The Pharmacological Characterization of a Novel Selective 5-Hydroxytryptamine<sub>1A</sub> Receptor Antagonist, NAD-299

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ABSTRACT

The pharmacological properties of a novel selective 5-hydroxytryptamine<sub>1A</sub> (5-HT<sub>1A</sub>) receptor antagonist, NAD-299 [(R)-3-N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-2H-1-benzo[4,5]cyclohexanecarboxamide hydrogen (2R,3R)-tartrate monohydrate] were examined in vitro and in vivo and compared with the reference 5-HT<sub>1A</sub> receptor antagonist, WAY-100635 [N-(2-(1-(4-(2-methoxyphenyl)piperazin-yl))ethyl)-N-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride]. The new compound had high affinity for 5-HT<sub>1A</sub> receptors in vitro with a K<sub>i</sub> value of 0.6 nM. The only other receptors for which NAD-299 had affinity less than 1 μM were alpha<sub>1</sub> and beta adrenoceptors with K<sub>i</sub> values of 260 and 340 nM, respectively. Thus, the selectivity of NAD-299 for 5-HT<sub>1A</sub> receptors was more than 400 times. WAY-100635 had considerably higher affinity than NAD-299 for alpha<sub>1</sub> adrenoceptors (K<sub>i</sub> = 45 nM) and dopamine D<sub>2</sub> and D<sub>3</sub> receptors (K<sub>i</sub> = 79 and 67 nM, respectively). Like WAY-100635, NAD-299 competitively blocked 5-HT-induced inhibition of vasoactive intestinal peptide-stimulated cAMP production in GH<sub>3</sub>ZD<sub>10</sub> cells and had no intrinsic activity. Both compounds were therefore 5-HT<sub>1A</sub> receptor antagonists in vivo and also behaved as such in in vivo experiments. Thus, they competitively antagonized the 8-hydroxy-2-(di-n-propylamino)tetralin-induced 5-HT behavioral effects, hypothermia, corticosterone secretion and inhibition of passive avoidance behavior without causing any actions of their own. The effective dose of NAD-299 varied between 0.03 and 0.35 μmol/kg s.c., depending on the test and the dose of 8-hydroxy-2-(di-n-propylamino)tetralin.

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ABBREVIATIONS: DA, dopamine; DOPA, L-3,4-dihydroxyphenylalanine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; 5-HT, 5-hydroxytryptamine; IBMX, 3-isobutyl-1-methylxanthine; 5-HTP, 5-hydroxytryptophan; NAD-299, (R)-3,N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-2H-1-benzo[4,5]cyclohexanecarboxamide hydrogen (2R,3R)-tartrate monohydrate; NSD 1015, 3-hydroxybenzylhydrazine dihydrochloride; (S)-UH-301, (S)-5-fluoro-8-hydroxy-2-(dipropylamino)tetralin; VIP, vasoactive intestinal peptide; WAY-100135, N-[2-(1-(4-(2-methoxyphenyl)piperazinyl))ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride; CHO, Chinese hamster ovary; FCS, fetal calf serum; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; AMPA, L-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; DHA, dihydrosphingosine; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; TBPS, tert-butylbicyclicophosphothionate; QNB, β-nitrobenzyl benzilate.
100635, have been useful in the characterization of 5-HT
receptor function.

We report here the basic biochemical and behavioral pharma-
ological characterization of a new selective 5-HT1A recep-
tor antagonist, NAD-299 (fig. 1). The synthesis of this sub-
stance has been reported elsewhere (Evenden et al., 1995).
WAY-100635 has been included in this study as a reference
5-HT1A receptor antagonist.

#### Materials and Methods

**Materials.** The GH4/ZD10 (rat pituitary tumor cells) cells containing
rat 5-HT1A receptors and the Ltk− (mouse fibroblast) cells
expressing human DA D2A (long isoform) receptors were obtained from
Dr. Olivier Civel (Vollum Institute for Advanced Biomedical Re-
search, Oregon Health Sciences University, Portland, OR). The CHO
cells expressing human D3, rat 5-HT6 and rat 5-HT7 receptors were
purchased from INSERM (Paris, France).

**Compounds.** The standard agonist used in the studies was 8-OH-
DPAT, from Research Biochemicals International Inc., Natick, MA.
NAD-299 and WAY-100635 were provided from the laboratories of
Astra Arcus AB. The test compounds were dissolved in saline, if not
otherwise stated. Ham's F10 medium, Earle's balanced salt solution
without Ca2+ and Mg2+, FCS, penicillin, streptomycin and HEPES
were obtained from Gibco Ltd., Paisley, Scotland, U.K. [3H]cAMP
and [3H]AMP were obtained from Amersham International plc, Amers-
hammad, U.K. Diazepam, dithiothreitol, genistein, NSD 1015, 5-hy-
droxytryptamine hydrochloride, IBMX, nicotine, oxtremorine, so-
dium glutamate, theophylline, tris/base and VIP were obtained from
Sigma Chemical Co., St. Louis, MO. Ascorbic acid was from Merck,
Darmstadt, Germany. (+)-Butaclamol hydrochloride, cimetidine, ga-
lanin, MK801 and pyrilamine were from Research Biochemicals
International, Inc. (2R,3S)-Alpenrolol hydrochloride was obtained from
Astra Hässle, Mölndal, Sweden; methiothepine was a gift from
Hoff-
man-LaRoche, Basel, Switzerland; methysergide was from Sandoz
AG, Basel, Switzerland; paroxetine was from SmithKlineBeecham
Pharmaceuticals, Betchworth, UK; and phenotolamine mesylate was
from Ciba-Geigy AG, Basel, Switzerland. All other compounds used
were of highest purity available.

The following radioactive ligands were used (Ci/mmol in paren-
theses): [3H]AMPA (53), [3H]DHA (59), [3H]flunitrazepam (82.5),
[3H]Igalanin (2200), [3H]8-OH-DPAT (130), [3H]ketanserin (64),
[3H]MK-801 (20.3), [3H]nicotine (63), [3H]prazosin (78), [3H]pyril-
amine (31.2), [3H]QNB (43), [3H]raclopride (80), [3H]SCH23390
(86), [3H]TBPS (69.2), and [3H]tiotidine (83.7), all purchased from
DuPont NEN, Boston, MA. [3H]Citalopram (85.7), [3H]5-hydroxy-
tryptamine (29.7) and [3H]RX821002 (60) were obtained from Amer-
sham International plc, UK.

**Subjects.** Male Sprague-Dawley rats (B&K strain, B&K Univer-
sal, Sollentuna, Sweden), weighing 150 to 350 g, were used. The animals
arrived in the laboratory at least 5 days before being used in the
experiments and were housed 5 per cage under controlled condi-
tions of temperature (21°C), relative humidity (55—65%) and light-
dark cycle (12:12 h, lights on at 6 a.m.). Food (R36, Ewos, Södertälje,
Sweden) and tap water were freely available in the home cage. The experiments were performed during the light phase, between 7 a.m.
and 5 p.m. All injections were subcutaneous (s.c.) unless otherwise
stated.

**Radioligand binding studies.** The rats were decapitated and the
various brain regions dissected out on ice (table 1). The brain
regions were frozen as tissues or homogenates in 0.32 M sucrose and
stored at −20°C or −70°C until the day of the experiment. The membranes were prepared and the binding studies performed essen-
tially as described previously (Chang et al., 1978; Speth et al., 1979;
Gajtkowski et al., 1983; Hall et al., 1986; Murphy et al., 1987; Cross
et al., 1989; Land et al., 1991; Rapier et al., 1990; Steele et al., 1992;
Jackson et al., 1995). The Ltk− cells expressing human DA D2A (long
isoform) receptors and the CHO cells expressing human D3, rat
5-HT6 and rat 5-HT7 receptors were grown and the cell membranes
prepared essentially as described by Malmberg et al. (1993). Protein
concentration was measured by the method of Markwell et al. (1978).
The compounds were dissolved and diluted in 0.1% ascorbic acid.
Table 1 summarizes the incubation conditions of the binding as-
says for the various receptors examined. The Kd values (inhibition
constants) of the test compounds were determined from inhibition
curves by the iterative nonlinear curve-fitting program LIGAND
(Munson and Rodbard, 1980). One- and two-site curve fitting was
tested in all experiments. The one-site model gave a better fit (F >
0.05; F test) unless otherwise stated. The Kd values (dissociation
constants) of the various radioligands used to calculate the Kd
values were determined by saturation studies and are given in table 1.

**Second messenger studies.** The GH4/ZD10 cells were cultured
in 175-cm2 flasks in Ham's medium with 1 mM t-glutamine supple-
mented with 10% FCS, 10 mM HEPES, penicillin and streptomycin
at 37°C. Cells in passages 8 to 11 were used. Genetin (G418 sulfate,
700 µg/ml) was used for selection of cells transfected with receptors.
The test compounds were dissolved in a 20 mM concentration in
dimethyl sulfoxide and stored at −20°C until used. The stock solu-
tions were further diluted in water containing 0.01% ascorbic acid and
0.1 mM IBMX. The 5-HT was freshly prepared in the solution above. The cAMP
assay was carried out according to Dorflinger and Schonbrunn
(1983) with some minor modifications (Fowler et al., 1992). The cells were
detached from the cultured flasks with Earle's balanced salt solution
supplement with 1 mM EDTA without Ca2+ and Mg2+. The cells
were suspended in FCS-free Ham's medium and the suspension was
centrifuged at 250 × g for 6 min at room temperature. The pellets
were resuspended to a density of 106 cells/ml in medium containing
0.01% ascorbic acid and 0.1 mM IBMX. Cells were preincubated in
this solution for 1 h at 37°C and then diluted to a final density of 106
cells/ml. Aliquots (0.4 ml) of the cell suspension were added to ASPET Journals on November 3, 2017 jpet.aspetjournals.org Downloaded from
samples were stored at and the regions dissected were immediately frozen on dry ice. The rats were sacrificed with a guillotine 30 min later. The rats were injected with (S)-5-HTP and DOPA accumulation. Groups of five rats were given 

5-HTP and DOPA uptake. Groups of five rats were given 

The quantitative data (EC50 and B) are based on at least two independent experiments.

**TABLE 1**

The experimental conditions used in the receptor binding assays

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand (Kp, nM)</th>
<th>Tissue (conc mg w.w./ml incubation volume) or cell (conc mg protein/ml incubation volume)</th>
<th>Incubation Time and Temperature (min/°C)</th>
<th>Incubation Buffer (in mM), (pH)</th>
<th>Nonspecific Binding (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>[3H]OH-8-DPAT (0.65)</td>
<td>Rat hippocampus</td>
<td>45/37*</td>
<td>50 Tris-HCl, 2 CaCl2, 1 MgCl2, 1 MnCl2 (pH 7.4)</td>
<td>10 WAY100635</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>[3H]Cyanopindolol (0.05)</td>
<td>Rat cortex</td>
<td>90/37</td>
<td>10 Tris-HCl, 154 NaCl, 0.01 pargyline, 0.06 (±)-isoprenaline (pH 7.4)</td>
<td>10 5-HT</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>[3H]Ketanserin (0.60)</td>
<td>Rat cortex</td>
<td>45/37</td>
<td>50 Tris-HCl (pH 7.7)</td>
<td>10 methysergide</td>
</tr>
<tr>
<td>5-HT3</td>
<td>[3H]5-HT (3.00)</td>
<td>CHO5-HTa</td>
<td>30/30</td>
<td>50 Tris-HCl, 4 MgCl2, 1 EDTA (pH 7.4)</td>
<td>10 methiothepin</td>
</tr>
<tr>
<td>5-HT7</td>
<td>[3H]5-HT (0.40)</td>
<td>CHO5-HTb</td>
<td>60/30</td>
<td>50 Tris-HCl, 4 MgCl2, 1 EDTA (pH 7.4)</td>
<td>10 methiothepin</td>
</tr>
<tr>
<td>5-HT uptake</td>
<td>[3H]Citalopram (0.78)</td>
<td>Rat cortex</td>
<td>120/25</td>
<td>50 Tris-HCl, 5 KCl, 120 NaCl (pH 7.4)</td>
<td>0.5 paroxetine</td>
</tr>
<tr>
<td>α1</td>
<td>[3H]Prazosin (0.04)</td>
<td>Rat cortex</td>
<td>60/25</td>
<td>50 Tris-HCl, 0.1% a.a. (pH 7.7)</td>
<td>100 phenolamine</td>
</tr>
<tr>
<td>α2</td>
<td>[3H]RX821002 (0.40)</td>
<td>Rat cortex</td>
<td>60/25</td>
<td>50 Tris-HCl, 0.1% a.a. (pH 7.7)</td>
<td>100 phenolamine</td>
</tr>
<tr>
<td>β</td>
<td>[3H]DHA (0.50)</td>
<td>Rat cortex</td>
<td>60/25</td>
<td>50 Tris-HCl, 120 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 0.01 pargyline, 0.1% a.a. (pH 7.6)</td>
<td>10 (±)-alpenol</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>[3H]QNB (0.17)</td>
<td>Rat cortex</td>
<td>60/25</td>
<td>50 Tris-HCl, 120 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 0.01 pargyline, 0.1% a.a. (pH 7.6)</td>
<td>100 oxotremorine</td>
</tr>
<tr>
<td>Nicotinic</td>
<td>[3H]Nicotine (0.34)</td>
<td>Rat brain-cerebellum</td>
<td>10/25</td>
<td>20 HEPES, 118 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4 (pH 7.4)</td>
<td>10 nicotine</td>
</tr>
<tr>
<td>D1</td>
<td>[3H]Sch23390 (0.29)</td>
<td>Rat striatum</td>
<td>60/25*</td>
<td>50 Tris-HCl, 120 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 0.01 pargyline, 0.1% a.a. (pH 7.6)</td>
<td>1 flupentixol</td>
</tr>
<tr>
<td>D2</td>
<td>[3H]Raclopride (1.20)</td>
<td>LtkhD2A</td>
<td>60/25</td>
<td>50 Tris-HCl, 120 NaCl, 5 KCl, 1.5 CaCl2, 4 MgCl2, 1 EDTA (pH 7.4)</td>
<td>1 butaclamol</td>
</tr>
<tr>
<td>D3</td>
<td>[3H]Raclopride (1.60)</td>
<td>CHOHD3</td>
<td>60/25</td>
<td>50 Tris-HCl, 120 NaCl, 5 KCl, 1.5 CaCl2, 4 MgCl2, 1 EDTA (pH 7.4)</td>
<td>1 butaclamol</td>
</tr>
<tr>
<td>Histamine H1</td>
<td>[3H]Pyrilamine (0.73)</td>
<td>Rat cortex</td>
<td>25/25</td>
<td>50 Na2HPO4/KH2PO4 (pH 7.4)</td>
<td>100 pyrilamine</td>
</tr>
<tr>
<td>Histamine H2</td>
<td>[3H]Tiotidine (17)</td>
<td>Guinea pig cortex</td>
<td>30/25</td>
<td>50 Na2HPO4/KH2PO4 (pH 7.4)</td>
<td>100 cimetidine</td>
</tr>
<tr>
<td>GABA A</td>
<td>[35S]TBPS (70)</td>
<td>Rat cortex</td>
<td>90/25</td>
<td>50 Tris-citrate, 200 NaCl (pH 7.4)</td>
<td>4.75 TBPS</td>
</tr>
<tr>
<td>NMDA</td>
<td>[3H]MK801 (11.99)</td>
<td>Rat cortex</td>
<td>15/25</td>
<td>5 Tris-HCl, 0.01 glutamate, 0.01 glycine, 0.1 Spermidine (pH 7.4)</td>
<td>100 MK801</td>
</tr>
<tr>
<td>AMPA</td>
<td>[3H]AMPA (82)</td>
<td>Rat forebrain</td>
<td>60/4</td>
<td>50 Tris-HCl (pH 7.4)</td>
<td>1000 glutamate</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>[3H]Flunitrazepam (2.1)</td>
<td>Rat cortex</td>
<td>30/0</td>
<td>50 Tris-HCl (pH 7.4)</td>
<td>100 diazepam</td>
</tr>
<tr>
<td>Galanin</td>
<td>[125I]Galanin (0.15)</td>
<td>Rat hypothalamus</td>
<td>40/37</td>
<td>20 HEPES, 2.5 MgCl2, 0.5 EDTA, 0.05% bovine serum albumin, 1 mg/ml bacitracin (pH 7.4)</td>
<td>1 galanin</td>
</tr>
</tbody>
</table>

* Preincubated 10 min at 37°C.

b a.a., ascorbic acid.

“efficacy” which indicates the ratio of the effect of the test compound to maximum response of 5-HT in percent. The same experimental model as described above was used for Schild plots (Schild, 1949). The quantitative data (EC50 and Kp) are based on at least two independent experiments.

**Determinaton of 5-HTP and DOPA accumulation.** Groups of five rats were given the test compound at the time noted before the injection of 8-OH-DPAT, 0.3 µmol/kg, or saline. NSD 1015, 100 mg/kg (in water solution with the pH brought to about 5 with sodium hydroxide), was injected 30 min later. The rats were sacrificed with a guillotine 30 min after the NSD 1015 injections. The brains were rapidly removed and the regions dissected were immediately frozen on dry ice. The samples were stored at −70°C until assayed.

**Antagonism of 8-OH-DPAT-induced secretion of corticosterone.** The method has been described previously (Kelder and Ross, 1992). Rats were given daily injections of saline for 5 to 7 days before...
the start of the experiment to habituate the animals to the injections and thereby avoid acute increases in serum corticosterone. The test compounds were administered at the time noted before the injection of 8-OH-DPAT, 0.75 μmol/kg. Each experiment consisted of eight groups of five animals, and controls were always included. The rats were sacrificed 60 min after the injection of 8-OH-DPAT. The experiments were performed from 9 A.M. to 1 P.M. The trunk blood was collected in plastic tubes, and the serum obtained was stored at −70°C. Corticosterone in rat serum was assayed with a corticosterone[3H] RIA kit from ICN Biomedicals Inc., Costa Mesa, CA.

**Flat body posture, forepaw treading and lower lip retraction.** The test apparatus was a clear plastic cage (Macrolon type IV cage, 34 × 56 × 19 cm), without sawdust, in which the rats were placed singly. The cages were placed in front of a mirror. The rats were first treated with the test compounds. Re´nyi body posture and lower lip retraction were expressed as the median sum of reciprocal forepaw treading, flat intensity. The experimenter was blinded to the treatment. Results are presented as the minimal effective dose, which is the lowest dose used that significantly blocked the effect of 8-OH-DPAT. The 5-HT syndrome and passive avoidance data were analyzed by Kruskal-Wallis ANOVA; when a significant difference was indicated by the ANOVA, between-groups analysis was performed with a Mann-Whitney U-test.

**Results**

**In Vitro Experiments.**

**Receptor binding profile in vitro.** Table 2 summarizes the binding affinities of NAD-299 for various receptors and compares them with those of WAY-100635. Both NAD-299 and WAY-100635 had high affinity for 5-HT1A receptors with Ki values less than 1 nM. A representative curve of the displacement of [3H]8-OH-DPAT from 5-HT1A receptors by NAD-299 is shown in figure 2. Apart from an affinity at alpha-1 adrenoceptors of 260 nM and at beta adrenoceptors of 340 nM the affinity of NAD-299 was less than 1000 nM for a range of other receptors, including serotonergic (5-HT1B, 5-HT2A, 5-HT3, 5-HT7, and 5-HT uptake site), alpha-2 adrenergic, cholinergic (muscarnic and nicotinic), dopaminergic (D1, D2 and D3), histamine (H1 and H2), GABA, NMDA, AMPA, benzodiazepine and galanin receptors. WAY-100635 showed considerably higher affinities than NAD-299 for alpha-1 adrenoceptors and DA D2 and D3 receptors.

**Second messenger studies.** The maximal suppressive effect of 5-HT on VIP-stimulated cAMP production was about TABLE 2

<table>
<thead>
<tr>
<th>Receptor</th>
<th>NAD-299 (K (nM))</th>
<th>WAY-100635 (K (nM))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>0.59 ± 0.08</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>D1</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>D2</td>
<td>67 ± 1</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>5-HT2B</td>
<td>&gt;1000</td>
<td>n.d.</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>&gt;1000</td>
<td>1100 ± 150</td>
</tr>
<tr>
<td>5-HT5</td>
<td>&gt;39000</td>
<td>n.d.</td>
</tr>
<tr>
<td>5-HT7</td>
<td>1900 ± 160</td>
<td>n.d.</td>
</tr>
<tr>
<td>5-HT uptake</td>
<td>&gt;10000</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mucarnic</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Nicotinic</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Histamine H1</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Histamine H2</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
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<tr>
<td>GABA A</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
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<tr>
<td>NMDA</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>AMPA</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
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<tr>
<td>Benzodiazepine</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Galalin</td>
<td>&gt;2600</td>
<td>&gt;2600</td>
</tr>
</tbody>
</table>

* n.d. = not determined.

Significantly better fit to a two-site model with K1 = 160 ± 34 and K2 = 16,300 ± 6,800 nM (P < .05; F test).
This was achieved at 1 μM 5-HT. Neither NAD-299 nor WAY-100635 exerted any intrinsic activity (efficacy) on the 5-HT1A receptor. This could be concluded from data (expressed as percent of VIP-stimulated cAMP production) obtained with the two putative antagonists at 0.1, 1 and 10 μM. NAD-299 exposure gave 101 ± 4.8, 101 ± 3.9 and 95 ± 3.7 (mean ± S.E., n = 6), respectively; and WAY-100635 exposure gave 103 ± 17, 104 ± 4 and 103 ± 8 (mean ± S.E., n = 2), respectively. The inhibitory effect of 5-HT was fully antagonized by both compounds tested (fig. 3), because the calculated apparent maximal inhibitory effects of NAD-299 (87%) and WAY-100635 (86%) were not significantly different from 100%. Calculation of EC50 values of the inhibition of 5-HT-induced suppression of cAMP formation indicates that WAY-100635 was about four times more potent than NAD-299 with EC50 values of 2 and 7 nM, respectively. The competitive nature of the compounds on 5-HT-inhibited cAMP production were investigated according to the description by Schild (1949). Both putative antagonists produced a parallel displacement to the right of the 5-HT concentration-response curve (fig. 4). The equiactive concentration ratios were then used for the Schild analysis. The slopes of the Schild plots yielded straight lines which did not significantly deviate from unity. The Ke values were 1 nM and 0.2 nM for NAD-299 and WAY-100635, respectively.

In Vivo Experiments

NAD-299 and WAY-100635 were examined for their abilities to antagonize 8-OH-DPAT-induced effects in various in vivo test models. Because the tests required different doses of 8-OH-DPAT to produce an almost maximal effect, the dose used in each test is given in table 3, which summarizes the

<table>
<thead>
<tr>
<th>Test</th>
<th>8-OH-DPAT</th>
<th>NAD-299</th>
<th>WAY-100635</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HTP accumulation</td>
<td>Striatum</td>
<td>0.3 ED50</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td>0.3 ED50</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Corticosterone secretion</td>
<td>0.75 ED50</td>
<td>0.04</td>
</tr>
<tr>
<td>Flat body posture</td>
<td>1.5 ED50</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>Forepaw treading</td>
<td>1.5 ED50</td>
<td>0.03</td>
<td>0.016</td>
</tr>
<tr>
<td>Lower lip retraction</td>
<td>1.5 ED50</td>
<td>0.35</td>
<td>0.09</td>
</tr>
<tr>
<td>Inhibition of cage leaving response</td>
<td>0.3 MED</td>
<td>0.3</td>
<td>0.05</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>0.9 ED50</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>Passive avoidance</td>
<td>0.6 MED</td>
<td>0.3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Values stated are micromoles per kilogram.
The effects of NAD-299 and WAY-100635 on DOPA accumulation in the hypothalamus and striatum in the rat brain

The test compound was injected 45 min before NSD 1015, 100 mg/kg s.c. (15 min before 8-OH-DPAT). 5-HTP accumulation (nmol/g ± S.E.) in saline-pretreated rats: hypothalamus, 0.81 ± 0.03; striatum, 0.40 ± 0.02. The values are means ± S.E. from five rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>µmol/kg s.c.</th>
<th>Hypothalamus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD-299</td>
<td>1</td>
<td>94 ± 5</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>NAD-299</td>
<td>31</td>
<td>99 ± 5</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>WAY-100635</td>
<td>1</td>
<td>99 ± 4</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>WAY-100635 + 8-OH-DPAT</td>
<td>5.5 ± 0.3</td>
<td>75 ± 3*</td>
<td>78 ± 3*</td>
</tr>
<tr>
<td>Saline + 8-OH-DPAT</td>
<td>0.3</td>
<td>55 ± 4*</td>
<td>62 ± 4**</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .01 vs. saline control (Dunnett’s t-test after ANOVA).

The results of these experiments. The ED50 values were estimated by interpolation of log dose-response curves.

Antagonism of 8-OH-DPAT-induced 5-HTP accumulation. 8-OH-DPAT (0.3 µmol/kg s.c.) produced an almost maximal decrease in the 5-HTP accumulation under the experimental conditions used (table 4). NAD-299 was 6 to 10 times less potent than WAY-100635 in antagonizing the 8-OH-DPAT-induced decrease in 5-HTP accumulation in hypothalamus and striatum (fig. 5). NAD-299 by itself, at doses 1 to 30 µmol/kg, had no effect on the 5-HTP accumulation (table 4). WAY-100635 by itself produced a decrease in the 5-HTP accumulation at 5.5 µmol/kg but not at lower doses. However, this effect does not seem to result from a direct action on the somatodendritic 5-HT1A receptors, because the same dose completely blocked the effect of 8-OH-DPAT on the 5-HTP accumulation (table 4).

DOPA accumulation. NAD-299 by itself did not significantly change the rate of DOPA synthesis in hypothalamus and striatum after DOPA decarboxylase inhibition (fig. 6). WAY-100635, on the other hand, produced a marked increase in the DOPA accumulation in striatum at the highest dose tested (5.5 µmol/kg) (fig. 6). However, the increase in DOPA accumulation induced by 8-OH-DPAT at 0.3 µmol/kg was antagonized by NAD-299 and WAY-100635 at 5-HT1A receptor-antagonizing doses.

Antagonism of 8-OH-DPAT-induced secretion of corticosterone. 8-OH-DPAT at 0.75 µmol/kg produced an almost maximal increase in the corticosterone secretion into the blood circulation (see fig. 8). WAY-100635 was about 3 times more potent than NAD-299 in antagonizing the 8-OH-DPAT-induced corticosterone secretion when injected 15 min before 8-OH-DPAT (0.3 µmol/kg) or saline and 45 min before NSD 1015 (100 mg/kg). The rats were sacrificed 30 min after the last injection, and the hypothalamus and striatum were rapidly dissected out and frozen on dry ice. The values are means ± S.E. (vertical bars) of five rats. The DOPA accumulation (nmol/g tissue) in saline-pretreated rats was: 1.64 ± 0.08 (hypothalamus) and 6.91 ± 0.34 (striatum). * denotes a significant difference, P < .05 vs. saline controls, **P < .05 vs. 8-OH-DPAT controls (Dunnett’s t-test after ANOVA).

![Fig. 5](image-url) The effect of NAD-299 (circles) and WAY-100635 (squares) on the 8-OH-DPAT-induced changes in 5-HTP accumulation in hypothalamus and striatum of the rat in vivo. The test compounds were injected 15 min before 8-OH-DPAT (0.3 µmol/kg) and 45 min before NSD 1015 (100 mg/kg i.p.). The rats were sacrificed 30 min after the last injection, and the hypothalamus and striatum were rapidly dissected out and frozen on dry ice. The values are means ± S.E. (vertical bars) of 5 to 10 rats. 5-HTP accumulation (nmol/g tissue) in saline controls: 0.93 ± 0.20 (hypothalamus) and 0.37 ± 0.07 (striatum); in 8-OH-DPAT controls: 0.55 ± 0.05 (hypothalamus) and 0.22 ± 0.02 (striatum).

flat body posture, forepaw treading and lower lip retraction. NAD-299 and WAY-100635, at the doses tested, did not exert any effects on the three behavioral measures (data not shown). In a dose finding study with 8-OH-DPAT it was found that lower lip retraction was induced already by 0.09 µmol/kg of 8-OH-DPAT, flat body posture was induced by an intermediate dose (0.4 µmol/kg), but a higher dose (1.5 µmol/kg) was needed to induce forepaw treading. Because 1.5
\[
\text{m} \text{mol/kg}
\]

8-OH-DPAT induced all components of the 5-HT behavioral effects, it was chosen for the study with the antagonists. NAD-299 at 0.03 \text{m} \text{mol/kg} significantly antagonized forepaw treading and flat body posture and at 0.3 \text{m} \text{mol/kg} the lower lip retraction (fig. 10). Complete block of all components of the syndrome induced by 8-OH-DPAT was obtained with NAD-299 at 3 \text{m} \text{mol/kg}.

**Inhibition of cage leaving response.** NAD-299 and WAY-100635 by themselves did not affect the cage leaving behavior in the dose range tested. The inhibition of the cage leaving response by 8-OH-DPAT (0.3 \text{m} \text{mol/kg}) was antagonized by both compounds. The minimal effective doses (the lowest tested dose that caused significant blockade of the effect of 8-OH-DPAT) was 0.3 \text{m} \text{mol/kg} for NAD-299 and 0.05 \text{m} \text{mol/kg} for WAY-100635.

**Hypothermia.** Neither of the antagonists themselves affected body temperature (data not shown). The hypothermia-inducing effects of 0.9 \text{m} \text{mol/kg} 8-OH-DPAT were blocked by the antagonists (fig. 11). WAY-100635 was 7 times more potent than NAD-299 in this test (table 3).

**Passive avoidance behavior.** 8-OH-DPAT and other 5-HT_{1A} receptor agonists block the acquisition of an avoidance response in the passive avoidance paradigm when administered before the training session (Johansson *et al.*, 1989; Carli *et al.*, 1992; Jackson *et al.*, 1994). None of the 5-HT_{1A} receptor antagonists studied had any effect by themselves in the passive avoidance test but antagonized the effect of 8-OH-DPAT (0.6 \text{m} \text{mol/kg}) (fig. 12). WAY-100635 was about 6 times more potent than NAD-299 when measured as the lowest dose that completely antagonized the effect of 8-OH-DPAT (table 3).

**Discussion**

The *in vitro* radioligand binding studies with the novel 5-HT_{1A} receptor antagonist, NAD-299 show that it is highly selective for the 5-HT_{1A} receptors. The only other receptors for which the compound had affinity less than 1 \text{m} \text{M} were alpha-1 and beta adrenoceptors. However, NAD-299 had a selectivity for 5-HT_{1A} receptors vs. the alpha-1 adrenoceptors and beta adrenoceptors of about 400 times. Although the
The reference antagonist, WAY-100635, is 3 times more potent than NAD-299, it is less selective because of its affinities for \( \alpha_1 \) adrenoceptors and DA D2A and D3 receptors. NAD-299 also differs in selectivity from the structurally related 5-HT1A receptor antagonist, \((S)-UH-301\), that has considerable affinity for D2 receptors (Hillver et al., 1990; Björk et al., 1991).

The 5-HT1A receptor antagonistic property of NAD-299 was demonstrated in the \textit{in vitro} experiments measuring the concentration-dependent block of the inhibitory effect of 5-HT on VIP-stimulated cAMP production in GH4ZD10 cells. The results of Schild analysis of this blockade was found to be consistent with a simple reversible and competitive antagonism. The \( K_B \) values were close to the \( K_i \) values calculated from the results of the binding experiments. Neither NAD-299 nor WAY-100635 had any intrinsic activity in this test, i.e., they were without any agonist effect. The \( \text{GH}_4 \text{ZD10} \) cell line was chosen because of its neuronal origin and because the expressed 5-HT1A receptors are a verified model of postsynaptic receptors in rat hippocampus (Fowler et al., 1992). This cell line expresses a low amount of receptors (<50 fmol/mg protein). The low expression level, however, was not the reason for the lack of intrinsic activity since similar results were obtained (data not shown) in a CHO cell line (obtained from Dr. Philip Strange, Canterbury University, UK) containing >1 pmol human 5-HT1A receptor/mg protein. This lack of intrinsic activity was verified in the various \textit{in vivo} experiments performed. Thus, no decrease in the 5-HTP accumulation in NSD 1015-treated rats, which would indicate stimulation of 5-HT1A receptors, was observed. Because of the large reserve of somato-dendritic 5-HT1A receptors, this test is very sensitive to partial 5-HT1A receptor agonists and several compounds, \textit{e.g.}, \((-)\)-pindolol and NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-pthalimido)butyl]piperazine hydrobromide), which behave as antagonists in tests of postsynaptic 5-HT1A receptors, have been found to be partial agonists of the somato-dendritic receptors (Hjorth and Carlsson, 1986; Hjorth and Sharp, 1990). This and other functional \textit{in vivo} tests, \textit{e.g.}, block of 8-OH-DPAT-induced behavioral effects, hypothermia and corticosterone secretion, confirm that NAD-299, like WAY-100635, is a “silent” 5-HT1A receptor antagonist.

Although these 5-HT1A receptor antagonists have no intrinsic activity, they are not always without any pharmaco-
In summary, the development and availability of the novel selective 5-HT₁₄ receptor antagonist, NAD-299, provides a breakthrough from a structurally new chemical class for studies of 5-HT₁₄ receptor pharmacology in animals and its clinical application in man.

Acknowledgments

The skillful technical assistance of Charlotte Ahlgren, Annelie Bengtsson, Ulla Haglund, Patricia Jimenez, Li-Marie Lindgren, Susanne Rosqvist, Maria Sålmark and Gun Torell-Svantesson is gratefully acknowledged. We thank Dr. Alan Cross, Astra Arcus AB, Rochester, NY, for some of the binding data.

References


Fletcher, A., Bill, D. J., Bill, S. J., Clepper, I. A., Dover, G. M., Forster, E. A., Hanks, J. T., Jones, D., Mansell, H. L. and Reilly, Y.: WAY-100135: a novel 5-HT₁₄ antagonist. The observation (table 4) that a high dose (5.5 μmol/kg) of WAY-100635 decreases the 5-HTP accumulation in hypothalamus and striatum may be caused by the effects of WAY-100635 on other neuron systems, e.g., DA or noradrenaline, because WAY-100635 has some affinities to D₂ receptors in vitro (table 2) and at this dose also in vivo (fig. 6) and to alpha₁ adrenoceptors in vitro (table 2). Combination of this dose of WAY-100635 with 0.3 μmol/kg 8-OH-DPAT abolished the decrease in the 5-HTP accumulation observed for both compounds alone. The origin of this interaction has not been examined in the present study.

logical effects under in vivo conditions. It has been reported that WAY-100635 can increase the firing rate of raphé 5-HT neurons in extracellular electrophysiological studies in guinea pigs (Mundey et al., 1996) and UH-301 in rats (Aerborelius and Svensson, 1992). These findings indicate that 5-HT may, under certain homeostatic conditions, exert a tonic inhibitory effect which can then be blocked by an antagonist. The observation (table 4) that a high dose (5.5 μmol/kg) of WAY-100635 decreased the 5-HTP accumulation in hypothalamus and striatum may be caused by the effects of WAY-100635 on other neuron systems, e.g., DA or noradrenaline, because WAY-100635 has some affinities to D₂ receptors in vitro (table 2) and at this dose also in vivo (fig. 6) and to alpha₁ adrenoceptors in vitro (table 2). Combination of this dose of WAY-100635 with 0.3 μmol/kg 8-OH-DPAT abolished the decrease in the 5-HTP accumulation observed for both compounds alone. The origin of this interaction has not been examined in the present study.

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