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Antidepressant-like actions of DOV 21,947: a "triple" reuptake inhibitor

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Abstract

DOV 21,947 [(+)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride] inhibits the reuptake of [^{3}H]serotonin, [^{3}H]norepinephrine, and [^{3}H]dopamine in human embryonic kidney (HEK) 293 cells expressing the corresponding human recombinant transporters (IC_{50} values of 12, 23, and 96 nM, respectively). This compound also inhibits [^{125}I]RTI 55 (3β-(4-iodophenyl)tropane-2β-carboxylic acid methyl ester) binding to the corresponding transporter proteins in membranes prepared from these cells (K_i values of 99, 262, and 213 nM, respectively). DOV 21,947 reduces the duration of immobility in the forced swim test (using rats) with an oral minimum effective dose of 5 mg/kg. This antidepressant-like effect manifests in the absence of significant increases in motor activity at doses of up to 20 mg/kg. DOV 21,947 also produces a dose-dependent reduction in immobility in the tail suspension test, with a minimum effective oral dose of 5 mg/kg. The ability of DOV 21,947 to inhibit the reuptake of three biogenic amines closely linked to the etiology of depression may result in a therapeutic profile different from antidepressants that inhibit the reuptake of serotonin and/or norepinephrine.

Keywords: DOV 21,947; "Broad-spectrum" antidepressant; Norepinephrine; Dopamine; 5-HT (5-hydroxytryptamine, serotinin); Reuptake inhibitor

1. Introduction

Most antidepressants in current use increase the synaptic availability of biogenic amines, with the majority of these drugs selectively inhibiting norepinephrine and/or serotonin reuptake (Briley and Moret, 1997; Skolnick and Krystal, 2002). These compounds include serotonin and norepinephrine (dual) reuptake inhibitors such as venlafaxine and milnacipran, serotonin-selective reuptake inhibitors including citalopram, fluoxetine and paroxetine, and norepinephrine-specific reuptake inhibitors such as reboxetine (Briley and Moret, 1997; Kent, 2000). While these "second" and "third" generation antidepressants are safer and easier to use than "first" generation agents such as imipramine (Skolnick, 1997), most double-blind, placebo-controlled studies indicate that these biogenic-amine-based therapies require ≥3 weeks of treatment to achieve clinically meaningful symptom relief. Further, a significant number of patients (estimated at 30–40%) do not benefit from these therapies (reviewed in Paul, 2001).

During the past decade, a number of strategies have emerged to reduce the so-called therapeutic lag and/or increase the efficacy of newly developed agents compared to compounds in current use (Skolnick, 1997; Skolnick et al., 2001; Paul, 2001; Vetulani and Nalepa, 2000). Among these strategies is the "broad-spectrum" antidepressant (Skolnick, 2002) capable of simultaneously inhibiting the reuptake of norepinephrine, serotonin, and dopamine. The rationale for this approach is grounded, in large part, on a body of clinical and preclinical evidence linking deficits in mesocorticolimbic dopaminergic function to anhedonia, a core symptom of depression (reviewed in D'Aquila et al., 2000; Naranjo et al., 2001; Willner, 2000).

In this report, we demonstrate that DOV 21,947 [(+)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride] (Fig. 1) is a potent inhibitor of serotonin, norepinephrine, and dopamine reuptake in human embryonic kidney (HEK) 293 cells expressing recombinant forms of

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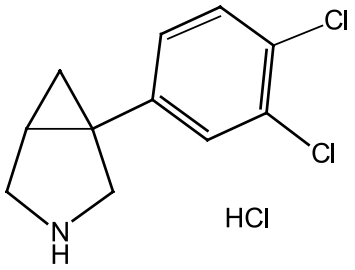

Fig. 1. Structure of DOV 21,947 ([(+)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride]).

the respective human transporters, and inhibits [^{125}I]RTI-55 (3β-(4-iodophenyl)tropane-2β-carboxylic acid methyl ester) binding to the corresponding transporter proteins in membranes prepared from the cells. Further, DOV 21,947 exhibits antidepressant-like actions in both the forced swim and tail suspension tests, preclinical procedures often used to predict activity in humans (Borsini and Meli, 1988; Porsolt and Lenegre, 1992).

2. Materials and methods

2.1. In vitro studies

2.1.1. Expression of the human dopamine (hDAT), serotonin (hSERT), and norepinephrine (hNET) transporters in HEK-293 cells

The cloning and characterization of the hDAT cDNA used in these experiments (pcDNA1-hDAT) was described previously (Eshleman et al., 1994, 1995). The cDNA for the hSERT (Ramamoorthy et al., 1993) and HEK cells expressing the hNET (HEK-hNET; Galli et al., 1995) were generously provided by Dr. Randy Blakely.

2.1.2. Inhibition of [^{3}H]neurotransmitter uptake in HEK-293 cells expressing the hDAT, hSERT, and hNET

Assays were conducted using procedures identical to those described by Eshleman et al. (1999). Cells were grown on 150-mm-diameter tissue culture dishes. The medium was removed, and plates were washed twice with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS). Fresh Ca^{2+}, Mg^{2+}-free PBS (2.5 ml) was then added to each plate. The plates were placed in a 25 °C water bath for 5 min and the cells were gently scraped from the plates. Cell clusters were separated by trituration with a pipette for 5–10 aspirations and ejections. Aliquots (50 μl) of the suspended cells were added to assay tubes (in triplicate) containing DOV 21,947 (31.6 nM to 10 μM in cells expressing hDAT and hSERT cells; 3.16 nM to 1 μM in cells expressing hNET) and Krebs–HEPES assay buffer in a final assay volume of 0.5 ml. Following a 10-min preincubation in a 25 °C water bath, [^{3}H]neurotransmitter (final concentration, 20 nM; specific activity of [^{3}H]dopamine, [^{3}H]serotonin, and [^{3}H]norepinephrine: 40–60, 23.7, and 56 Ci/mmol, respectively) was added and incubated for 10 min. The reaction was terminated by filtration through Wallac filtermat A filters, presoaked in 0.05% polyethylenimine, using a 96-well Tomtec cell harvester. Scintillation fluid was added to each filtered spot, and radioactivity remaining on the filters was measured by liquid scintillation spectrometry. Specific uptake was defined as the difference in uptake observed in the absence and presence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT).

2.1.3. Inhibition of [^{125}I]RTI-55 binding

Assays were conducted using procedures identical to those described by Eshleman et al. (1999). HEK-293 cells expressing the recombinant hDAT and hSERT cells were incubated in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% calf bovine serum, 0.05 U penicillin/streptomycin, and puromycin (2 μg/ml). Cells expressing the hNET were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, and geneticin (300 μg/ml). Cells were grown until confluent on 150-mm-diameter tissue culture dishes in a humidified 10% CO_2 environment at 37 °C. Medium was removed from culture dishes, and cells were washed with 10 ml of lysis buffer (PBS containing 2 mM HEPES and 1 mM EDTA). The culture dishes were then placed on ice for 10 min. Cells were scraped from these culture dishes and centrifuged for 20 min at 30,000 × *g*. The resulting pellet was resuspended in 6–24 ml of 0.32 M sucrose with a Polytron disruptor (setting 7) for 5 s.

Assays contained an aliquot of membrane preparation (ranging from ~ 12 to 30 μg protein, which, depending on the cell line, resulted in binding < 10% of the added radioactivity), DOV 21,947, and [^{125}I]RTI-55 (40–80 pM final concentration) in a final volume of 250 μl. Krebs–HEPES assay buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 μM pargyline, 100 μM tropolone, 2 mg glucose/ml, 0.2 mg ascorbic acid/ml, pH 7.4) was used for all assays. Specific binding was defined as the difference in binding observed in the presence and absence of 5 μM mazindol (HEK-hDAT and-NET) or 5 μM imipramine (HEK-hSERT). Membranes were preincubated with DOV 21,947 (21.6 nM to 10 μM) at room temperature for 10 min before the addition of [^{125}I]RTI-55, unless indicated otherwise. The reaction (performed in duplicate at each concentration of DOV 21,947) was incubated for 90 min at room temperature in the dark and was terminated by filtration through Wallac Filtermat A filters using a 96-well Tomtec cell harvester. Radioactivity remaining on the filters was determined as described for uptake assays (above).

2.2. In vivo studies

In vivo studies were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals (revised 1996). These studies were approved by the Animal

Care and Use Committee of the Institute of Pharmacology, Polish Academy of Sciences.

2.2.1. Animals

Male Wistar rats (~ 250 g) were obtained from the breeding facility of the Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland. Male C57/Bl mice (~ 25 g) were obtained from the breeding facility of the University Children's Hospital, Prokocim, Cracow, Poland. Animals were group-housed in standard laboratory cages and maintained in a temperature-controlled colony room (21 ± 2 °C) with a 12-h light/dark cycle (lights on: 07:00). Commercial food and tap water were freely available.

2.2.2. Forced swim test

Rats were placed in transparent, cylindrical 10-l glass beakers (height ~ 40 cm, internal diameter ~ 19 cm) containing water (24–25 °C) to a level of 22 cm, as described by Porsolt et al. (1977). Two to three rats, separated by opaque screens, were exposed simultaneously. Water was always changed between trials. The procedure consisted of a pretest and test separated by 24 h (Porsolt et al., 1978). During the pretest, rats (adapted to the experimental room for no less than 1 h) were placed in the glass beakers for 15 min. Following this initial exposure, the rats were wiped dry with paper towels and transferred to a "drying cage" with an IR lamp suspended over this cage. Fifteen minutes later, rats were administered either drugs or vehicle and transferred to their home cages. The following day, rats were transferred to the experimental room and reacclimated for at least 1 h. Rats were administered either drugs or vehicle (imipramine and DOV 21,947 were administered 30 and 60 min before testing, respectively) and placed in the test chambers. The time each animal spent immobile was measured during a 5-min period by an observer unaware of the treatment conditions, using the "PORSOLT" program (Layer et al., 1995). As described by Porsolt et al. (1977), a rat was considered immobile when floating motionless or making only those movements necessary to keep his head above water surface.

2.2.3. Tail suspension test

This procedure was performed essentially as described by Steru et al. (1985). C57/Bl mice were individually suspended 75 cm above the tabletop with an adhesive tape placed ~ 1 cm from the tip of the tail. Immobility duration was recorded for 6 min. Mice were considered immobile only when they hung passively and completely motionless. A trained observer, using the "PORSOLT" program (Layer et al., 1995), measured the duration of immobility.

2.2.4. Locomotor activity

Rats were transferred into the testing room at least 2 h prior to drug administration. Sixty minutes after administration of MK-801 or DOV 21,947, rats were placed in a custom fabricated, 50 × 50 × 25 cm (l × w × h) activity chambers. Locomotor activity was recorded for a total of 30 min in 10-min epochs.

2.3. Data analysis

Prism 3.0 (GraphPad Software, San Diego, CA) was used for data analysis. Immobility time (in seconds) was used for statistical analyses of data in the forced swim and tail suspension tests. Data from vehicle and imipramine treatment were pooled across several independent experiments to create one "negative" and "positive" control. Locomotor activity of rats calculated as area under the curve (AUC) on measurements 0–30 min (at 10-min intervals) after drug administration with the use of the trapezoidal rule.

2.4. Drugs

DOV 21,947 was prepared from a racemic mixture using simulated moving bed chromatography (Huthmann and Juza, 2000) by CarboGen, Laboratories (Aarau, Switzerland). MK-801 was the gift of Prof. Kenner Rice, NIH. Other compounds were purchased from Sigma-Aldrich (St. Louis, MO). DOV 21,947 (dissolved in deionzed water) was administered orally via feeding tube. Imipramine and MK-801 (dissolved in saline) were administered intraperitoneally. Drugs were administered to rats and mice in volumes of 1 and 10 mg/kg, respectively.

3. Results

3.1. Inhibition of [^{3}H]biogenic amine reuptake

DOV 21,947 produced a concentration-dependent inhibition of [^{3}H]norepinephrine, [^{3}H]serotonin, and [^{3}H]dopamine reuptake in HEK 293 cells transfected with the respective amine transporters. Under the conditions described in Materials and methods, the IC_{50} values for DOV 21,947 to inhibit [^{3}H]amine uptake were 22.8 ± 3.3, 12.3 ± 2.8, and 96 ± 20 nM, respectively (Table 1).

3.2. Inhibition of [^{125}]RTI-55 binding

DOV 21,947 inhibited [^{125}]RTI-55 binding to membranes prepared from HEK 293 cells expressing recombinant human norepinephrine, serotonin, and dopamine transporters with K_i values of 262 ± 41, 99 ± 16, and 213 ± 56 nM, respectively; Hill slopes did not differ from unity (Table 1).

3.3. Effects of DOV 21,947 in the forced swim test

Sixty minutes following oral administration, DOV 21,947 produced a dose-dependent reduction in immobility in the forced swim test (Fig. 2, top panel). The MED of DOV 21,947 was 5 mg/kg, with a maximum reduction in immobility at 20 mg/kg comparable to that produced

Table 1
Inhibition of [^{3}H]neurotransmitter uptake and [^{125}I]RTI-55 binding to neurotransmitter transporters by DOV 21,947: comparison with other antidepressants

Compound	hDAT, binding	[^{3}H]DA uptake	hSERT, binding	[^{3}H]5-HT uptake	hNET, binding	[^{3}H]NE uptake
DOV 21,947	213 ± 56	96 ± 20	99 ± 16	12.3 ± 2.8	262 ± 41	22.8 ± 3.3
Desmethylimipramine	>10,000	>10,000	55 ± 4	64 ± 17	13.9 ± 1.5	4.2 ± 1.1
Fluoxetine	6670 ± 850	>10,000	1.1 ± 0.5	7.3 ± 2.9	1560 ± 30	1020 ± 180
Imipramine	>10,000	>10,000	3.3 ± 0.4	8.0 ± 2.3	215 ± 72	70 ± 21
Nomifensine	87 ± 23	72 ± 9	2350 ± 200	2780 ± 650	119 ± 16	42 ± 15

Values are expressed in nM (K_i for inhibition of radioligand binding, IC_{50} for inhibition of neurotransmitter uptake) and represent the $X \pm$ S.E.M. of at least three independent experiments for DOV 21,947. Comparative values for the other antidepressants are taken from Eshleman et al. (1999) and represent the $X \pm$ S.E.M. of three to eight experiments assayed under conditions identical to those employed in the present study. Hill slopes were not different from unity. Abbreviations: hDAT, human dopamine transporter; DA, dopamine; hSERT, human serotonin transporter; 5-HT, serotonin; hNET, human norepinephrine transporter; NE, norepinephrine.

by the positive control, an intraperitoneal dose of imipramine (15 mg/kg) administered 15 min prior to testing (Fig. 2, top panel).

3.4. Effects of DOV 21,947 on locomotor activity

DOV 21,947 did not produce a statistically significant increase in locomotor activity at doses (10 and 20 mg/kg) active in the forced swim test (Fig. 2, bottom panel). By contrast, the positive control in this study, MK-801 (0.15 mg/kg, i.p.), significantly increased locomotor activity (Fig. 2, bottom panel).

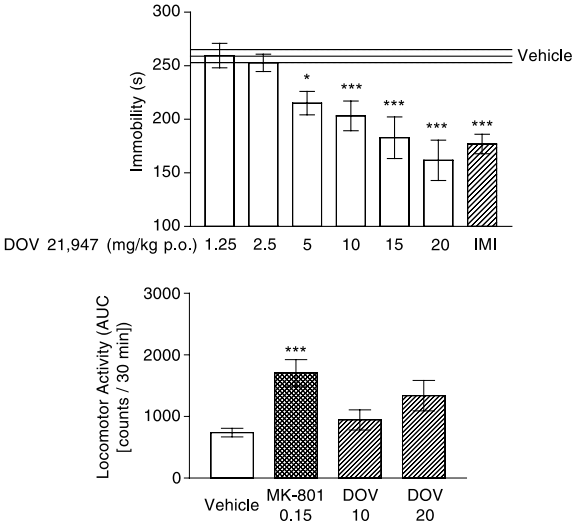

Fig. 2. Effects of DOV 21,947 on immobility in the forced swim test (top panel) and locomotor activity (bottom panel). Top panel: DOV 21,947 was administered to male Wistar rats as described in Materials and methods. Imipramine (15 mg/kg, i.p.) was included as a positive control. Values represent $X \pm$ S.E.M. of 8–18 rats/group. The solid horizontal lines flanked by two dotted lines represent immobility times ($X \pm$ S.E.M.) of saline-treated rats. The hatched bar represents imipramine-treated rats. ANOVA demonstrates significant effects among treatments: $F(7,90) = 9.13$, $P < 0.001$. Asterisks indicate statistically significant difference compared with the control group that received saline (i.p.). Symbols: $^*P < 0.05$; $^{***}P < 0.001$, Dunnett's multiple comparison test. Bottom panel: DOV 21,947 and MK-801 were administered to male Wistar rats as described in Materials and methods. MK-801 was selected as a positive control because it produces a reliable increase in motor activity in rodents. Values represent the $X \pm$ S.E.M. of 7–9 rats/group. Data is expressed as the AUC between 0 and 30 min as described in Materials and methods. ANOVA: $F(3,32) = 6.11$, $P < 0.01$. Symbol: $^{***}P < 0.001$, Dunnett's multiple comparison test compared with saline-treated (intraperitoneal) rats.

3.5. Effects of DOV 21,947 in the tail suspension test

DOV 21,947 produced a dose-dependent reduction in immobility in the tail suspension test with an MED of 5 mg/kg,

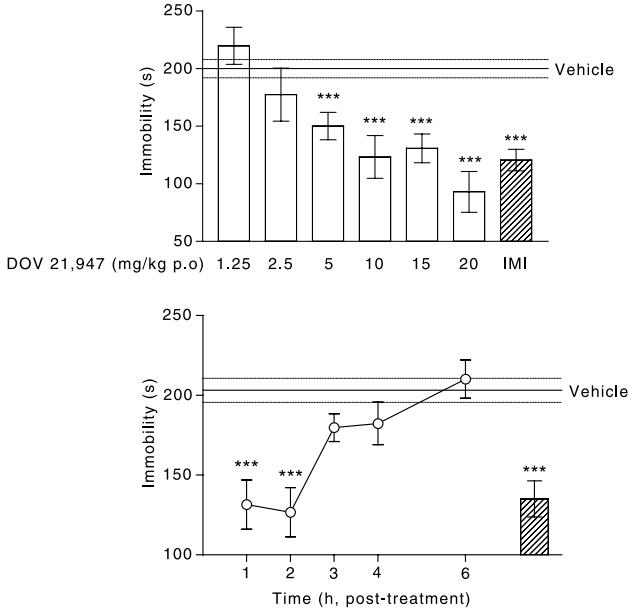

Fig. 3. Effects of DOV 21,947 on immobility in the tail suspension test. (Upper panel) C57Bl/6J mice were orally administered DOV 21,947 1 h prior to testing. Values represent the $X \pm$ S.E.M. of 6–22 mice/group. The solid horizontal line flanked by two dotted lines represents immobility times ($X \pm$ S.E.M.) of saline-treated mice. The hatched bar represents immobility times of imipramine-treated mice. ANOVA demonstrates significant effects among treatments: $F(7,82) = 9.74$, $P < 0.001$. Asterisks indicate a statistically significant difference toward control group that received saline i.p. $^{***}P < 0.001$, Dunnett's multiple comparison test. (Bottom panel) Groups of mice were administered DOV 21,947 (20 mg/kg, p.o.) 1–6 h prior to testing. Imipramine (20 mg/kg, i.p., administered 30 min prior to testing) was included as a positive control (hatched bar). Values represent the $X \pm$ S.E.M. of 8–28 mice/group. The solid horizontal lines flanked by two dotted lines represent immobility times ($X \pm$ S.E.M.) of saline-treated mice. ANOVA demonstrates significant effects among groups: $F(6,121) = 9.61$, $P < 0.001$. Symbols: $^{***}P < 0.001$, Dunnett's multiple comparison test compared to mice that received saline (i.p.) or water (p.o.).

p.o. (Fig. 3, top panel). The maximum reductions in immobility produced by 10–20 mg/kg of DOV 21,947 were comparable to that produced by the positive control, an intraperitoneal dose of imipramine (20 mg/kg) administered 30 min prior to testing. DOV 21,947 (20 mg/kg, p.o.) significantly reduced immobility in the tail suspension test for at least 2 h, with immobility times returning to control values within 6 h (Fig. 3, bottom panel).

4. Discussion

Anhedonia is a core symptom of depression, and both preclinical and clinical studies have demonstrated that dopaminergic pathways are integral to reward, or hedonic, processes (reviewed in D'Aquila et al., 2000; Naranjo et al., 2001; Willner, 2000). These data have led to the proposal that hypofunction of the mesocorticolimbic dopaminergic system may mediate both the anhedonia and loss of motivation typically associated with major depressive disorder (reviewed in Willner, 2000; Zacharko and Anisman, 1991). A compound capable of increasing synaptic dopamine concentrations could have an immediate effect to reduce symptoms of anhedonia, and when combined with an increased synaptic availability of norepinephrine and serotonin, result in a greater efficacy and/or more rapid onset than conventional antidepressants. While this hypothesis has not been fully explored in the clinic, several studies have demonstrated that the adjunctive use of dopamine agonists (e.g., pergolide, pramipexole) produces an improvement in ~40–50% of depressed individuals refractory to monotherapy with conventional agents (Boukoms and Mangini, 1993; Izumi et al., 2000; Sporn et al., 2000). Further, as monotherapies, dopamine agonists (e.g., pramipexole, bromocriptine) are antidepressant (Corrigan et al., 2000; Sitland-Marken et al., 1990; Willner, 2000), but do not appear to be dramatically different from "traditional" antidepressants with respect to either efficacy or onset of action.

There is also a body of preclinical evidence consistent with the hypothesis that the therapeutic profile of an antidepressant concurrently increasing the synaptic availability of dopamine, norepinephrine, and serotonin will differ from a compound selectively affecting norepinephrine and/or serotonin reuptake. Thus, it has been known for more than 20 years (Serra et al., 1979) that chronic antidepressant treatments enhance dopamine receptor function in the mesolimbic system (reviewed in D'Aquila et al., 2000). This phenomenon has been independently observed by many laboratories, and across a wide range of antidepressants (reviewed in D'Aquila et al., 2000). The apparent requirement for chronic antidepressant treatment indicates this enhanced sensitivity to dopamine receptor stimulation is an adaptive phenomenon. This adaptation may occur via projections into the nucleus accumbens from other limbic areas more traditionally associated with antidepressant action (Willner, 1997). While the molecular mechanism(s) responsible for this increased sensitivity to dopamine are unknown, several laboratories have reported that chronic antidepressant administration increases the expression of mRNA encoding dopamine D2 and D3 receptors in the mesolimbic system (Ainsworth et al., 1998; Dziedzicka Wasylewska et al., 1997; Lammers et al., 2000). If these antidepressant-induced changes in dopamine receptor sensitivity are required for a therapeutic effect, then increasing the synaptic availability of dopamine (together with norepinephrine and serotonin) may initiate this process, resulting a rapid onset of action and/or high efficacy.

DOV 21,947 inhibits both [^{125}I]RTI-55 binding to the serotonin, norepinephrine, and dopamine transporters and reuptake of the respective biogenic amines (Table 1). The optimum potency ratio for inhibiting these three biogenic amines is unknown, but there is a wide variation in potency ratios among clinically active dual reuptake inhibitors (Table 1 and Briley and Moret, 1997). While DOV 21,947 has not yet entered clinical trials, plasma levels of the racemic form of this molecule are sufficient to saturate each of the transporters at doses that are well tolerated by normal volunteers (unpublished observations). Further, DOV 21,947 is orally active in both the forced swim and tail suspension tests, procedures that are predictive of antidepressant activity in humans (Borsini and Meli, 1988; Porsolt and Lenegre, 1992). Since these preclinical procedures do not yield useful information about either onset of action or efficacy, the antidepressant properties of such a broad-spectrum agent merits study in the clinic.

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