold decrease in the IC_{50}-value. All data in **C** and **D** are assessed by displacement of [^{3}H]WIN 35428 binding by the indicated modafinil enantiomer performed on intact COS-7 cells transiently transfected with DAT WT or mutant. Data are means ± SEM of 3–8 experiments performed in triplicate.

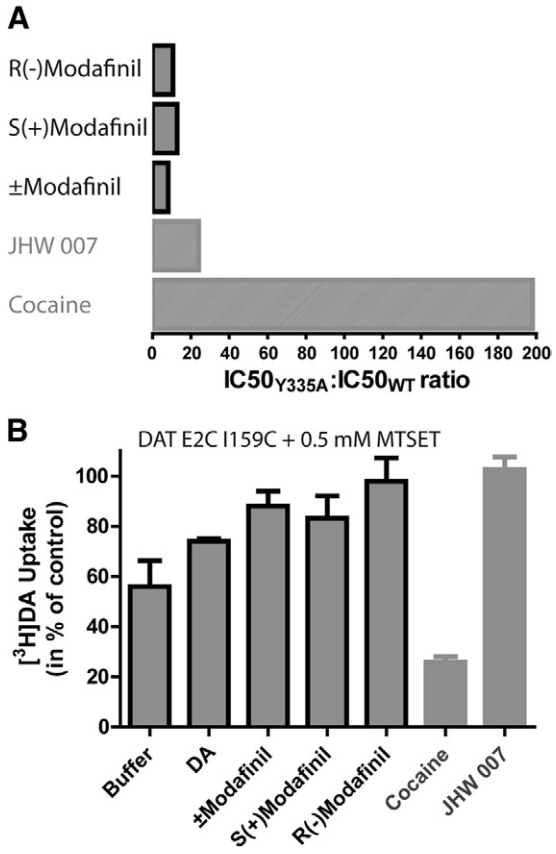

Figure 4. R- and S-modafinil bind to a dopamine transporter (DAT) conformation that differs from cocaine. **(A)** Effect of the Y335A mutation on IC_{50} values for inhibition potency of [^{3}H] dopamine (DA) uptake by (±)-modafinil and enantiomers, JHW007 and cocaine, compared with the DAT wild-type (WT). The calculated difference in inhibition potency (IC_{50}) of [^{3}H]DA uptake by (±)-modafinil and enantiomers in the DAT Y335A mutant relative to WT is displayed as an IC_{50}Y335A/IC_{50}WT ratio. The data for JHW007 and cocaine are shown in grey for comparison and are in agreement with previously determined data (36). **(B)** Effect of (±)-modafinil and enantiomers on [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET) (.5 mmol/L) inhibition of [^{3}H]DA uptake on the DAT E2C I159C (a mutant in which two endogenous cysteines, Cys90 and Cys306, have been changed to alanines, rendering it insensitive to MTSET [36], data not shown). Data are shown as mean ± SEM of the effect of preincubating with the indicated drug on the MTSET reactivity. One hundred percent activity is set as the preincubation of drug alone followed by vehicle only. All experiments are performed on COS7 cells transiently expressing DAT WT or mutant of at least three experiments performed in triplicate.

regulating conformational isomerization in the transport cycle (34,35,45) and that mutation of this residue (Y335A) shifts the conformational equilibrium toward an inward-facing conformation. This suggestion has recently been supported by the crystallization of the inward-facing conformation of LeuT with the cognate mutation (46). Thus, the Y335A mutation can be used as a tool to probe whether a drug favors an inward-facing or outward-facing conformation (36). To assess this for the modafinil enantiomers, we investigated their [^{3}H]DA uptake inhibition potency in the DAT Y335A mutant and compared it with WT (Figure 4A). As previously determined, DAT inhibitors such as cocaine bind preferentially to the outward-facing conformation of the DAT. This results in a large decrease in potency for [^{3}H]DA uptake inhibition between DAT WT and the Y335A mutant as determined in Figure 4A for cocaine, resulting in an almost 200-fold change in IC_{50}. On the contrary, the nonstimulant atypical DAT inhibitor JHW 007 shows only a minor, approximately 20-fold, change in IC_{50}, suggesting that it binds to a conformation that differs from that preferred by cocaine (Figure 4A). Performing the same experiments on the R- and S-modafinil enantiomers gave Y335A/WT IC_{50} ratios (10.6 and 14.8, respectively) that approximate that for JHW 007 (Figure 4A). This suggests that these DA uptake inhibitors bind to a DAT conformation distinct from the cocaine-induced conformation and closer to the one observed for JHW 007.

The second assay is based on the reactivity of a cysteine inserted into position 159 in TM3 of the DAT located in the vicinity of the ligand binding site on the extracellular side. Previous observations in DAT (35,36,47,48), NET, and SERT (49) have suggested that the accessibility of this position is dependent on the conformational state of the transporter: it is accessible to the extracellular environment when the DAT is in the outward-facing conformation and inaccessible in the closed or inward-facing conformation. Importantly, reaction of an inserted cysteine in this position (I159C) with MTSET results in inactivation of the DAT, allowing the use of DA uptake as a functional read-out for I159C reactivity (36). The I159C mutant was generated in a MTSET insensitive DAT background (E2C) in which the two external endogenous cysteines were mutated to alanines (C90A-C306A). Incubation of .5 mmol/L MTSET for 10 min in buffer resulted in an inactivation to approximately 60% of the initial [^{3}H]DA transport capacity. The addition of cocaine (30 μmol/L) together with MTSET resulted in a marked increase in inactivation to approximately only 20% remaining transport capacity, whereas DA (100 μmol/L) and, in agreement with previous results (36), JHW 007 (5 μmol/L) caused a protection from the MTSET reactivity (Figure 4B). The addition of the R- or S-modafinil enantiomers (100 μmol/L) caused a similar protection of Cys159 from MTSET reactivity as observed for JHW 007 (98 ± 9% and 83 ± 9% for R- and S-modafinil, respectively, compared with 102 ± 5% for JHW 007, $n = 4–8$) (Figure 4B). This further suggests that both modafinil enantiomers induce a conformation of the DAT in which the extracellular vestibule is closed, thus protecting Cys159 from reacting with the added MTSET.

Microdialysis Studies with Cocaine, (±)-, R-, and S-Modafinil

To investigate the comparative pharmacology of modafinil and cocaine, we measured extracellular DA concentrations in the mouse NAc shell (see Figure 5 for statistical analyses). R-modafinil (30, 100, 300 mg/kg IP) significantly stimulated DA levels to approximately 300% of DA basal levels at 40–60 min after injection. These levels were maintained throughout the 6 hours of measurement (Figure 5A). Similar effects were obtained with S-modafinil (Figure 5B). As in previous reports (43,50), cocaine (10–55 mg/kg) significantly stimulated the extracellular levels of DA, with rapid onsets and offsets of action. The DA reached a maximum of approximately 700% of basal levels at approximately 30 min after cocaine injection (Figure 5C). The maximal increases in DA levels were strongly related to the dose of cocaine, whereas the slopes of the dose-effect curves for modafinil and its enantiomers were much more shallow (Figure 5D), indicating a limited dose-dependency in effects of modafinil and a lower level of maximal stimulation of DA, compared with cocaine (43).

Cocaine Drug Discrimination

Cocaine produced a dose-related increase in the percentage of drug-appropriate responses in mice trained to discriminate cocaine (10 mg/kg) from saline injections (Figure 6, filled symbols). (±)-

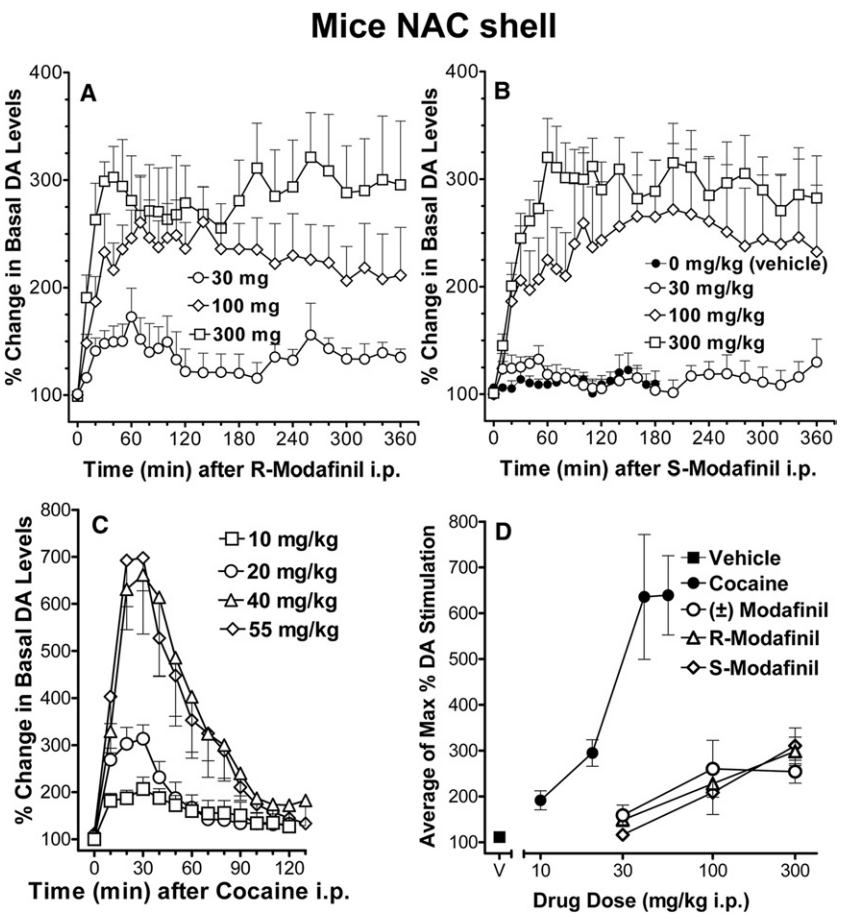

Figure 5. (A, B) Effects of R- and S-modafinil, respectively, on extracellular levels of dopamine (DA) in dialysates from the nucleus accumbens (NAc) shell in mice. **(A–C)** Ordinates represent the change in extracellular DA concentration as a percentage of basal values. Abscissae represent the time after drug administration. Drugs were administered at doses of 30, 100, and 300 mg/kg intraperitoneal (i.p.). Basal DA values were 50.4 ± 3.9 ($n = 5$), 50.3 ± 3.8 ($n = 6$), and 40.4 ± 4.6 ($n = 5$) fmoles/sample for respective doses of R-modafinil; and 70.0 ± 9.4 ($n = 5$), 52.6 ± 4.8 ($n = 7$), and 44.1 ± 5.4 ($n = 5$) fmoles/sample, respectively, for S-modafinil. Two-way analysis of variance (ANOVA) indicated main effects of R-modafinil dose [$F(3,16) = 8.652$, $p < .01$], time [$F(24,384) = 7.776$, $p < .001$], and their interaction [$F(72,384) = 2.212$, $p < .001$]. An ANOVA for S-modafinil indicated main effects of dose [$F(3,17) = 5.259$, $p < .01$], time [$F(24,408) = 6.441$, $p < .001$], and their interaction [$F(72,408) = 2.385$, $p < .001$]. **(C)** Dose-dependent effects of acute administration of cocaine on extracellular DA levels in the NAc shell in mice at doses of 10–55 mg/kg. Group size, $n = 5$ for all groups; basal DA values were 48.5 ± 8.1, 57.4 ± 12.2, 29.8 ± 6.3, and 34.2 ± 6.0 fmoles/sample, for respective doses of cocaine. A two-way ANOVA indicated main effects of cocaine dose [$F(3,17) = 6.636$, $p < .01$], time [$F(12,204) = 39.189$, $p < .001$], and their interaction [$F(12,36) = 5.376$, $p < .001$]. Each point represents the mean DA levels in 10-min dialysate samples, expressed as a percentage of basal values. For panel **D**, ordinates represent the change in extracellular DA concentration as a percentage of basal values during the 30-min period after drug administration in which maximal stimulation of DA was observed. Abscissae, dose of drug in milligrams/kilogram (log scale). Vertical bars in all panels represent SEM. V, Vehicle, 10% dimethyl sulfoxide + 15% Tween 80 in sterile water.

Modafinil and both enantiomers fully substituted for cocaine, although with approximately one-tenth the potency of cocaine on a molar basis (Table 2). There were no significant differences in potency between the enantiomers of modafinil, similar to the observations in the microdialysis studies.

Discussion

(±)-Modafinil and its R- and S-enantiomers bind with relatively low affinity to the human DAT and inhibit DA uptake in COS7 cells, with the R- slightly more potent than the S-enantiomer. As reported previously in rat brain tissue (17), neither enantiomer showed measurable binding to SERT or NET.

Both the R- and S-enantiomers docked at a common DAT binding pocket significantly overlapping with the S1 binding site for DA and cocaine (37) as well as for the atypical DAT inhibitors (e.g., JHW 007) (38). Although a significant overlap in binding of the modafinil enantiomers was apparent, a unique residue Tyr156 that coordinated differently with the R- and S-enantiomers was identified (Figure 3A, B). To verify these models, we investigated the interaction of the enantiomers with Tyr156, through a single point mutation. According to the model, both enantiomers interact with Tyr156, but only the R-enantiomer interacts with the Tyr-OH group. Thus, we hypothesized that removal of the OH-group with mutation of Tyr156 to a phenylalanine would disrupt R-modafinil but have little effect on S-modafinil binding. Indeed, R-modafinil showed a 14-fold decrease in affinity for the DAT Y156F mutant, as compared with DAT WT protein (Figure 3C; Table 1), whereas only a minor difference in affinities was obtained with S-modafinil. Moreover, the removal of the OH-group disrupts a hydrogen bond to Asp79 in transmembrane helix 1, which presumably alters the orientation of the residue and therefore only slightly affects coordination to the S-modafinil.

We have previously provided evidence that DAT Tyr335 is critical for regulating conformational isomerization in the transport cycle (34,35,45). Tyr335 is located in the third intracellular loop and is 100% conserved throughout the family of neurotransmitter/sodium symporter proteins (51). Mutation of this residue changes the conformational equilibrium of the DAT, resulting in a transporter residing preferentially in an inward-facing conformation (52). The crystal structure of LeuT, a bacterial homolog of DAT, supports the suggestion of Tyr335 as part of an intracellular gate (46,53). Our experiments revealed that the R- and S-modafinil enantiomers gave Y335A/WT IC_{50} ratios that were similar to those for JHW 007 and in contrast to cocaine. This suggests that these DAT inhibitors bind differently from the cocaine-induced conformation and closer to the one observed for JHW 007.

Previous observations of DAT (36), NET, and SERT (49) have suggested that Cys159 is accessible to the extracellular environment when the transporter is in an outward-facing conformation but becomes less accessible when the extracellular gate closes and the DAT isomerizes toward an inward-facing conformation. Importantly, reaction of Cys159 with the sulfhydryl reactive MTSET results in inactivation of the transporter, allowing the use of DA uptake as a functional read-out for I159C reactivity (36). In this experiment, both R- and S-modafinil protected Cys159 from reacting with MTSET, further supporting their binding mode as preferring a more extracellular occluded conformation of the DAT, unlike cocaine (Figure 4B). These findings are consistent

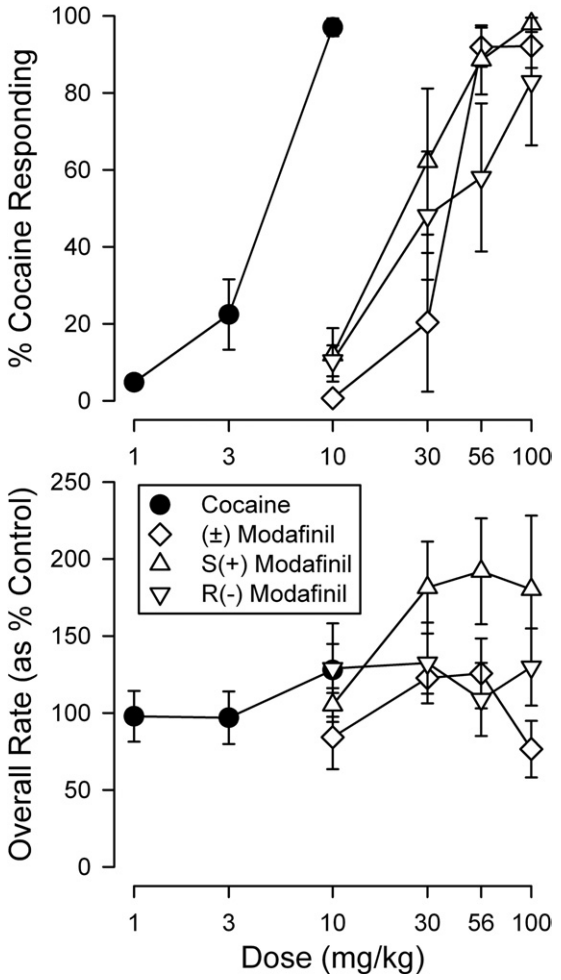

Figure 6. Effects of various doses of cocaine and modafinil enantiomers in mice trained to discriminate injections of cocaine (10 mg/kg) from saline. Cocaine was administered 5 min before and the modafinil enantiomers were administered 60 min before testing. Ordinates for the top panel indicate percentage of responses on the cocaine-appropriate key, and ordinates for the bottom panel indicate the rates at which responses were emitted (as a percentage of response rates after saline administration). Abscissae: drug dose in milligrams/kilogram (log scale). Each point represents average effects of six mice, with the exception of the highest dose of R-modafinil, which was examined in only five subjects. Note the dose-effect curve for R-modafinil has been "nudged" to the left to ensure that it can be discerned in the graphic presentation of the data.

with the recently reported study of W84L and D313N DAT mutants wherein (±)-modafinil displayed WT/mutant ratios different from cocaine and more like those of the DAT inhibitors benztropine, GBR 12909, and bupropion (54).

Although these in vitro experiments suggested that the modafinil enantiomers might be more like atypical DAT inhibitors, several reports of (±)-modafinil in models of psychostimulant abuse suggest a cocaine-like pharmacological profile (4–6). Cocaine, modafinil, and its enantiomers stimulated DA levels in the NAc shell—a finding consistent with the literature—suggesting that these drugs might produce reinforcing effects like those produced by other abused drugs (39–41). However, cocaine administration showed a time- and dose-related stimulation of DA levels that differed from that produced by modafinil and its enantiomers. Because a temporal contingency between drug-injection and drug-effects is an important feature of the reinforcing effects of drugs, this predicts that modafinil and its enantiomers will have lower liability for abuse in humans. Moreover, the highest doses of modafinil and its enantiomers reached similarly lower maximal effects on DA levels than those with cocaine, suggesting that, although they block DA reuptake, they do so differently than cocaine. Also, at variance with cocaine, the DA elevations produced by modafinil and its enantiomers were obtained at doses 10–15 times higher than effective cocaine doses. Taken together, these data suggest a low abuse liability for (±)-modafinil and its enantiomers in humans.

Finally, although (±)-modafinil has been evaluated in several species, including humans, the cocaine-like discriminative-stimulus effects of the enantiomers have not previously been described. We found that both the R- and S-enantiomers of modafinil fully substituted for cocaine in mice, at a pretreatment time of 60 min, and were approximately 8- to 11-fold less potent than cocaine. In addition, as with the microdialysates, no enantioselectivity was observed.

There might be several explanations for the differences we observe in the computational/in vitro studies and behavior in mice, as compared with the benztropine-like DAT inhibitors. First, the modafinil enantiomers bind with relatively low affinity to the DAT, and their binding affinities at NET and SERT were too low to quantify, although NET binding has been reported previously (15). Although the modafinil enantiomers share the diphenyl moiety of the benztropines, previously reported structure-activity relationships suggest that the modafinil analogues might bind somewhat differently at the DAT (55). Furthermore, the modeling studies described herein clearly show that R-modafinil interacts with the Tyr156–OH, and this interaction likely causes this molecule to prefer a more occluded conformation of the DAT, as opposed to cocaine, wherein the 2-carbomethoxy group prevents an H-bond from forming between Asp79 and Tyr 156 and thus keeps this "gate" open (37). Finally, the modafinil enantiomers are non-amine DAT inhibitors (56,57) in that they have no basic nitrogen to interact with the Asp79, like most DAT inhibitors. Thus, modafinil and its enantiomers might not be "atypical"—defined as having high affinity for the DAT with a preference for a more occluded conformation and devoid of significant cocaine-like behaviors (32). However, they are certainly different from cocaine in both binding mode and pharmacological effects and show strong preference for a DAT conformation different from cocaine and more similar to the benztropines.

The present data are comparable to reported discriminative stimulus effects of (±)-modafinil in rats (22) and primates (23). With self-administration procedures, Gold and Balster (19) found reinforcing effects of modafinil in rhesus monkeys trained to self-ad-

Table 2. Comparisons of ED_{50} Values and Potencies

Compound	ED_{50} Value (μmol/kg)	Potency Relative to Cocaine
Cocaine	11.3[a] (9.60–13.5)	—
(±)-Modafinil	125[a] (99.2–157)	.0896[a] (.0677–.120)
S-modafinil	89.0 (64.0–115)	.127[b] (.0881–.191)
R-modafinil	132 (77.9–226)	.0883 (.0513–.159)

Comparisons of ED_{50} values and potencies relative to cocaine of the enantiomers of modafinil in substituting for cocaine in mice trained to discriminate cocaine from saline injections. Values in parentheses are 95% confidence limits.

[a]Significant deviation from linearity.

[b]Significant effect of preparations.

minister cocaine. In contrast, Deroche-Gamonet *et al.* (58) failed to find reinforcing effects of (±)-modafinil in rats without a history of cocaine self-administration. Interestingly, one study found chronic administration of (±)-modafinil to decrease cocaine self-administration (23), supporting its use as a potential substitute therapy in human cocaine abusers.

(±)-Modafinil and its R-enantiomer (armodafinil) are clinically available, and the racemate is currently being evaluated for treatment of attention-deficit/hyperactivity disorder, cocaine, and methamphetamine addiction (59). Despite clinical availability, there are no reports of their abuse, and the literature for the racemate predicts a low abuse liability (24,25,60). Importantly, (±)-modafinil did not serve as a reinforcer in cocaine abusers, making it an attractive candidate for treatment of this population (61). R-modafinil does not seem to be significantly different from the racemate in preclinical studies, but reports indicate an improved pharmacokinetic profile and duration of action in humans (28,31,62,63). Thus, we suggest that R-modafinil might be a promising candidate for a well-designed and compliance controlled clinical trial (8,64) for cocaine or methamphetamine addiction. The therapeutic dose for R-modafinil is typically 50–250 mg orally/day (31), which is lower than (±)-modafinil (200–600 mg orally/day), and might translate into an improved side-effect profile. Furthermore, the recently reported cognitive-enhancing actions of (±)-modafinil, especially in methamphetamine abusers (12), suggest that additional benefit of R-modafinil might be realized in this patient population.

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