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ANTIDEPRESSANT BIOCHEMICAL PROFILE OF THE NOVEL BICYCLIC COMPOUND Wy-45,030, AN ETHYL CYCLOHEXANOL DERIVATIVE

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Abstract—The novel bicyclic compound Wy-45,030 [1-2-(dimethylamino)-1-(4-methoxyphenyl)ethyl cyclohexanol, hydrochloride] exhibited a neurochemical profile predictive of antidepressant activity. Like the tricyclic antidepressants, it inhibited rat brain imipramine receptor binding and synaptosomal monoamine uptake (dopamine as well as norepinephrine and serotonin). It did not inhibit monoamine oxidase. Unlike the tricyclic antidepressants, it was not antimuscarinic in the guinea pig ileum, nor did it have any appreciable affinity for brain α-1 adrenergic or histamine-1 binding sites. Wy-45,030 was also without affinity for α-2 or β adrenergic, benzodiazepine, serotonin-1, serotonin-2, dopamine-2, and opiate receptors. Such a profile is predictive of antidepressant activity devoid of the side-effects common to tricyclic therapy.

Tricyclic antidepressants have been observed to displace [^{3}H]imipramine from rat brain cortical binding sites with potencies paralleling their abilities to inhibit neuronal serotonin uptake [1, 2]. We have exploited this correlation to screen for probable monoamine uptake inhibitors among novel compounds by examining their abilities to inhibit [^{3}H]imipramine binding in rat cortical membranes. Having thus identified Wy-45,030 [Fig. 1; 1-2-(dimethylamino) - 1 - (4 - methoxyphenyl)ethyl cyclohexanol, hydrochloride] [3], we subsequently examined this compound and its enantiomers for other *in vitro* evidence of antidepressant activity. Specifically, the compounds were examined for their inhibition of synaptosomal norepinephrine, serotonin, and dopamine uptake; their inhibition of monoamine oxidase activity; and their interaction with several other drug and neurotransmitter receptor sites in rat brain (serotonin-1, serotonin-2, dopamine-2, α-1, α-2 and β-adrenergic, muscarinic cholinergic, histamine-1, benzodiazepine, and opiate receptors) and in the guinea pig ileum (muscarinic cholinergic and histamine-1).

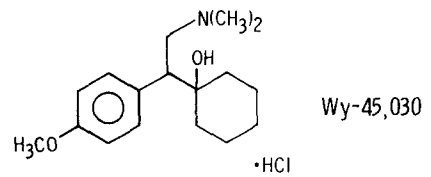

Fig. 1. Structure of Wy-45,030 [1-2-(dimethylamino)-1-(4-methoxyphenyl)ethyl cyclohexanol, hydrochloride].

METHODS

General receptor binding. Several rats were decapitated and the brains were removed rapidly. Dissected cortical tissue (imipramine, serotonin-2, noradrenergic, and benzodiazepine binding), striatal tissue (dopamine-2 and muscarinic cholinergic binding), or whole brain minus the cerebellum (serotonin-1 and histamine-1 binding) was homogenized on ice in buffer (see Table 1) using a Polytron homogenizer. The homogenates were centrifuged and resuspended in fresh buffer several times to wash out endogenous ligands. The protein concentration of the final tissue suspension was determined by the method of Lowry *et al.* [4] and, except for serotonin-1 tissue, the suspension was stored at −70° until use. Aliquots of tissue suspension (0.2 to 0.3 mg protein/sample) were incubated with the appropriate tritiated ligand (Table 1) and various concentrations of test compound. Following incubation, cold buffer was added to each sample, and the contents were rapidly vacuum-filtered through Whatman GF/B glass-fiber filters. The filters were then rapidly washed three times with 50 mM sodium phosphate buffer (pH 7.4), placed in scintillation vials, and shaken for 15 min with 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ) scintillation mixture. The vials were then counted in a Packard 460CD scintillation counter. Specific binding was defined as total binding less binding in the presence of displacing agent (Table 1). Binding in the presence of various concentrations of test compound was expressed as a percent of specific binding with no drug present. These results were then plotted as logit percent binding versus log concentration of test compound. Linear regression analysis then yielded a straight line with 95% confidence limits from which an IC_{50} was inversely predicted. K_i values were then

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Table 1. Receptor binding protocols

Receptor	Ligand			Buffer	Incubation		Displacing agent	Reference
	[^{3}H]-Ligand	Molarity (nM)	Specific activity (Ci/mmole)		Time	Temp. (°C)		
Imipramine	Imipramine HCl	5.0	20–40	A*	1 hr	0–4°	0.4 μM Imipramine HCl	[1]
Serotonin-1	5-Hydroxytryptamine binoxalate	5.0	15–30	See text	30 min	37°	1 μM 5-Hydroxy-tryptamine creatinine sulfate	[5]
Serotonin-2	Spiperone	0.8	20–40	B†	10 min	37°	10 μM (+)Butaclamol	[6, 7]
Dopamine-2	Spiperone	0.3	20–40	B†	10 min	37°	1 μM (+)Butaclamol	[6, 7]
Adrenergic:								
α-1	WB 4101	0.5	15–30	50 mM Tris–HCl, pH 7.7	30 min	25°	10 μM Norepinephrine bitartrate	[8]
α-2	*p*-Aminoclonidine	4.0	40–60	50 mM Tris–HCl, pH 7.7	30 min	25°	10 μM Norepinephrine bitartrate	[8]
β	Dihydroalprenolol HCl	4.0	90–120	50 mM Tris–HCl, 3 mM $MgCl_2$, pH 8.0	10 min	37°	20 μM Propranolol HCl	[7]
Muscarinic cholinergic	Quinuclidinyl benzilate	0.06	30–60	50 mM Tris–HCl, pH 7.7	1 hr	25°	100 μM Oxotremorine	[9]
Histamine-1	Pyrilamine	2.0	>20	50 mM phosphate, pH 7.5	30 min	25°	10 μM Chlorpheniramine maleate	[10]
Benzodiazepine	Diazepam	3.5	>60	A*	1 hr	0–4°	3 μM Lorazepam	[11, 12]
Opiate	Naloxone	1.3	40–60	50 mM Tris–HCl, pH 7.4	30 min	0–4°	2 μM Morphine	[13]

* A = 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4.
† B = 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, 10 μM pargyline HCl, pH 7.1.

calculated using the following formula [14]:

$$K_i = \frac{IC_{50}}{1 + \frac{[^3\text{H-ligand}]}{K_D}}$$

where K_D = the dissociation constant for the ^{3}H-ligand:receptor complex under consideration.

Serotonin-1 receptor binding. Serotonin-1 binding was performed using an adaptation of the method of Savage *et al.* [5]. Rat brain tissue was homogenized in 10 ml of buffer "1" (280 mM sucrose, 25 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4) using a Teflon/glass homogenizer. The homogenate was centrifuged at 800 *g* for 15 min. The supernatant fraction was recentrifuged at 30,000 *g* for 10 min. This pellet was resuspended in 7.14 ml of distilled water and kept on ice for 20 min with occasional mixing. It was then centrifuged again at 30,000 *g* for 10 min. The resulting pellet was resuspended in 10 ml of buffer "2" (50 mM Tris–HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.4) and incubated at 30° for 15 min. The suspension was then centrifuged at 30,000 *g* for 10 min, and the pellet was resuspended in 2.57 ml of incubation buffer (buffer "2" + 10 μM pargyline HCl and 1.1 mM ascorbic acid, pH 7.4). The protein content was assayed by the method of Lowry *et al.* [4]. Remaining methodology was the same as in the preceding section, "General Receptor Binding."

Synaptosomal uptake studies. These studies were performed using the modified methodology of Wood and Wyllie [15]. Five rats were decapitated, and the brains were removed rapidly and placed on ice. All subsequent steps were performed at 0–4° unless otherwise specified. After weighing, the brains were homogenized in 10 vol. of 0.32 M sucrose using a Potter–Elvehjem Teflon homogenizer (8 strokes at 840 rpm, clearance 0.009–0.010 in.). The homogenate was centrifuged at 1000 *g* for 10 min. The resulting supernatant fraction was centrifuged at 10,000 *g* for 20 min. The resulting pellet was resuspended in a Dounce homogenizer in 1 ml of 0.32 M sucrose/g original wet weight of tissue. Aliquots (5 ml) of the suspension were layered onto sucrose density gradients (7 ml of 3.0 M sucrose, 12 ml of 1.2 M sucrose, 10 ml of 0.8 M sucrose in Sorvall 34-ml polycarbonate centrifuge tubes, No. 3141) and centrifuged in the vertical rotor of a Sorvall OTD-65B ultracentrifuge (acceleration rate control setting 4 to 201,000 *g* for 25 min; total time not including deceleration, 40 min). A 7-ml sample of the synaptosomal fraction (at the 0.8 M/1.2 M interface) was collected from each tube and resuspended in 25 ml of 0.32 M sucrose. This synaptosomal preparation was centrifuged in the angle rotor of the ultracentrifuge at 97,000 *g* for 30 min, and the resulting pellet was resuspended using a Dounce homogenizer in 2 ml of 0.32 M sucrose/g original wet weight of tissue. Protein content of this preparation was determined by the method of Lowry *et al.* [4].

For uptake studies, all components were dissolved in the following buffer: 136 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 1 mM ascorbic acid, 20 mM Tris, pH 7.4, gassed with O_2 for 30 min prior to use. Various concentrations of test drug were preincubated with 0.1 μM [^{3}H]dopamine or 0.1 μM [^{3}H]norepinephrine (130,000 dpm/tube) and 0.1 μM [^{14}C]serotonin (7,500 dpm/tube) in 0.9 ml buffer for 5 min at 37°. One-tenth milliliter of synaptosomal preparation was added to each tube and incubated for a further 4 min at 37°. The reaction was terminated by the addition of 2.5 ml buffer, after which the mixture was filtered under vacuum using cellulose acetate filters (0.45 μm pore size). The filters were washed once with an additional 2.5 ml buffer, removed and shaken in scintillation vials with 10 ml Hydrofluor, and counted in a Packard 460CD scintillation counter equipped with dual-label dpm data reduction. Sodium-dependency of uptake was determined by running equivalent samples in buffer with NaCl replaced by LiCl. Blank samples contained either no tissue or osmotically-shocked (ruptured) synaptosomes. All samples were run in triplicate.

Results were expressed as pmoles uptake/mg protein/min. The IC_{50} values for uptake inhibition were calculated by linear regression of logit [percent of Na^+-dependent uptake] vs log [concentration of test drug].

Monoamine oxidase (MAO) inhibition. MAO activity was determined using a modification of the method of Wurtman and Axelrod [16]. Several rats were decapitated, and the brains were removed and homogenized (minus the cerebellum) in 10 vol. of 0.15 M KCl. The protein content of this homogenate was determined by the method of Lowry *et al.* [4]. Fifty microliter (0.6 mg protein) aliquots of the homogenate were incubated with 30 μM [^{14}C]tryptamine (6000 dpm) and various concentrations of test compound in a final volume of 0.5 ml of 1.25 M potassium phosphate buffer, pH 7.4. After a 20-min incubation at 37°, the reaction was acidified and stopped by the addition of 0.5 ml of 2 N HCl. Toluene (6 ml) was then added to each sample, and the radioactive oxidative products (primarily indoleacetic acid) were extracted into the organic phase. Five milliliters of the toluene phase was transferred to scintillation vials containing 10 ml of Hydrofluor, shaken, and counted in a Packard 460CD scintillation counter. Following transformation of the results to a percent of basal enzyme activity, an IC_{50} for the test compound was inversely predicted from the linear regression of logit [percent basal activity] on log [concentration of test compound].

Antagonism of guinea pig ileal contraction. Male Charles River Hartley guinea pigs, weighing 250–300 g, were killed by cervical dislocation. Segments of terminal ileum immediately proximal to Peyer's patch were transferred to petri dishes containing aerated 37° Tyrode's solution of the following composition: 137 mM NaCl; 3.4 mM KCl; 0.1 mM $MgCl_2 \cdot 6H_2O$; 12 mM $NaHCO_3$; 1.4 mM $CaCl_2$; 5.0 mM glucose; and 0.4 mM NaH_2PO_4, pH 7.4.

The ileal segments were suspended in 10 ml of 37° Tyrode's solution in a tissue bath and aerated with room air. Tissues were placed under 1 g tension and allowed to equilibrate for 1 hr. By plotting grams tension versus the log dose of stimulant, control dose responses to histamine and carbachol were obtained in separate groups of four ileal segments each. The baths were then replaced with baths containing a 1 μM concentration of the drug to be tested, tissues

were allowed to equilibrate in the presence of the drug for 20 min, and the dose responses were repeated. Following estimation of IC_{50} values, an antagonist K_B was calculated by the formula:

$$K_B = [\text{Antagonist}]\left[\frac{IC_{50}\text{ with test drug}}{\text{Control } IC_{50}} - 1\right]$$

Drugs and chemicals. All radioactive chemicals were purchased from New England Nuclear, Boston, MA. Desipramine and propranolol were the gifts of Merrell-Dow Pharmaceuticals and Ayerst Laboratories, Inc., respectively. Lorazepam, Wy-45,030, Wy-45,651, and Wy-45,655 were synthesized at Wyeth. All other compounds and reagents were purchased from standard commercial sources.

RESULTS

Receptor affinities (Table 2). With a K_i of 90 nM, Wy-45,030 was slightly more potent than desipramine in inhibiting [^{3}H]imipramine binding in rat brain cortex. Surprisingly, the two enantiomers of the racemic Wy-45,030 (Wy-45,651 and Wy-45,655) also inhibited imipramine binding with similar affinities. Unlike desipramine, Wy-45,030 exhibited no significant affinity for the rat brain muscarinic cholinergic, α-1 adrenergic, or histamine-1 binding sites. Neither Wy-45,030 nor desipramine showed appreciable affinity for α-2 or β adrenergic receptors. Wy-45,030 was also without appreciable affinity for benzodiazepine, serotonin-1, serotonin-2, dopamine-2, or opiate receptors (data not shown).

Synaptosomal uptake (Table 2). In synaptosomes prepared from rat brain, Wy-45,030 inhibited the uptake of norepinephrine, serotonin, and dopamine, with respective IC_{50} values of 0.64, 0.21 and 2.8 μM. In parallel with imipramine binding studies, the enantiomers Wy-45,651 and Wy-45,655 also inhibited the uptake of norepinephrine and serotonin (see Table 2). For comparison, desipramine inhibited monoamine uptake with IC_{50} values of 0.15 μM (norepinephrine) and 1.5 μM (serotonin). Inhibition of dopamine uptake by desipramine was weak ($IC_{50} > 20$ μM).

Monoamine oxidase inhibition. Wy-45,030 exhibited no inhibition of rat brain monoamine oxidase at concentrations up to 10 μM. For comparison, pargyline exhibited a K_i of 1.1 μM when [^{14}C]tryptamine was used as the substrate.

Guinea pig ileal contraction. Responses of the isolated guinea pig ileum to carbachol, histamine, and potassium chloride were not altered by the presence of 1 μM Wy-45,030; nor was the potassium chloride response altered by imipramine. In contrast, imipramine shifted the dose–response curves to both carbachol and histamine to the right. Approximate K_B values for imipramine were determined to be 1×10^{-7} M (against carbachol) and 8×10^{-9} M (against histamine).

DISCUSSION

Wy-45,030 exhibited an *in vitro* biochemical profile predictive of antidepressant activity. It competed with [^{3}H]imipramine for cortical binding sites, and inhibited the uptake of norepinephrine and serotonin in rat brain synaptosomal preparations. The uptake inhibitory property has been cited as a mechanism for the observed inhibitory effects of Wy-45,030 on locus coeruleus noradrenergic neuronal firing rate [17]. Wy-45,030 is not a monoamine oxidase A or B inhibitor, since it does not inhibit the oxidation of [^{14}C]tryptamine, a nonspecific monoamine oxidase substrate [18].

Interestingly, both the [−] (Wy-45,651) and [+] (Wy-45,655) enantiomers of Wy-45,030 were active in inhibiting synaptosomal uptake and [^{3}H]imi-

Table 2. Comparison of neurochemical profiles of desipramine (DMI), Wy-45,030, Wy-45,651, and Wy-45,655 in rat brain

	DMI	Wy-45,030	Wy-45,651	Wy-45,655
	Receptor binding K_i (nM) (95% C.I.)			
Imipramine	130 (110–150)	90 (10–3000)	140 (100–200)	109 (71–198)
Muscarinic cholinergic	50 (36–68)	>10 μM		
Histamine-1	124 (77–198)	>10 μM		
α-1 Adrenergic	300 (150–750)	>10 μM		
α-2 Adrenergic	>10 μM	>10 μM		
β Adrenergic	>10 μM	>10 μM		
	Neurotransmitter uptake: IC_{50} (μM) (95% C.I.)			
Norepinephrine	0.15 (0.07–0.38)	0.64 (0.50–0.84)	0.76 (0.61–0.99)	3.14 (2.87–3.45)
Serotonin	1.5 (1.1–2.3)	0.21 (0.15–0.28)	0.19 (0.16–0.23)	0.10 (0.09–0.12)
Dopamine	>20	2.8 (1.8–5.1)		

pramine binding, albeit with somewhat different potencies. Such lack of stereospecificity at neuronal monoamine uptake sites has been observed previously [19], but its significance is unclear.

A possibly important difference between Wy-45,030 and the standard tricyclic antidepressants is the ability of Wy-45,030 to inhibit synaptosomal dopamine uptake. Whereas the tricyclics and some of the newer antidepressants in clinical use show relative specificities for norepinephrine (maprotiline), serotonin (fluoxetine), or dopamine uptake inhibition (nomifensine; also norepinephrine uptake) [20], Wy-45,030 exhibited uptake inhibition for all three monoamines. While the clinical significance of this property is unknown, such a profile is unique among antidepressants.

A more immediately advantageous property of Wy-45,030 was its lack of affinity at muscarinic cholinergic, histamine-1, and α-1 adrenergic receptors. Unlike the tricyclic antidepressants, exemplified by desipramine, Wy-45,030 ought not to cause side-effects mediated by the above receptors, such as dry mouth, constipation, urinary retention, visual disturbances, and sedation.

Recently, clinically used antidepressants have been observed to cause down-regulation of serotonin-2 receptors in rodent brain after chronic treatment [21], despite a lack of acute interaction with these receptors. The novel antidepressant mianserin, however, does bind to these receptors *in vitro* [22]. For this reason, the ability of Wy-45,030 to inhibit serotonin receptor binding was assessed. Wy-45,030, like most antidepressants except mianserin, showed no inhibition of either [^{3}H]spiroperidol binding to serotonin-2 receptors in rat frontal cortex or [^{3}H]serotonin binding to serotonin-1 receptors in whole rat brain. The effect of chronic treatment with Wy-45,030 on serotonin-2 receptor density has not been examined yet.

In summary, Wy-45,030 is a novel bicyclic compound that possesses an antidepressant biochemical profile. Although it inhibited monoamine uptake like the classical tricyclic drugs, it was devoid of other drug or neurotransmitter receptor affinities. These results suggest that Wy-45,030 will have antidepressant activity comparable to the tricyclic agents without their associated side-effects.

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