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NEUROCHEMICAL PROPERTIES OF AHR-9377: A NOVEL INHIBITOR OF NOREPINEPHRINE REUPTAKE

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Abstract—A novel potential antidepressant, AHR-9377, was evaluated for its inhibition of norepinephrine (NE), serotonin (5-HT) and dopamine (DA) reuptake in hypothalmic, cortical, and striatal rat synaptosomal preparations. AHR-9377 was found to be a potent, selective, noncompetitive inhibitor of NE reuptake. In addition, repeated injections of AHR-9377 caused a decrease in the density of beta adrenergic receptors in rat cerebral cortex. Little displacement by AHR-9377 at beta, alpha$_1$ and alpha$_2$ adrenergic, histaminergic, muscarinic, GABA-ergic, benzodiazepine of imipramine sites was observed. These pharmacological properties indicate that AHR-9377 may have clinical antidepressant activity with few side effects.

Norepinephrine (NE) reuptake plays a major role in terminating the effects of NE released into the synapse [1]. Stimulating the release or blocking the reuptake system causes an increase in NE levels at cerebral cortical synapses and, if this elevation persists for 1 week or longer, it will reduce the number of beta adrenergic receptors on those synaptic membranes [2].

It has been suggested that the time required for the reduction in number of beta adrenergic receptors could account for the lag time between administering antidepressants and lessening the symptoms of depression [2]. Moreover, the inhibition of NE and serotonin (5-HT) reuptake by tricyclic antidepressants correlates well with their clinical antidepressant effects [3, 4]; in contrast, imipramine and desimipramine are weaker inhibitors of dopamine reuptake.

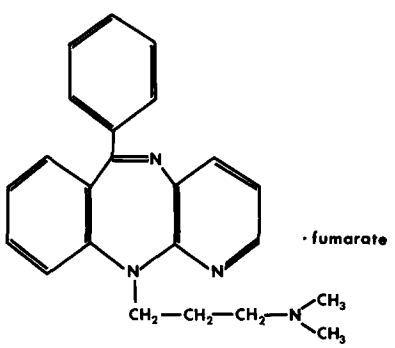
The present report is a description of the neurochemical properties of a new and selective NE-reuptake inhibitor, AHR-9377 (*n*,*N*-dimethyl-6-phenyl-11-*H*-pyrido[2,3-*b*][1,4]benzodiazepine-11-propanamine, (E)-butendedioate (1:1) (Fig. 1). This compound was evaluated for its selectivity in inhibiting NE reuptake, its potential for biogenic amine release, and its ability to decrease beta adrenergic receptors. In addition, the potency of AHR-9377 against muscarinic, alpha$_1$ and alpha$_2$ adrenergic and histaminergic receptors was investigated to assess possible side effects associated with antagonizing those receptors.

These results indicate that AHR-9377 may have clinical antidepressant activity without anticholinergic, antihistaminergic and antiadrenergic side effects.

MATERIALS AND METHODS

Synaptosomal preparation. Synaptosome-rich homogenates were prepared according to the method of Snyder and Coyle [5]. Briefly, male Sprague–Dawley rats, 150 g, were killed by decapitation. The hypothalamus, striatum, or cortex was rapidly removed and placed in a Potter–Elvehjem homogenizer with 10 vol. of ice-cold 0.32 M sucrose, pH 7.0, homogenized with eight complete strokes. The homogenate was centrifuged at 1000 *g* for 10 min at 4°. The supernatant fraction containing the synaptosomes was decanted and used in reuptake and release studies; hypothalamus, cerebral cortex, and striatum were used, respectively, for NE, 5-HT and dopamine (DA) reuptake and release studies.

Reuptake and release studies. Reuptake experiments were done according to the procedure of Snyder and Coyle [5]. A 0.05-ml aliquot (60–100 mg



AHR-9377

Fig. 1. Structural formula of AHR-9377.

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protein/ml) of the synaptosomal preparation was incubated in a mixture consisting of 0.80 ml of modified Krebs–Ringer solution (118 mM NaCl, 4 mM KCl, 1.3 mM $CaCl_2$, 1.12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM glucose, 1 mM ascorbic acid, 0.15 mM EDTA, 1.2 μM nilamide, 24 mM $NaHCO_3$, pH 7.4), 0.1 ml of drugs, and 0.1 ml of a 1 μM tritiated amine ([^{3}H]NE, 8.8 Ci/mmole; [^{3}H]5-HT, 15.9 Ci/mmole; [^{3}H]DA, 13 Ci/mmole) for a total volume of 1 ml. The incubation mixture was preincubated at 37° under 95% O_2/5% CO_2 for 5 min in a Dubnoff Metabolic Shaking Incubator before adding the tritiated biogenic amine; the incubation was then continued for 10 min. At the end of the incubation, synaptosomes were collected by filtration [6], and their radioactivity was determined by liquid scintillation counting.

The release of biogenic amines from synaptosomes was determined according to the procedure of Vosmer *et al.* [7]. The procedure was similar to the one described for reuptake except that synaptosomes were incubated with the biogenic amine without drug. Subsequently, synaptosomes were harvested by centrifugation at 20,000 *g* for 10 min, and the pellet was resuspended in the Krebs–Ringer solution. The drug was then added, and the mixture was incubated for an additional 10 min. Synaptosomes were harvested by filtration, and radioactivity was determined.

Chronic injection and [^{3}H]*dihydroalprenolol binding.* Male Sprague–Dawley rats (150 g) were injected intraperitoneally twice daily for 3 weeks with 10 mg/kg of desmethylimipramine (DMI) or AHR-9377 dissolved in saline; control animals received saline. The animals were killed 24 hr after the last injection, and the cerebral cortex was removed.

The cerebral cortex was homogenized in 50 vol. of Tris–HCl (pH 8.0) with a Polytron tissue grinder at a setting of 6 for 15 sec to give a membrane preparation. Binding of [^{3}H]dihydroalprenolol (DHA) to these membranes was measured according to the method described by Kinnier *et al.* [8]; concentrations of 0.5 to 6 nM [^{3}H]DHA were used.

Other binding assays. The number of muscarinic receptors was measured [9] with 0.5 nM [^{3}H] quinuclidinyl benzilate (QNB) (60 Ci/mmole).

The number of $alpha_1$ and $alpha_2$ adrenergic receptors was determined by the procedure of U'Prichard *et al.* [10] with 0.5 nM [^{3}H]WB-4101 (30 Ci/mmole) and 1.0 nM [^{3}H]aminoclonidine (40 Ci/mmole). Imipramine binding sites were measured with 2 nM [^{3}H]imipramine (90 Ci/mmole) according to the procedure of Kinnier *et al.* [11]. Benzodiazepine binding was determined with 1.5 nM [^{3}H]diazepam (86.6 Ci/mmole) according to the procedure of Williams and Risley [12]. Muscimol binding was done with 5.0 nM [^{3}H]muscimol (10.1 Ci/mmole) according the method of Enna *et al.* [13].

Chemicals. Desmethylimipramine, imipramine and clomipramine, amitriptyline and protriptyline, doxepine, paroxitine and femoxatine, and citalopram were supplied by the USV Pharmaceutical Corp.; the Ciba Geigy Corp.; Merck Sharp & Dohme; Pfizer; Ferrosan; and Lundbeck respectively. All other chemicals were reagent grade.

All ^{3}H-labelled compounds were purchased from the New England Nuclear Corp., and the scintillation mixture, EP Ready Sol, was purchased from Beckman.

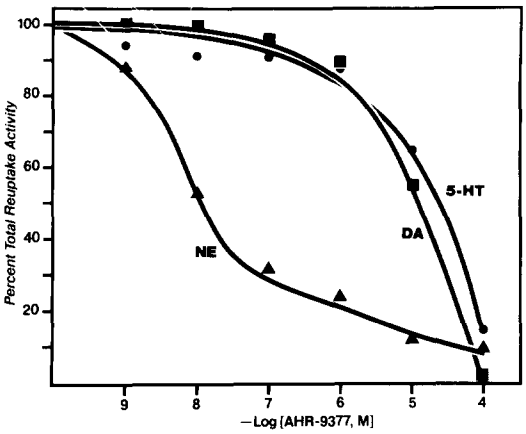

Fig. 2. Inhibition by AHR-9377 of the reuptake of [^{3}H]-NE, [^{3}H]5-HT and [^{3}H]DA into hypothalmic, cortical and striatal synaptosomes respectively. Data are expressed as a percent of the total reuptake. Each point represents the mean of triplicate determinations. Incubations were carried out at 37° for 10 min.

RESULTS

The effects of AHR-9377 on the reuptake of [^{3}H]NE, [^{3}H]5-HT and [^{3}H]DA into rat hypothalmic, cortical and striatal synaptosomes are presented in Fig. 2. AHR-9377 was a potent inhibitor of [^{3}H]-NE reuptake in hypothalamic synaptosomes (IC_{50} = 0.06 μM); it was, however, only weakly effective in blocking [^{3}H]5-HT (IC_{50} = 73 μM) and [^{3}H]DA (IC_{50} = 12.9 mM) reuptake (Fig. 2, Table 1). Based on these data, AHR-9377 was more selective for [^{3}H]NE than for [^{3}H]5-HT and [^{3}H]DA.

A comparison of AHR-9377 with other reuptake inhibitors is shown in Table 1. AHR-9377 was at least three times more potent than imipramine in inhibiting [^{3}H]NE reuptake. DMI, however, with an IC_{50} of 0.003 μM, was twenty times more effective than AHR-9377 in blocking [^{3}H]NE reuptake. The order of potency was DMI > AHR-9377 > protriptyline > imipramine > amitriptyline > clomipramine > femoxatine > citalopram.

Kinetic analysis of the effect of AHR-9377 on [^{3}H]-NE reuptake is shown in Fig. 3. AHR-9377 is a noncompetitive inhibitor of [^{3}H]NE and had a K_i of 0.2 μM when analyzed by means of a Dixon plot [14] AHR-9377 inhibition was also found to be reversible.

When rats were treated b.i.d. for 21 days with 10 mg/kg i.p. of AHR-9377 or DMI, a significant decrease ($P < 0.05$) in the specific binding of [^{3}H]-DHA was observed. Scatchard analysis showed that the decrease was due to a reduction in the maximum number of beta adrenergic receptors. A 15% reduction was observed with AHR-9377 and a 30% reduction with DMI (Fig. 4), whereas no apparent change was seen in the dissociation constant (K_d

Table 1. Comparison of IC_{50} values of AHR-9377 and other reuptake inhibitors in blocking the reuptake of 0.1 μM [^{3}H]NE, [^{3}H]5-HT and [^{3}H]DA into hypothalamic, cortical, and striatal synaptosomes respectively*

Drug	IC_{50} (μM)		
	Hypothalamus (norepinephrine)	Cortex (serotonin)	Striatum (dopamine)
AHR-9377	0.06 ± 0.01	73.2 ± 20.9	12.9 ± 2.9
Imipramine	0.22 ± 0.03	0.57 ± 0.07	9.3 ± 0.3
Desimipramine	0.003 ± 0.0004	2.9	ND†
Clomipramine	1.7	0.12	ND
Amitriptyline	0.41	1.8	ND
Protriptyline	0.18	2.7	ND
Doxepin	ND	6.9	ND
Paroxetine	ND	0.12	ND
Citalopram	86	0.40	ND
Femoxatine	6.5	1.20	ND

* IC_{50} Values were determined by logit analysis. Incubations were carried out at 37° for 10 min. The means ± S.E.M. are of three to seven separate determinations conducted in triplicate at five concentrations. All other values are single determinations conducted in triplicate at five concentrations.

† ND, not determined.

of 1 nM). Since micromolar amounts of AHR-9377 were needed to displace [^{3}H]DHA (see Table 3), it is unlikely that the decrease in specific binding was due to residual AHR-9377 binding to the beta adrenergic receptors.

Table 2 shows the micromolar concentrations of AHR-9377 and imipramine required to stimulate the release of biogenic amines by 50% from synaptosomes prepared from selected brain areas. Based on the RC_{50} values, the concentrations of imipramine required to release these amines were less than those of AHR-9377, but these differences were not statistically significant. The RC_{50} value of AHR-9377 did not change in the presence of 30 mM KCl nor did it inhibit K^+-stimulated release. The relevance of these release values was not considered important since the concentration of drug needed to cause 50% release of NE was greater than the concentration required for 100% inhibition of NE reuptake.

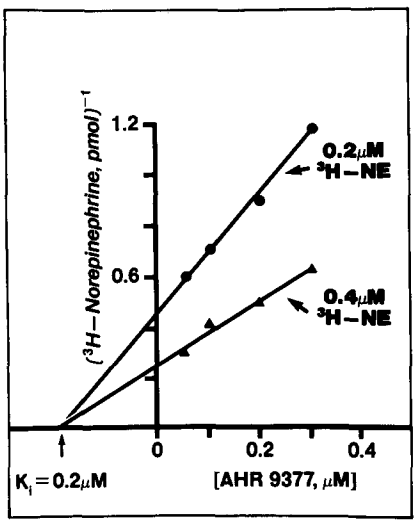

Fig. 3. Dixon plot of [^{3}H]NE reuptake into hypothalamic synaptosomes inhibited by AHR-9377. Incubations were carried out at 37° for 5 min with either 0.2 or 0.4 μM [^{3}H] NE. Each point is the average of triplicates.

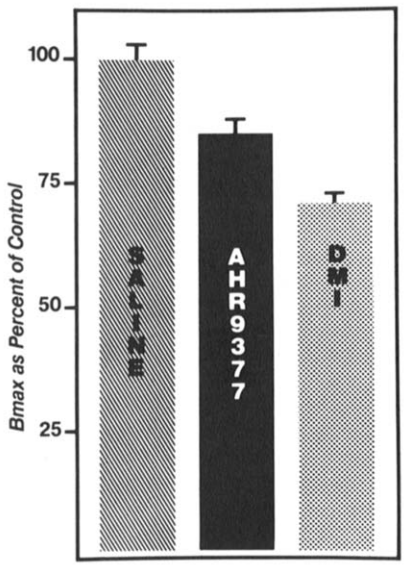

Fig. 4. Effect of repeated injections of AHR-9377 compared with DMI (10 mg/kg, i.p., b.i.d., for 21 days) on the density of beta adrenergic receptors. Each histogram is the average B_{max} ± S.E.M. from three rat brain cortices expressed as a percent of the control. Saline-treated controls typically had a K_d of 1.0 nM and a B_{max} of 12 pmoles/g of tissue as determined by Scatchard analysis (0.5 to 6.0 nM). Student's *t*-test was used for statistical analysis.

Table 2. Comparison of potency of AHR-9377 and imipramine on the release of 0.1 μM [^{3}H]NE, [^{3}H]5-HT and [^{3}H]DA from preloaded hypothalamic, cortical and striatal synaptosomes respectively*

	Mean RC_{50} (μM)	
Biogenic amine	AHR-9377	Imipramine
Norepinephrine	24.63	92.00
Dopamine	26.50	21.33
Serotonin	29.33	8.56

* RC_{50} Values were determined by logit analysis where RC_{50} is the concentration required to increase release by 50%. Incubations were carried out at 37° for 10 min. Values are the mean of three separate determinations done in triplicate.

AHR-9377 was tested in eight different receptor systems and compared with DMI and imipramine by ligand binding techniques. The IC_{50} values for these comparisons are shown in Table 3. AHR-9377 was a weaker displacer of the ^{3}H-ligand from the alpha$_1$ adrenergic, alpha$_2$ adrenergic, histaminergic, muscarinic, and imipramine recognition sites than either DMI or imipramine.

Because of the benzodiazepine structure of AHR-9377, its displacement of [^{3}H]diazepam was also examined. Concentrations as high as 100 μM had no effect on [^{3}H]diazepam binding. Furthermore, it neither enhanced nor decreased [^{3}H]muscimol binding. AHR-9377 (IC_{50} = 48,000 nM) showed only weak activity on [^{3}H]DHA binding at beta adrenergic recognition sites.

DISCUSSION

The results of this study demonstrate that AHR-9377 is an effective inhibitor of [^{3}H]NE reuptake into hypothalamic synaptosomes, and it has a potency between DMI and protriptyline. AHR-9377 appears to be a highly selective [^{3}H]NE-reuptake inhibitor based upon its weak inhibition of [^{3}H]5-HT and [^{3}H]DA reuptake. Furthermore, AHR-9377 is a noncompetitive inhibitor—a novel difference from other antidepressants, most of which are competitive inhibitors [15]. These properties of AHR-9377 should allow a more precise titration of the symptoms of depression.

It was shown that repeated injections of AHR-9377 reduced the density of [^{3}H]DNA binding sites without altering [^{3}H]DHA affinity. As has been shown previously [2] and repeated here for comparison, DMI decreased the number of binding sites in a similar manner, but it was twice as efficacious as AHR-9377. The precise mechanism by which AHR-9377 reduces the numbers of [^{3}H]DHA binding sites is unknown; the results of this study, however, in which AHR-9377 was found not to release NE *in vitro* effectively and not to inhibit MAO activity (data not shown) indicate that AHR-9377 probably elevates NE levels primarily by blocking NE reuptake. Thus, the nerve terminal compensates for the prolonged exposure to NE by decreasing the beta adrenergic receptors.

In addition to its noncompetitive inhibition of NE reuptake, AHR-9377 has other neurochemical characteristics that distinguish it from other antidepressants. AHR-9377 does not displace [^{3}H]imipramine until high concentrations of it are attained. This is further supported by the lack of 5-HT-reuptake inhibition since [^{3}H]imipramine binding is thought to be associated with 5-HT reuptake in the brain [16].

AHR-9377 had little affinity for alpha$_1$ adrenergic, beta adrenergic, histaminergic or muscarinic receptors, and it showed weak anticholinergic activity. This indicates that there would be less likelihood for anticholinergic side effects such as urinary retention and dryness of mouth; anti-alpha$_1$ adrenergic side effects such as sedation and cardiovascular properties; and antihistaminergic side effects such as sedation [17].

In conclusion, the present study shows that AHR-9377 was a potent, reversible, noncompetitive

Table 3. Inhibition of receptor ligand binding by AHR-9377, DMI and imipramine*

	IC_{50} (nM)		
Receptor	AHR-9377	DMI	Imipramine
Alpha$_1$ adrenergic	15,617	697	342
Alpha$_2$ adrenergic	38,826	5,932	6,821
Beta adrenergic	48,000	42,427	8,143
Benzodiazepine	>100,000	>100,000	>100,000
γ-Aminobutyric acid	>100,000	>100,000	>100,000
Histamine$_1$	10,000	548	53
Imipramine	3,600	46	7
Muscarinic	87,612	8,716	3,715

* Specific binding was determined as described in Materials and Methods. The IC_{50} values were obtained from log probit analysis by using six concentrations (10^{-9} to 10^{-4} M). Values are the mean from duplicate experiments, each done in triplicate.

inhibitor that was selective for NE reuptake, down-regulated beta adrenergic receptors, and had little antagonism of cholinergic, adrenergic or histaminergic receptors. These neurochemical properties of AHR-9377 suggest that it may have superior clinical antidepressant activity.

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