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Alniditan, a New 5-Hydroxytryptamine$_{1D}$ Agonist and Migraine-Abortive Agent: Ligand-Binding Properties of Human 5-Hydroxytryptamine$_{1D\alpha}$, Human 5-Hydroxytryptamine$_{1D\beta}$, and Calf 5-Hydroxytryptamine$_{1D}$ Receptors Investigated with [^{3}H]5-Hydroxytryptamine and [^{3}H]Alniditan

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SUMMARY

Alniditan is a new migraine-abortive agent. It is a benzopyran derivative and therefore structurally unrelated to sumatriptan and other indole-derivatives and to ergoline derivatives. The action of sumatriptan is thought to be mediated by 5-hydroxytryptamine (5-HT)$_{1D}$-type receptors. We investigated the receptor-binding profile *in vitro* of alniditan compared with sumatriptan and dihydroergotamine for 28 neurotransmitter receptor subtypes, several receptors for peptides and lipid-derived factors, ion channel-binding sites, and monoamine transporters. Alniditan revealed nanomolar affinity for calf substantia nigra 5-HT$_{1D}$ and for cloned h5-HT$_{1D\alpha}$, h5-HT$_{1D\beta}$, and h5-HT$_{1A}$ receptors (K_i = 0.8, 0.4, 1.1, and 3.8 nM, respectively). Alniditan was more potent than sumatriptan at 5-HT$_{1D}$-type and 5-HT$_{1A}$ receptors. Alniditan showed moderate-to-low or no affinity for other investigated receptors; sumatriptan showed additional binding to 5-HT$_{1F}$ receptors. Dihydroergotamine had a much broader profile with high affinity for several 5-HT, adrenergic and dopaminergic receptors. In signal transduction assays using cells expressing recombinant h5-HT$_{1D\alpha}$, h5-HT$_{1D\beta}$, or h5-HT$_{1A}$ receptors, alniditan (like 5-HT) was a full agonist for inhibition of stimulated adenylyl cyclase (IC_{50} = 1.1, 1.3, and 74 nM, respectively, for alniditan). Therefore, in functional assays, the potency of alniditan was much higher at 5-HT$_{1D}$ receptors than at 5-HT$_{1A}$ receptors. We further compared the properties of [^{3}H]alniditan, as a new radioligand for 5-HT$_{1D}$-type receptors, with those of [^{3}H]5-HT in membrane preparations of calf substantia nigra, C6 glioma cells expressing h5-HT$_{1D\alpha}$, and L929 cells expressing h5-HT$_{1D\beta}$ receptors. [^{3}H]Alniditan revealed very rapid association and dissociation binding kinetics and showed slightly higher affinity (K_d = 1–2 nM) than [^{3}H]5-HT. We investigated 25 compounds for inhibition of [^{3}H]alniditan and [^{3}H]5-HT binding in the three membrane preparations; K_i values of the radioligands were largely similar, although some subtle differences appeared. Most compounds did not differentiate between 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors, except methysergide, ritanserin, ocaperidone, risperidone, and ketanserin, which showed 10–60-fold higher affinity for the 5-HT$_{1D\alpha}$ receptor. The K_i values of the compounds obtained with 5-HT$_{1D}$ receptors in calf substantia nigra indicated that these receptors are of the 5-HT$_{1D\beta}$-type. We demonstrated that alniditan is a potent agonist at h5-HT$_{1D\alpha}$ and h5-HT$_{1D\beta}$ receptors; its properties probably underlie its cranial vasoconstrictive and antimigraine properties.

Alniditan {(−)-(*R*)-*N*-[(3,4-dihydro-2*H*-1-benzopyran-2-yl)-methyl]*N*′-(1,4,5,6-tetrahydro-2-pyrimidinyl)-1,3-propanediamine dihydrochloride} is a novel 5-HT$_{1D}$ receptor agonist. The compound has a unique chemical structure (Fig. 1) (1); it does not belong to the family of 5-HT$_{1D}$ receptor agonists with an indole structure of which sumatriptan is the prototype, nor is it an ergoline-derivative such as dihydroergotamine. Its 5-HT$_{1D}$-like receptor agonistic properties emerged from *in vitro* pharmacological studies that showed it to be a potent constrictor of dog saphenous vein and pig basilar artery.[1] In anesthetized dogs, alniditan specifically reduced carotid arterial blood flow at a low dose and over a wide range

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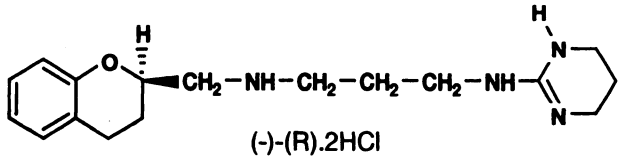
[1] W. Janssens, manuscript in preparation.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; c5-HT, calf 5-hydroxytryptamine; DMSO, dimethylsulfoxide; h5-HT, human 5-hydroxytryptamine; IFN, interferon; m5-HT, murine 5-hydroxytryptamine; p5-HT, porcine 5-hydroxytryptamine; [^{125}I]GTI, 5-hydroxytryptamine-5-*O*-carboxymethylglycyl-[^{125}I]iodotyrosinamide; LSD, *d*-lysergic acid diethylamide.



(-)-(R).2HCl

Alniditan

(-)-(R)-*N*-[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]-*N*'-(1,4,5,6-tetrahydro-2-pyrimidinyl)-1,3-propanediamine dihydrochloride

Fig. 1. Chemical structure of alniditan.

(0.63–80 mg/kg intravenous) with relatively little effect on other cardiovascular variables, including coronary, mesenteric, and renal arterial blood flow; systemic and pulmonary vascular resistance; and airway resistance (2). In early clinical studies, alniditan proved to be well tolerated and highly efficacious in the treatment of migraine (82% responders after 1.4 mg subcutaneous administration), with considerably less migraine recurrences (only 16%) than that reported for sumatriptan (3). In *in vitro* and *in vivo* pharmacological studies and in clinical studies, alniditan was found to be more potent than sumatriptan, which was supposed to exert its therapeutic action via a stimulatory effect on 5-HT_{1D}-like receptors in cranial blood vessels (4, 5).

The 5-HT_{1D} receptor-binding sites were first detected with the use of [^{3}H]5-HT and later with the use of various labeled indole derivatives in bovine, porcine, canine, guinea pig, and human brain (6–9). The 5-HT_{1D} receptors in these species were thought to be functional homologues of the 5-HT_{1B} receptors in rat brain; the distinction in name resulted from observed differences in pharmacological properties of the receptors between the rat and other mammalian species (10). The 5-HT_{1D} receptors belong to the superfamily of G protein-coupled receptors; via coupling to G_i, they inhibit adenylyl cyclase (11). The 5-HT_{1D} receptors were shown to play a role in inhibition of release of 5-HT and norepinephrine from nerve endings in both brain slices and vascular preparations (12, 13). The 5-HT_{1D}-like receptors mediate vasoconstriction, particularly in cranial blood vessels and the saphenous vein (14–16). They may also be involved in the regulation of release of inflammatory neuropeptides such as substance P and calcitonin gene-related peptide (17).

Gene cloning revealed the existence of two distinct 5-HT_{1D} receptor genes encoding protein monomers with seven putative transmembrane domains, as expected for G protein-coupled receptors (18). It was proposed that the human receptor subtypes be called 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (19). These receptor subtypes show only 58% identity in amino acid sequence, but the expressed proteins revealed a remarkably similar drug-binding pharmacology (20–23). The 5-$HT_{1D\alpha}$ receptor genes from dog (24) and guinea pig (25) and the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor genes from rabbit (26) have also been cloned. For these species, the pharmacology of the heterologously expressed receptors seem to be in large part similar to the pharmacology of the human receptor subtypes. Also, for the rat, genes homologous to the h5-$HT_{1D\alpha}$ and h5-$HT_{1D\beta}$ receptor genes were cloned. The rat receptor subtypes were called 5-HT_{1B} (homologous to 5-$HT_{1D\beta}$) and 5-HT_{1D} (homologous to 5-$HT_{1D\alpha}$). The heterologously expressed rat receptor proteins showed a different pharmacology than the human receptor subtypes despite an amino acid sequence identity of >90% (27, 28). Sumatriptan in particular showed a lower affinity for the rat receptor subtypes, whereas certain β-adrenergic receptor blockers showed a higher affinity for the rat receptors than for the 5-HT_{1D} receptors in other species. The difference in pharmacology between the rat 5-HT_{1B} and the h5-$HT_{1D\beta}$ receptor could be accounted for by a single amino acid difference at a homologous position (rat Asp351/human Thr355) in the seventh transmembrane domain (29, 30).

Until today, no selective compounds have been reported that can adequately distinguish between the 5-$HT_{1D\alpha}$ and the 5-$HT_{1D\beta}$ receptors within a given species, although the receptor subtypes have a relatively low sequence identity.

We report on the receptor-binding profile of alniditan compared with sumatriptan and dihydroergotamine, which involves 14 5-HT receptor subtypes [for a review of 5-HT receptors, see Hoyer *et al.* (19)], seven adrenergic receptor subtypes, five dopamine receptor subtypes, the histamine H_1 receptor, the muscarinic cholinergic receptor, several peptide receptors, lipid-derived factor receptors, ion channel-binding sites, and monoamine transporters (Table 1). Alniditan bound with nanomolar affinity to the calf brain 5-HT_{1D} receptor and to the h5-$HT_{1D\alpha}$, h5-$HT_{1D\beta}$, and h5-HT_{1A} receptors.

We studied the receptor-binding properties of [^{3}H]alniditan compared with [^{3}H]5-HT using membrane preparations of calf substantia nigra (under conditions that ensured selective 5-HT_{1D} receptor binding) and of mammalian cells expressing the cloned h5-$HT_{1D\alpha}$ and h5-$HT_{1D\beta}$ receptors. [^{3}H]Alniditan proved to be a new radioligand for these receptor subtypes with advantageous binding properties. We investigated a large series of compounds to compare the pharmacology of the 5-HT_{1D} receptor subtypes labeled by the two ligands in the different tissue preparations. The full agonistic properties of alniditan on h5-$HT_{1D\alpha}$, h5-$HT_{1D\beta}$, and h5-HT_{1A} receptors were demonstrated by its inhibitory effect on cAMP formation in cells expressing the cloned receptor.

Experimental Procedures

Materials. Alniditan is an original product of Janssen Pharmaceutica (1).

[^{3}H]Alniditan (41.4 Ci/mmol or 1.52 TBq/mmol) was obtained through catalytic tritiation of (*R*)-(−)-R091273, the pyrimidine analog, to yield (−)-(*R*)-*N*-[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]*N*'-(1-monohydro-3,4,5-tritritio-2-pyrimidinyl)-1,3-propanediamine dihydrochloride. The compound was purified with high performance liquid chromatography to yield a radiochemical purity of 98.6% (31). [^{3}H]5-HT (88 Ci/mmol or 3.25 TBq/mmol) was obtained from Amersham (Paisley, UK). [^{3}H]8-OH-DPAT (41.0 Ci/mmol or 4.79 TBq/mmol) was purchased from DuPont-New England Nuclear (Dreieich, Germany). The other radioligands listed in Table 1 were obtained from Amersham or DuPont-New England Nuclear, except for [^{125}I]5-iodo-R91150 {[^{125}I]-4-amino-*N*-[1[3-(4-fluorophenoxy)-propyl]-4-methyl-4-piperidinyl]-5-iodo-2-methoxybenzamide}, a new radioligand for 5-HT_{2A} receptors (32), which was labeled by J. Mertens (Free University of Brussels, Brussels, Belgium).

The compounds listed in Table 3 were obtained from various commercial sources or kindly donated by the companies of origin.

Lipophilic compounds were dissolved and further diluted in DMSO; the last 20-fold dilution step, which was carried out just before the addition of the compound to the incubation medium, was performed in distilled, deionized water; the dilution in the assay mixture was 10-fold. 5-HT and tryptamine-derivatives were dissolved and further diluted in 5% DMSO; the DMSO concentration in

TABLE 1

Receptor binding profile *in vitro* of alniditan compared with sumatriptan and dihydroergotamine: inhibition of radioligand binding expressed as pIC_{50} value and derived apparent equilibrium inhibition constant (K_i)

Receptor	Ligand	Tissue or transfected cell	Alniditan				Sumatriptan				Dihydroergotamine			
			pIC_{50}	SD	n	K_i	pIC_{50}	SD	n	K_i	pIC_{50}	SD	n	K_i
			−log (M)			*nM*	*−log (M)*			*nM*	*−log (M)*			*nM*
Serotonin														
c5-HT_{1D}	[^{3}H]5-HT	Calf substantia nigra	8.69	0.15	3	0.8	7.48	0.20	4	12	8.58	0.11	2	1.0
h5-$HT_{1D\alpha}$	[^{3}H]5-HT	Human 5-$HT_{1D\alpha}C_6$ glioma cells	9.03	0.13	3	0.4	8.05	0.05	2	3.5	8.25	0.12	3	2.5
h5-$HT_{1D\beta}$	[^{3}H]5-HT	Human 5-$HT_{1D\beta}$L929sA cells	8.65	0.14	2	1.1	7.51	0.14	2	15	8.86	0.09	2	0.6
r5-HT_{1B}	[^{3}H]5-HT	Rat striatum	6.92	0.14	3	49	7.18	0.10	2	25	7.50			14
h5-HT_{1A}	[^{3}H]8-OH-DPAT	Human 5-HT_{1A} HeLa cells	8.32	0.18	7	3.8	6.30	0.07	2	370	9.22	0.24	2	0.5
h5-HT_{1E}	[^{3}H]5-HT	Human 5-HT_{1E} L929sA cells	6.20	0.17	3	240	5.40	0.07	2	1600	5.78	0.12	2	605
h5-HT_{1F}	[^{3}H]5-HT	Human 5-HT_{1F} COS-7 cells	6.20	0.10	2	360	7.65	0.20	2	14	6.68	0.05	2	110
r5-HT_{2A}	[^{3}H]Ketanserin	Rat frontal cortex	5.70	0.06	3	590	<5.00							
h5-HT_{2A}	[^{125}I]5-IR091150	Human 5-HT_{2A} L929sA cells	<5.00				<5.00				8.15	0.13	3	2.9
p5-HT_{2C}	[^{3}H]Mesulergine	Pig choroid plexus	5.38	0.15	3	2930	<5.00				7.27	0.15	2	37
m5-HT_3	[^{3}H]GR65630	NxG 108CC15 cells	<5.00				<5.00				<5.00			
Guinea pig 5-HT_4	[^{3}H]GR113808	Guinea pig striatum	5.15	0.16	2	5120	<5.00				6.37	0.12		304
r5-HT_6	[^{125}I]- LSD	Rat 5-HT_6HEK 293 cells	5.21	0.26	2	5470	4.97	0.03	2	8800	7.90	0.16	2	10
r5-HT_7	[^{125}I]-LSD	Rat 5-HT_7HEK 293 cells	6.50	0.10	2	260	5.87	0.09	2	1020	7.80	0.15	2	15
Adrenergic														
Rat α_1	[^{3}H]Prazosin	Rat cortex	6.66	0.18	3	97	<5.00				7.60	0.05	2	10
Rat α_2	[^{3}H]Clonidine	Rat cortex	5.91	0.05	3	460	<5.00				7.60	0.20	2	10
Human α_{2A}	[^{3}H]Rauwolscine	Human α_{2A} CHO cells	7.17	0.06	3	37	<5.00				8.50	0.02	2	1.7
Human α_{2B}	[^{3}H]Rauwolscine	Human α_{2B} CHO cells	6.63	0.27	3	170	<5.00				7.89	0.09	2	8.3
Human α_{2C}	[^{3}H]Rauwolscine	Human α_{2C} CHO cells	7.19	0.04	3	18	<5.00				8.39	0.07	2	1.1
Human β_1	[^{125}I-Cyanopindolol	Human β_1 *E. coli*	<5.00				<5.00				5.00	0.05	2	5400
Human β_2	[^{125}I-Cyanopindolol	Human β_2 *E. coli*	<5.00				<5.00				<5.00			
Dopamine														
Rat D_1	[^{3}H]SCH 23390	Rat striatum	<5.00				<5.00				5.17	0.07	2	4800
Rat D_2	[^{3}H]Haloperidol	Rat striatum	6.05	0.10	3	360	<5.00							
Human D_{2L}	[^{3}H]Spiperone	Human D_{2L} CHO cells	5.21	0.16	3	1950	<5.00				7.68	0.09	3	6.2
Human D_3	[^{125}I]Iodosulpride	Human D_3 CHO cells	6.42	0.12	3	280	<5.00				8.06	0.10	3	6.4
Human $D_{4.2}$	[^{3}H]Spiperone	Human $D_{4.2}$ CHO cells	6.68	0.12	3	80	<5.00				7.69	0.36	3	8
Histamine														
Guinea pig H_1	[^{3}H]Pyrilamine	Guinea pig cerebellum	5.06	0.01	2	3830	<5.00				<5.00			
Human H_1	[^{3}H]Pyrilamine	Human H_1 CHO cells					<5.00				<5.00			
Cholinergic muscarinic	[^{3}H]Dexetimide	Rat striatum	<5.00				<5.00				<5.00			
Opiate														
Rat μ	[^{3}H]Sufentanil	Rat forebrain	<5.00				<5.00				5.00	0.10	2	2000
Guinea pig κ	[^{3}H]U 69593	Guinea pig cerebellum	<5.00				<5.00				<5.00			
Murine δ	[^{3}H]DPDPE	NxG 108CC15 cells	<5.00				<5.00				<5.00			
Guinea pig haloperidol-sensitive σ1	[^{3}H]Haloperidol	Guinea pig medulla oblongata	6.97	0.07	3	40	<5.00				<5.00			
Peptide														
Guinea pig neurotensin	[^{3}H]Neurotensin	Guinea pig forebrain	<5.00				<5.00							
Rat CCK_A	[^{3}H]CCK_8	Rat pancreas	<5.00				<5.00				<5.00			
Guinea pig CCK_B	[^{3}H]CCK_8	Guinea pig cortex	<5.00				<5.00				5.04			3300
Calf substance P	[^{3}H]Substance P	Calf striatum	<5.00				<5.00							
Human substance P	[^{3}H]Substance P	Human substance P CHO cells	<5.00				<5.00				<5.00			
Human bradykinin B_2	[^{3}H]Bradykinin	Human B_2 CHO cells	<5.00				<5.00				<5.00			
Lipid-derived factor														
Human thromboxane A_2	[^{3}H]SQ 29548	Intact human platelets	<5.00				<5.00				<5.00			
Guinea pig leukotriene D_4	[^{3}H]LTD_4	Guinea pig lung	<5.00				<5.00				<5.00			
Rabbit PAF	[^{3}H]PAF	Rabbit platelets	<5.00				<5.00				<5.00			
Ion channel ligand sites														
Rat Ca^{2+} channel-binding site	[^{3}H]Nitrendipine	Rat cortex	<5.00				<5.00				<5.00			
Rat Na^+ channel-binding site	[^{3}H]Batrachotoxin	Rat cortex	<5.00				<5.00				5.34	0.01	2	4450
Rat $GABA_A$ benzodiazepine site	[^{3}H]Flunitrazepam	Rat forebrain	<5.00				<5.00							
Monoamine transporters														
Rat dopamine	[^{3}H]WIN 35428	Rat striatum	<5.00				<5.00				<5.00			
h5-HT	[^{3}H]Paroxetine	Human platelets	<5.00				<5.00				<5.00			
Rat norepinephrine	[^{3}H]Nisoxetine	Rat cortex	<5.00				<5.00				<5.00			
Rat vesicular	[^{3}H]Ketanserin	Rat striatum	6.43	0.19	2	365								

HEK, human embryonic kidney; DPDPE, [D-Pen^2, D-Pen^5]enkephalin; PAF, platelet-activating factor.

the assay was 0.5%. In all control assays, solvent was added to a final concentration of 0.5% DMSO.

Receptor-binding profile investigation. Inhibition by alniditan, sumatriptan, and dihydroergotamine of radioligand binding *in vitro* was investigated for the various receptors listed in Table 1 with radioligands and membrane preparations. Assay conditions were as reported in Schotte *et al.* (33).

Some recombinant cell systems were obtained from the laboratories who originally described the systems: the h5-HT_{1A} (M. Caron, Duke University), human bradykinin B_2 (J. Dumont, Free University of Brussels), and human β_1- and β_2-adrenergic (A. D. Strosberg, Hôpital Cochin, Paris, France) receptors. Membranes of cells expressing the cloned human dopamine D_4 and r5-HT_6 and r5-HT_7 receptors were purchased from Receptor Biology (Baltimore, MD).

For the remaining cloned human receptors listed in Table 1, the receptor DNA was amplified by PCR, cloned in a plasmid, and sequenced as described below. When the DNA sequence was not identical to the published sequence, the necessary corrections were made. The cloned cDNAs for the 5-HT_{1F}, α_{2A}, α_{2B}, α_{2C}, D_{2L}, D_3, H_1, and substance P receptors were transferred into the pRc/CMV expression vector, which contains a neomycin resistance gene (InVitrogen, Leek, The Netherlands), and the receptor cDNAs for the 5-HT_{1E} and 5-HT_{2A} receptors were cloned into the pSP64MxpA vector (34). The vector with the receptor DNA was transfected into the mammalian cell lines indicated in Table 1 according to the calcium phosphate transfection method (35). The pSP64MxpA vector was cotransfected with the pSV2neo vector for introduction of a neomycin-resistant gene. G-418 (800 μg/ml) was added to the cultures to select for resistant stable cell lines. Clonal cell colonies stably expressing the receptor were isolated.

Membranes of tissues and cells were prepared and radioligand-binding procedures were performed as described below.

Cloning and expression of h5-$HT_{1D\alpha}$ and h5-$HT_{1D\beta}$ receptors. C6 glioma cell lines stably expressing h5-$HT_{1D\alpha}$ receptors were obtained from Dr. J. Naranjo (Instituto Cajal, Madrid, Spain). The stable lines were obtained through G-418 selection of C6 glioma cells cotransfected with the pXTI plasmid (Stratagene, La Jolla, CA) and the p514 (Stratagene) expression vector containing the full h5-$HT_{1D\alpha}$ coding region. This insert sequence has been verified and found to be identical to the published sequence (20). For the h5-$HT_{1D\beta}$ receptor, PCR primers were designed based on a published sequence (21) to amplify the entire (intronless) coding region of the h5-$HT_{1D\beta}$ receptor from genomic DNA. At the 5′ end of the 5′ PCR primer, a Kozak consensus sequence was added, and recognition sites for restriction endonucleases were added to the 5′ end of both 5′ (ATAGCTAGCAGGCCTGCCACCATGGAGGAACCGGGTGCTCAG) and 3′ (GCGTCAACTTGTGCACTTAAAACGTATC) PCR primers. PCR-amplification products of the expected size were obtained and purified on a low-melting-point agarose gel. After excision from the gel, the DNA fragment was blunted with Klenow, phosphorylated, and ligated into *Sma*I-linearized and dephosphorylated pUC18. Two recombinants with the desired restriction pattern were selected for sequence verification. Both showed a single (but different) deletion, and based on intact segments from the two clones, a recombinant was reconstructed with a sequence verified to be identical to the published one. The insert from this intact pUC18 clone was transferred to the pSP64MxpA expression vector, and these expression constructs were verified by restriction analysis. In this expression vector, the insert is under control of the promoter of the murine *Mx1* gene (36), a 1600-base-pair fragment of which confers IFN-inducible expression on the insert (37). The expression construct was transfected according to the calcium phosphate method into murine L929 cells, which possess an endogenous IFN receptor. Clonal cell colonies were isolated, and the inducibility of receptor expression was verified (for details on the pSP64MxpA expression vector, transfection, and induction method, see Ref. 34). The use of this inducible expression system for the expression of the h5-$HT_{1D\beta}$ receptor was chosen because attempts had failed to obtain stable expression of the h5-$HT_{1D\beta}$ receptor in C6 glioma cells transfected with the pRc/CMV expression vector containing the receptor DNA.

Cell culture. The mouse fibrosarcoma cell line L929sA was grown in Dulbecco's modified Eagle's medium with 5% heat-inactivated (30 min at 56°) fetal calf serum, 5% heat-inactivated newborn calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. C6 glioma cells were grown in the same medium except for the serum, which was 2.5% heat-inactivated fetal calf serum and 15% heat-inactivated horse serum. The selection medium for L929sA cells expressing the h5-$HT_{1D\beta}$ receptor was supplemented with 500 μg/ml G-418, whereas the selection medium for C6 glioma cells expressing the h5-$HT_{1D\alpha}$-receptor was supplemented with 800 μg/ml G-418. Cells were grown at 37° in a humidified atmosphere containing 5% CO_2.

Calf brain tissue. Calf substantia nigra was obtained from a local slaughterhouse; the tissue was homogenized with an Ultra Turrax homogenizer (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) in 50 mM Tris·HCl, pH 7.4; a total membrane fraction was collected and washed by three subsequent centrifugation runs (20 min at 25,000 × *g* at 4°). An intermediate incubation of 15 min at 37° was carried out for elimination of endogenous 5-HT; otherwise, the tissue preparation was kept on ice during the entire procedure.

Cell membrane preparation. Cells were washed with phosphate-buffered saline; scraped from the culture plates; suspended in 50 mM Tris·HCl, pH 7.4; and collected through centrifugation (10 min at 16,000 × *g* at 4°). The cells were lysed in 5 mM hypotonic Tris·HCl, pH 7.4, and homogenized with an Ultra Turrax homogenizer, and membranes were collected through centrifugation (20 min at 25,000 × *g* at 4°). The preparation was kept on ice during the entire procedure.

Radioligand binding. For binding studies with [^{3}H]5-HT, membranes were suspended in 50 mM Tris·HCl, pH 7.4, containing 4 mM $CaCl_2$ and 10 μM pargyline.

For binding studies with [^{3}H]alniditan, membranes were suspended in 50 mM Tris·HCl, pH 7.7, containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 μM pargyline (Tris salt buffer).

For binding studies using calf substantia nigra, 30 nM 8-OH-DPAT and 30 nM mesulergine were added to the incubation mixture to occlude 5-HT_{1A} and 5-HT_{2C} receptor sites, respectively. Nonspecific binding of the radioligands was estimated in the presence of 10 μM sumatriptan. Incubations were run for 30 min at 37° in a volume of 0.5 ml containing ~80 μg of protein for calf substantia nigra, 50–100 μg of protein for C6 glioma cells expressing h5-$HT_{1D\alpha}$, and 25–50 μg of protein for L929sA cells expressing h5-$HT_{1D\beta}$ receptors. The incubation was stopped by rapid filtration under suction over GF/B glass-fiber filters presoaked in 0.1% polyethyleneimine, followed by a rinsing three times with 3 ml of ice-cold Tris·HCl buffer (50 mM; pH 7.4). Radioactivity collected on the filters was counted in a scintillation counter.

For ligand concentration binding isotherms, 10–12 concentrations of the radioligands, in a range of 0.1–10 nM, were used. Experiments were repeated independently four to six times. For inhibition of radioligand binding, compounds were added at 8–10 concentrations, spanning 3 orders of magnitude around the IC_{50} value; experiments were repeated independently two to eight times.

Ligand concentration binding isotherms (rectangular hyperbola) and sigmoidal inhibition curves were calculated by nonlinear regression analysis according to algorithms described by Oestreicher and Pinto (38). The B_{max} and apparent K_d values of the radioligand and pIC_{50} of the inhibitor were free parameters for the curve fitting. Apparent K_i values were calculated as $K_i = IC_{50}\cdot[1 + C/K_d],^{-1}$ where C is concentration of the radioligand (39).

Adenylyl cyclase assay. C6 glioma cells expressing the h5-$HT_{1D\alpha}$ receptor, L929sA cells expressing the h5-$HT_{1D\beta}$ receptor as well as the mock-transfected cells, and HeLa cells expressing the h5-HT_{1A} receptor were plated at 150,000 cells/well in a 24-well plate. The next day, C6 glioma cells and HeLa cells were directly assayed, whereas L929sA cells were first induced for 18–24 hr with 1000

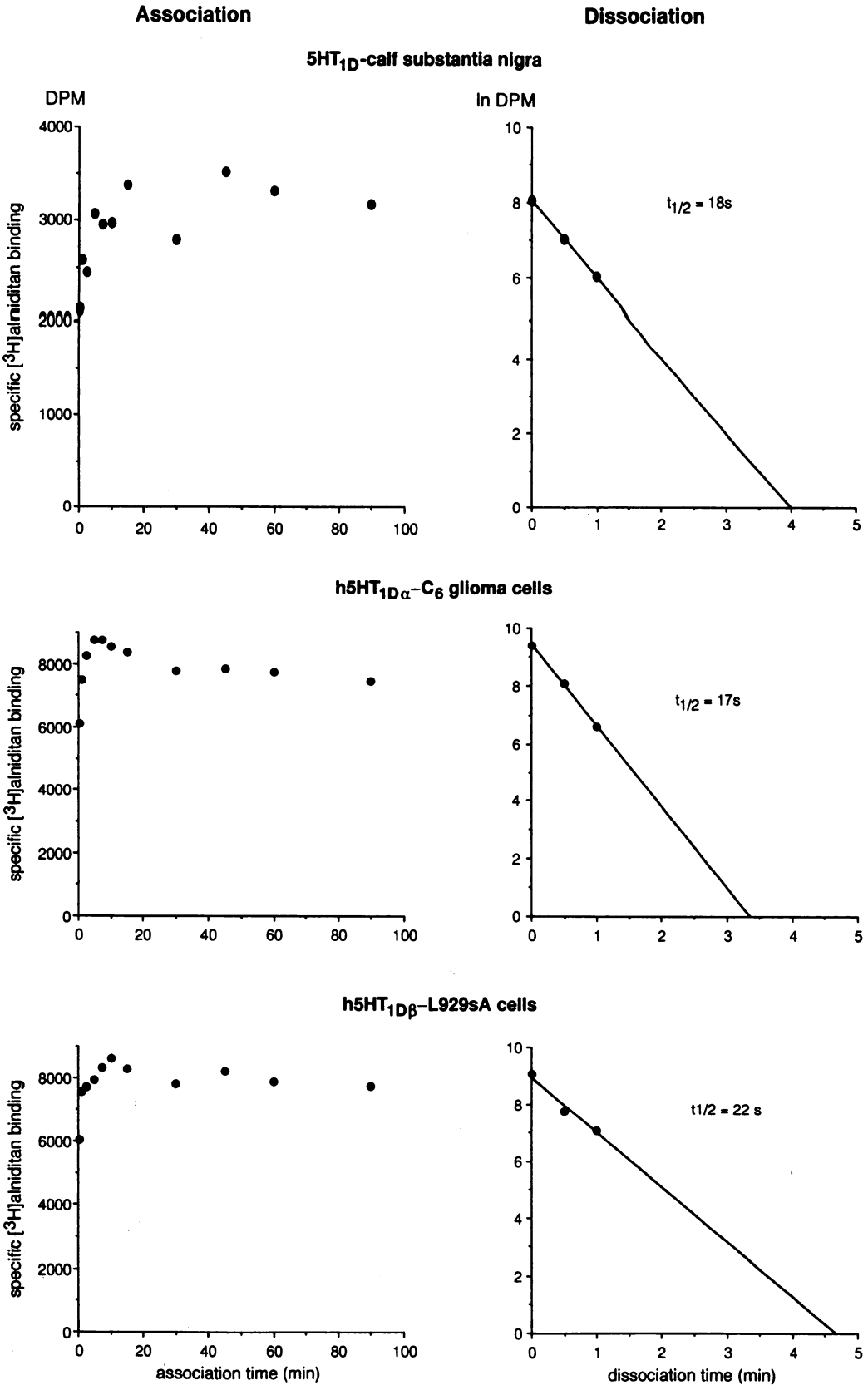


Fig. 2. Time curves of (left) association and (right) dissociation of [^{3}H]alniditan binding (37°) to 5-HT$_{1D}$ receptors in calf substantia nigra, h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells, and h5-HT$_{1D\beta}$ receptors expressed in L929sA cells. [^{3}H]Alniditan (4 nM) was incubated at 37° with the tissue or cell membrane preparations for various times (association) or for 30 min before dissociation of the radioligand was initiated by the addition of excess sumatriptan (10 μM) followed by further incubation for various time periods. Reactions were stopped by the addition of 5 ml of ice-cold buffer and rapid filtration under suction as described in Experimental Procedures. Receptor/ligand dissociation was graphically analyzed according to first-order kinetics with plots of [bound ligand] reaction versus time. *Straight line through points of initial dissociation*, calculated by linear regression analysis, and the slope yielded the dissociation rate constant k_{-1}. The half-time of dissociation was calculated as $t_{1/2} = \ln 2/k_{-1}$.

units/ml recombinant murine IFNβ. The cells were washed with controlled salt solution (120 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 0.04 mM phenol red in 25 mM Tris·HCl, pH 7.4) and then incubated for 20 min at 37° with 0.5 ml of controlled salt solution containing 1 mM 3-isobutyl-1-methylxanthine, 1 μM pargyline, 1 μM paroxetine, and either 100 μM forskolin, 1 μM isoproterenol, or solvent in the absence or presence of defined concentrations of 5-HT or alniditan. The incubation was stopped by the addition of 0.1 ml of 1 N ice-cold $HClO_4$, followed by neutralization to pH 7.5 with 0.1 ml of 0.5 M ice-cold KOH/K_3PO_4, pH 13.5. When the

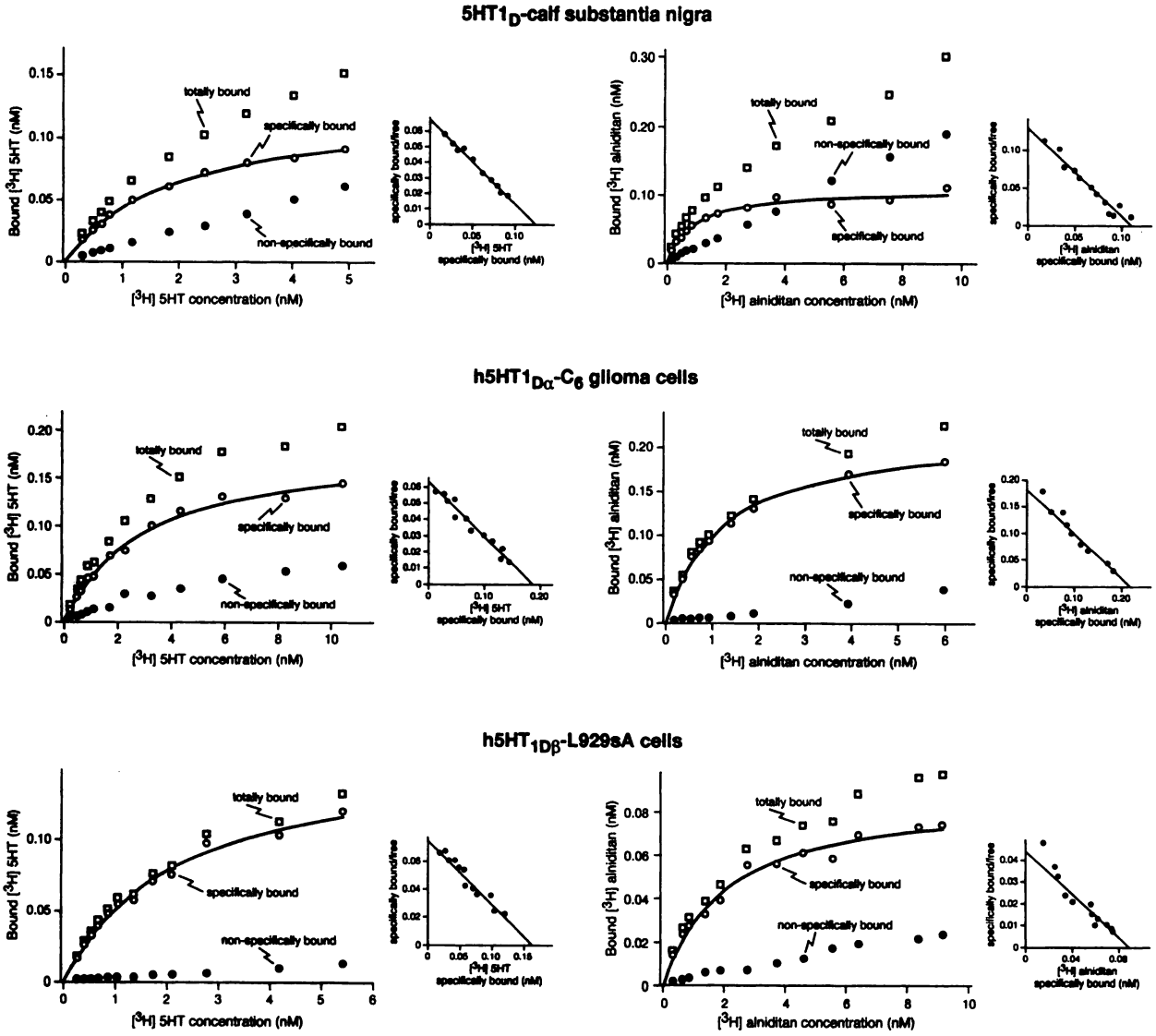


Fig. 3. Concentration binding isotherms (37°) of (left) [^{3}H]5-HT and (right) [^{3}H]alniditan to 5-HT$_{1D}$ receptors in calf substantia nigra, h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells, and h5-HT$_{1D\beta}$ receptors expressed in L929sA cells. K_d and B_{max} values were derived by nonlinear regression analysis of the specific binding in individual experiments. Experiments were repeated two to seven times with membrane preparations of different cell clones. Mean values are presented in Table 2. Scatchard plots are shown for verification of linearity.

$KClO_4$ precipitate was formed, plates were centrifuged for 5 min at 2000 × *g*, and the supernatant was assayed for cAMP content with a commercial radioimmunoassay kit using ^{125}I-cAMP (Immunotech International, Marseille, France) according to the manufacturer's directions. cAMP levels measured in assays with 5-HT or alniditan were expressed as percentage of the forskolin- or isoproterenol-stimulated cAMP levels in the absence of agonist and plotted against the drug concentration on a log scale. Sigmoidal curves of best fit were calculated by nonlinear regression analysis using GraphPAD software (San Diego, CA).

Results

Receptor-binding profile of alniditan. Table 1 shows the binding affinities of alniditan, sumatriptan, and dihydroergotamine (pIC_{50} and K_i values) measured in 48 radioligand-binding assays, including neurotransmitter receptor subtypes, receptors for peptide- and lipid-derived factors, ion channel-binding sites, and monoamine transporters.

Alniditan binds with nanomolar affinity to 5-HT$_{1D}$ receptors in calf substantia nigra and to h5-HT$_{1D\alpha}$ and h5-HT$_{1D\beta}$ receptors in transfected cell lines (K_i = 0.8, 0.4, and 1.1 nM, respectively). It has 4–10-fold lower affinity for h5-HT$_{1A}$ receptors (K_i = 3.8 nM) and a 50-fold lower affinity for 5-HT$_{1B}$ receptors in rat striatum. Its affinity for the other 5-HT receptor subtypes was >200-5000-fold lower. Alniditan bound stronger than sumatriptan to c5-HT$_{1D}$ (15-fold), h5-HT$_{1D\alpha}$ (9-fold), h5-HT$_{1D\beta}$ (14-fold), and h5-HT$_{1A}$ receptors (97-fold), but it bound 2-fold weaker than sumatriptan to r5-HT$_{1B}$ receptors. Sumatriptan, but not alniditan, showed a relatively high affinity for h5-HT$_{1F}$ receptors.

Alniditan, but not sumatriptan, showed moderate affinity for human α_2-adrenergic receptor subtypes, for haloperidol-sensitive σ sites, and for dopamine D_4 receptors; the potency difference with 5-HT$_{1D}$ receptor binding was 20–70-fold. Alniditan and sumatriptan interacted only very weakly with other neurotransmitter receptor subtypes. They did not bind to receptors for peptide- or lipid-derived factors, to ion channel-binding sites, or to monoamine transporters. Only weak

TABLE 2

Apparent equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) measured with [^{3}H]5-HT and [^{3}H]alniditan for binding to 5-HT$_{1D}$-type receptors in brain tissue and transfected cells

		[^{3}H]5-HT[a]		[^{3}H]Alniditan[b]	
		K_d	B_{max}	K_d	B_{max}
		nM	fmol/mg of protein	nM	fmol/mg of protein
5-HT$_{1D}$	Calf substantia nigra	1.9 ± 0.7 (4)	764 ± 14 (4)	1.08 ± 0.05 (3)	740 ± 240 (3)
h5-HT$_{1D\alpha}$	C6 glioma cells				
	Clone 22	2.9 ± 0.1 (3)	870 ± 360 (3)	1.10 ± 0.30 (5)	990 ± 370 (5)
				0.70 ± 0.30 (7)[a]	970 ± 590 (7)[a]
	Clone 6			1.40 ± 0.40 (2)	623 ± 30 (2)
	Clone 11			1.49 ± 0.04 (2)	450 ± 120 (2)
	Clone 14			1.60 ± 0.40 (2)	710 ± 10 (2)
h5-HT$_{1D\beta}$	L929sA cells				
	Clone 3	3.9 ± 0.1 (4)	2000 ± 180 (4)	1.85 ± 0.45 (5)	1845 ± 340 (5)
	Clone 5	3.2	1700	2.26 ± 0.45 (6)	1970 ± 435 (6)

[a] Assayed in Tris · HCl buffer containing 4 nm $CaCl_2$.
[b] Assayed in Tris · salt buffer.
Values are mean ± standard deviation (n).

binding of alniditan to the vesicular monoamine transporter was observed.

Dihydroergotamine clearly displayed a much broader receptor-binding profile than alniditan and sumatriptan (Table 1). Dihydroergotamine shows nanomolar binding affinity for c5-HT$_{1D}$, h5-HT$_{1D\alpha}$, and h5-HT$_{1D\beta}$ receptors and subnanomolar affinity for h5-HT$_{1A}$ receptors. In addition, it binds with high affinity to r5-HT$_{1B}$, h5-HT$_{2A}$, p5-HT$_{2C}$, r5-HT$_6$, and r5-HT$_7$ receptors. Furthermore, it binds at nanomolar concentrations to α_1- and α_2-adrenergic receptor subtypes and to human D_{2L}, D_3, and D_4 receptors.

Binding of [^{3}H]alniditan and [^{3}H]5-HT to 5-HT$_{1D}$ receptor subtypes. The association and dissociation of [^{3}H]alniditan to 5-HT$_{1D}$ receptors in calf substantia nigra, to h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells, and to h5-HT$_{1D\beta}$ receptors expressed in L929sA cells was very rapid; time curves are shown in Fig. 2. Full association was reached by ≤10 min, and the half-time of dissociation was 13–20 sec.

The concentration binding isotherms of [^{3}H]alniditan and [^{3}H]5-HT to calf substantia nigra 5-HT$_{1D}$, h5-HT$_{1D\alpha}$, and h5-HT$_{1D\beta}$ receptors are shown in Fig. 3. Derived K_d and B_{max} values are presented in Table 2. With both radioligands, regular rectangular hyperbolae were obtained (goodness of fit of the individual curves, r^2 = >0.96), resulting in linear Scatchard plots. Saturation of specific binding was reached in the nanomolar concentration range. For both ligands, the nonspecific binding, measured in the presence of 10,000-fold excess sumatriptan, was quite low; with membranes of transfected cells, it represented <10% of total binding at the K_d concentration of the ligand. The K_d value of [^{3}H]alniditan for the 5-HT$_{1D}$ receptor subtypes was 1–2 nM; this was 2–3-fold lower than that of [^{3}H]5-HT. Measured B_{max} values were similar with both ligands. In calf substantia nigra, it amounted to ~750 fmol/mg of protein. Different cell clones of C6 glioma cells were assayed with B_{max} values for h5-HT$_{1D\alpha}$ receptors of 450-1000 fmol/mg of protein. In two clones of recombinant murine IFNβ-induced L929sA cells, the density of h5-HT$_{1D\beta}$ receptors was ~1800 fmol/mg of protein (Table 2). Note that in accordance with several previous studies, [^{3}H]5-HT binding was measured in Tris buffer with 4 mM $CaCl_2$, whereas in the current study, [^{3}H]alniditan binding was measured in Tris salt buffer. However, when [^{3}H]alniditan binding was measured in the Tris-$CaCl_2$ buffer, the same binding parameters were obtained (Table 2).

Ligand-binding pharmacology of 5-HT$_{1D}$ receptor subtypes. For a large series of compounds, we investigated inhibition of [^{3}H]alniditan and [^{3}H]5-HT binding in the three 5-HT$_{1D}$ receptor preparations. Compounds and apparent K_i values are listed in Table 3. The applied curve-fitting program (see Experimental Procedures) provided information on the goodness of fit to a regular sigmoidal curve and pIC_{50} values, and Hill coefficients (a measure for the steepness of the curve) were derived. Apparent K_i values were further calculated from the pIC_{50} values. Because of the large amount of data obtained in three tissues with the use of two radioligands, we report only the apparent K_i values.

None of the compounds produced inhibition curves with Hill coefficients that were smaller than unity. Most compounds produced inhibition curves with Hill coefficients that were close to unity, indicating involvement of a single binding site. For only two compounds consistent exceptions were noted. With dihydroergotamine, Hill coefficients that were considerably greater than unity (1.4–2.8) were found for inhibition of both [^{3}H]5-HT and [^{3}H]alniditan binding to calf substantia nigra 5-HT$_{1D}$ and h5-HT$_{1D\alpha}$ receptors. The antagonist GR127935 produced steep inhibition curves (Hill coefficients, 1.5–2.7) in all assays.

To facilitate potency comparison, we calculated the ratios of K_i values in the various tissue preparations (Table 4). Alniditan, 5-carboxamidotryptamine, LSD, and dihydroergotamine are equally potent and show the highest binding affinity for the 5-HT$_{1D}$ receptor subtypes in the three preparations. Alniditan, the indole derivatives, and the ergoline derivatives (except methysergide) do not distinguish between the 5-HT$_{1D}$ receptor subtypes. Methysergide, risperidone, ritanserin, and ocaperidone have a 10–30-fold higher affinity and ketanserin has a 60-fold higher affinity for h5-HT$_{1D\alpha}$ than for h5-HT$_{1D\beta}$ receptors. K_i values of compounds for the c5-HT$_{1D}$ receptors were most similar to those for the h5-HT$_{1D\beta}$ receptors (see Tables 3 and 4).

Signal transduction mediated by h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells. The transfected C6 glioma cells cultured onto 24-well plate and treated with solvent revealed basal cAMP in a cell extract (0.5 ml) of 7 ± 1 pmol/well (mean ± standard error, 6 experiments). Direct

TABLE 3

Apparent equilibrium inhibition constants (K_i values) of drugs for 5-HT_{1D}-type receptors

	5-HT_{1D} calf substantia nigra						h5-$HT_{1D\alpha}$ C6 glioma cells						h5-$HT_{1D\beta}$ L929sA cells					
	[^{3}H]5-HT (4 nM)			[^{3}H]Alniditan (2 nM)			[^{3}H]5-HT (4 nM)			[^{3}H]Alniditan (2 nM)			[^{3}H]5-HT (4 nM)			[^{3}H]Alniditan (2 nM)		
	K_i (nM)	SD	n	K_i (nM)	SD	n	K_i (nM)	SD	n	K_i (nM)	SD	n	K_i (nM)	SD	n	K_i (nM)	SD	n
Alniditan	0.8	0.2	3	1.5	0.2	3	0.4	0.1	3	2.1	0.6	3	1.1	0.4	2	1.7	0.6	2
Indole derivatives																		
5-Carboxamidotryptamine	0.9	0.1	4	1.1	0.2	3	1.1	0.5	3	2.4	0.7	5	2.2	0.7	2	4.8	0.2	2
Naratriptan	2.1	0.7	3	2.8	0.2	3	0.9	0.1	2	3.7	1.3	6	1.2	0.1	3	3.9	0.6	3
5-HT	2.4	0.4	2	3.7	0.9	5	2.9	0.9	4	8.8	1.5	6	4.6	0.6	3	12	5	4
5-Methoxytryptamine	3.2	0.3	3	9.6	1.3	2	3.2	0.6	2	36	7	3	9.4	0.7	3	47	16	4
Bufotenine	10			43	20	2	8.9	3.5	4	21	9	5	13	7	3	37	13	5
Sumatriptan	12	5	4	13	2	4	3.5	0.1	2	9.7	1.6	4	15	5	2	50	5	3
N-Methyltryptamine	25	2	4	100	65	3	25	10	4	75	23	3	30	12	4	130	87	6
Tryptamine	34	3	3	91	20	2	42	2	4	141	68	3	58	32	4	150	90	3
Dimethyltryptamine	80	15	3	298	66	3	53	10	2	106	34	6	91	39	2	350	130	5
α-Methylserotonin	84	11	2	223	60	2	228	25	2	800	220	3	250	45	2	610	230	3
2-Methylserotonin	710	125	2	1700	700	2	600	180	2	2100	450	3	1590	13	2	4230	1100	3
CP-93129	1260	470	2	1200	600	3	1560	700	2	2940	500	3	930	290	2	2320	200	2
Ergoline derivatives																		
d-Lysergic acid	0.8	0.1	2	0.7	0.3	2	1.7	0.8	3	1.1	0.5	3	1.7	0.4	2	3.4	2	4
Metergoline	1.0	0.2	2	4.4	0.6	3	2.5	0.6	3	2.8	0.9	3	2.6	0.6	2	6.7	2	2
Dihydroergotamine	1.2	0.2	2	1.3	0.1	2	0.7	0.2	2	0.6	0.3	4	0.7	0.2	2	0.6	0.2	4
Yohimbine	8.7	1.2	4	13	2	2	19	10	2	25	9	5	33		1	65	41	3
Methysergide	9.5	3	3	6.2	0.7	3	1.3	0.6	4	2.8	0.6	4	15	1.5	3	27	17	3
Tetraline derivative																		
8-OH-DPAT	210	95	2	215	28	2	48	29	3	138	27	3	160	14	2	600	5	2
Putative antagonists																		
GR 127935	3.2	0.1	2	3.9	0.8	2	2.6	0.2	2	4.7	1.2	5	2.8	1	2	1.1	0.5	5
Ocaperidone	11	2	3	23	7	3	1.5	0.5	3	1.8	0.3	3	34	10	2	54	13	3
Metitepine	29	8	4	30	5	3	28	5	3	6.3	0.8	4	9.3	0.6	2	6.8	2.1	3
Risperidone	53	14	4	100	28	2	15	2	3	11	4	4	85	23	2	126	45	4
Ritanserin	137	17	4	480	100	2	26	5	3	24	6	2	156	53	3	250	200	3
Ketanserin	970	370	2	2000	650	5	31	19	6	37	14	8	2070	880	5	2060	1630	6

activation of adenylyl cyclase with 100 μM forskolin (20 min) yielded cAMP in the cell extract of 1260 ± 130 pmol/well (6 experiments), corresponding to an 180-fold stimulation relative to base-line levels. C6 glioma cells contain an endogenous β-adrenergic receptor that is positively coupled to adenylyl cyclase; stimulation of the cells with 1 μM isoproterenol resulted in cAMP of 470 ± 180 pmol/well (4 experiments); this was an 67-fold stimulation.

The effects of 5-HT and alniditan on cAMP production in the C6 glioma cells expressing the h5-HT$_{1D\alpha}$ receptor and stimulated with forskolin or isoproterenol are presented in Fig. 4. Neither compound affected the basal amount of cAMP. In mock-transfected cells stimulated with either forskolin or isoproterenol, 5-HT or alniditan, tested at several concentrations, did not affect the cAMP amounts (results not shown). In cells expressing the h5-HT$_{1D\alpha}$ receptor, a concentration-dependent inhibition of stimulated cAMP formation was observed with both compounds (Fig. 4). The potencies of the compounds were identical on stimulation of adenylyl cyclase with forskolin or isoproterenol (IC$_{50}$ values for stimulation with forskolin or isoproterenol: 5-HT, 2.5 and 1.1 nM alniditan, 1.2 and 0.8 nM, respectively). Antagonism of the 5-HT-mediated inhibition in these cells was investigated with ocaperidone; results are presented in Fig. 5. In cells stimulated with forskolin, 100 nM 5-HT produced a strong inhibition of the adenylyl cyclase activity. This inhibition was concentration-dependently reversed by ocaperidone; 50% reversal was obtained at 26 nM.

Signal transduction mediated by h5-HT$_{1D\beta}$ receptors expressed in L929sA cells. The murine IFNβ-induced transfected L929sA cells cultured onto 24-well plates and treated with solvent revealed a basal cAMP level in the cell extract (0.5 ml) of 4 ± 1 pmol/well (mean ± standard error, n = 4). Direct activation of adenylyl cyclase with 100 μM forskolin (20 min) yielded a cAMP amount of 41 ± 9 pmol/well (n = 4), corresponding to a 10-fold stimulation relative to base-line levels. Similar basal and forskolin-stimulated levels were found in noninduced cells, indicating that the IFNβ stimulation did not affect the adenylyl cyclase activity.

The effect of 5-HT and alniditan on cAMP formation stimulated with 100 μM forskolin in murine IFNβ-induced and noninduced L929sA cells expressing h5-HT$_{1D\beta}$ receptors is shown in Fig. 6. A concentration-dependent inhibition of cAMP formation was observed with both compounds (IC$_{50}$ values in noninduced and murine IFNβ-induced cells: 5-HT, 22 and 5.1 nM; alniditan, 6.2 and 1.0 nM, respectively). Therefore, under noninduced conditions, the low level of h5-HT$_{1D\beta}$ receptor expression (200–300 fmol/mg of protein) seems sufficient to give a full-blown signal transduction response. However, the potencies of the agonists were apparently lower in the noninduced cells, which have a lower receptor expression. In mock-transfected cells, neither 5-HT nor alniditan affected the forskolin-stimulated cAMP formation.

Signal transduction mediated by h5-HT$_{1A}$ receptors expressed in HeLa cells. HeLa cells expressing the h5-HT$_{1A}$ receptor (clone Ha6) revealed a basal cAMP amount in the extract of a well in a 24-well plate of 3 ± 1 pmol/well (n = 4). After stimulation with 100 μM forskolin, cAMP of 244 ± 57 pmol/well (n = 4) was found, corresponding to a 81-fold stimulation over base-line. In h5-HT$_{1A}$ receptor-expressing cells, alniditan and 5-HT produced a concentration-dependent inhibition of forskolin-induced cAMP formation. The

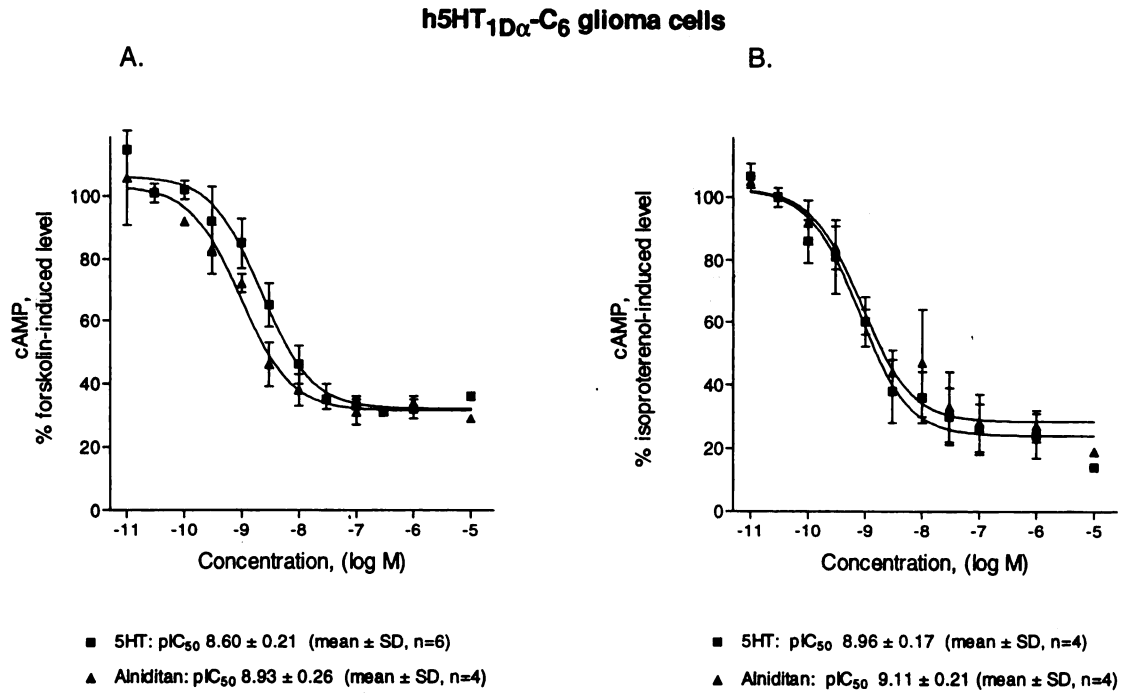


Fig. 4. Signal transduction mediated by h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells. Cells were grown onto 24-well plates. Adenylyl cyclase was activated by the addition of (A) 100 μM forskolin or B 1 μM isoproterenol, and cells were incubated for 20 min in the presence of 3-isobutyl-1-methylxanthine (1 mM); cAMP (per well) was measured by radioimmunoassay in extracts of these cells and was taken as 100% (for absolute values, see text). The simultaneous addition of 5-HT or alniditan caused a concentration-dependent inhibition of the stimulated adenylyl cyclase. cAMP measured in extracts of cells that were incubated with the agonists were expressed as percentage of the cAMP amounts (per well) obtained in the presence of forskolin or isoproterenol only. Sigmoidal inhibition curves were fitted by nonlinear regression analysis, and pIC$_{50}$ values were derived.

TABLE 4
Ratio of K_i values

	[^{3}H]5-HT			[^{3}H]Alniditan
	c5-HT$_{1D}$/ h5-HT$_{1D\alpha}$	c5-HT$_{1D}$/ h5-HT$_{1D\beta}$	h5-HT$_{1D\beta}$/ h5-HT$_{1D\alpha}$	h5-HT$_{1D\beta}$/ h5-HT$_{1D\alpha}$
Alniditan	1.9	0.7	2.8	0.8
Indole derivatives				
5-Carboxamido-tryptamine	0.8	0.4	2.0	2.0
Naratriptan	2.2	1.8	1.3	1.1
5-HT	0.8	0.5	1.6	1.4
5-Methoxytryptamine	1.0	0.3	2.9	1.3
Bufotenine	1.1	0.8	1.5	1.8
Sumatriptan	3.4	0.8	4.3	5.2
N-Methyltryptamine	1.0	0.8	1.2	1.7
Tryptamine	0.8	0.6	1.4	1.1
Dimethyltryptamine	1.5	0.9	1.7	3.3
α-Methylserotonin	0.4	0.4	1.0	0.8
2-Methylserotonin	1.5	0.4	3.4	2.0
CP-93129	0.6	1.8	0.4	0.8
Ergoline derivatives				
LSD	0.5	0.5	1.0	3.1
Metergoline	0.4	0.4	1.0	2.4
Dihydroergotamine	1.7	1.7	1.0	1.0
Yohimbine	0.5	0.3	1.7	2.6
Methysergide	7.3	0.6	11.5	9.6
Tetraline derivative				
8-OH-DPAT	4.3	1.4	1.9	4.3
Putative antagonists				
GR 127935	1.2	1.1	1.1	0.2
Ocaperidone	7.3	0.3	23	30.0
Metitepine	1.0	3.1	0.3	1.1
Risperidone	3.5	0.6	5.6	11.5
Ritanserin	5.3	0.9	6	10.4
Ketanserin	31.3	0.5	67	56

concentration-effect curves are shown in Fig. 7 (derived IC_{50} values: alniditan, 74 nM; 5-HT, 56 nM).

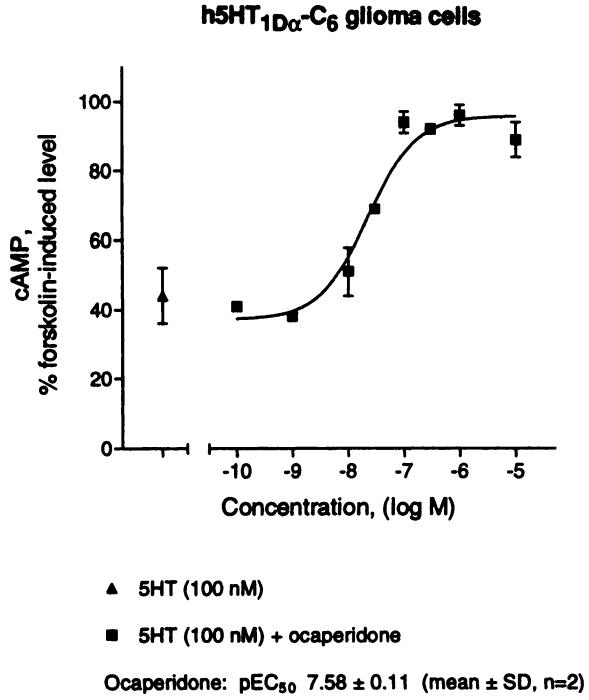


Fig. 5. Antagonism by ocaperidone of inhibition of adenylyl cyclase caused by the agonist action of 5-HT on h5-HT$_{1D\alpha}$ receptors expressed in C6 glioma cells. Cells were grown onto 24-well plates. Adenylyl cyclase was activated by the addition of forskolin (100 μM), and cells were incubated for 20 min in the presence of 3-isobutyl-1-methylxanthine (1 mM); cAMP (per well) was measured in extracts of these cells and was taken as 100%. The simultaneous addition of 5-HT (100 nM) resulted in a decrease in cAMP to 20% of the forskolin-stimulated levels. In the additional presence of ocaperidone, cAMP was increased with increasing concentrations of ocaperidone to reach the 100% level at 10^{-6} M. The sigmoidal curve was fitted by nonlinear regression analysis, and the dose producing 50% reversal of inhibition (EC_{50} value) was derived.

Discussion

Receptor interactions of alniditan. Alniditan was shown to be a very potent 5-HT$_{1D}$ receptor agonist with an almost equally high binding affinity for calf brain 5-HT$_{1D}$, h5-HT$_{1D\alpha}$, and h5-HT$_{1D\beta}$ receptors (K_i = 0.8, 0.4, and 1.1 nM, respectively). The binding affinity of alniditan for these receptor subtypes is 9–15-fold higher than that of sumatriptan and comparable to that of dihydroergotamine (Table 1). The potency difference between alniditan and sumatriptan seems to be somewhat higher *in vitro* than *in vivo*. In studies in dogs *in vivo*, alniditan was a~3-fold more potent than sumatriptan for reducing cardiac arterial blood flow (2). Alniditan fully inhibits stimulated adenylyl cyclase in cells expressing the h5-HT$_{1D\alpha}$ and h5-HT$_{1D\beta}$ receptors, with potencies corresponding to its binding affinity for the receptors (IC_{50} = ~1.0 nM). It shows a slightly higher potency and the same intrinsic activity as the natural agonist 5-HT (Figs. 4 and 6).

For h5-HT$_{1D\alpha}$ receptors expressed in C6-glioma cells, similar inhibitory effects of the agonists were observed after stimulation of adenylyl cyclase with forskolin and via β-receptor activation with isoproterenol (Fig. 4). Therefore, the agonist potency and the inhibition of the adenylyl cyclase after 5-HT$_{1D\alpha}$ receptor stimulation seemed to be equally effective regardless of the method of cyclase activation. For h5-HT$_{1D\beta}$ receptors expressed in L929sA cells, agonistic action of alniditan and 5-HT was investigated in cells with lower (200–300 fmol/mg of protein) and higher (1800–2000 fmol/mg of protein) receptor expression (Fig. 6). In both types of cells, the compounds revealed full agonistic action. However, a somewhat lower potency was observed in the cells with lower receptor expression. This is in accordance with studies on 5-HT$_{1A}$ receptors expressed in cells in which it was shown that the level of receptor expression affects the agonistic properties of compounds so that agonistic action becomes more facilitated with higher receptor expression (40). The ratio of receptor to G protein in the cells will increase with higher receptor expression, resulting in a larger pool of what can be considered "spare receptors." Also, in pharmacological studies it is a known phenomenon that in tissues with spare receptors agonistic effects are facilitated.

Interaction of alniditan with 5-HT$_{1D}$ receptors probably accounts for its potent constrictive action on dog saphenous vein (ED_{50} = 9.1 nM) and on pig basilar artery (ED_{50} = 23 nM).[1] Constriction of cerebral arteries has been attributed to agonistic action on 5-HT$_{1D\beta}$-type receptors (16). Hamel *et al.* (41) also reported the specific detection of 5-HT$_{1D\beta}$ receptor mRNA in human and bovine cerebral arteries, whereas 5-HT$_{1D\alpha}$ receptor mRNA could not be detected. As was suggested for sumatriptan (4, 5), the migraine-abortive properties of alniditan could be related to the cranial vasoconstrictive action. The role of 5-HT$_{1D\alpha}$ receptors is still unclear. Moskowitz (42) suggested that neurogenic effects may be

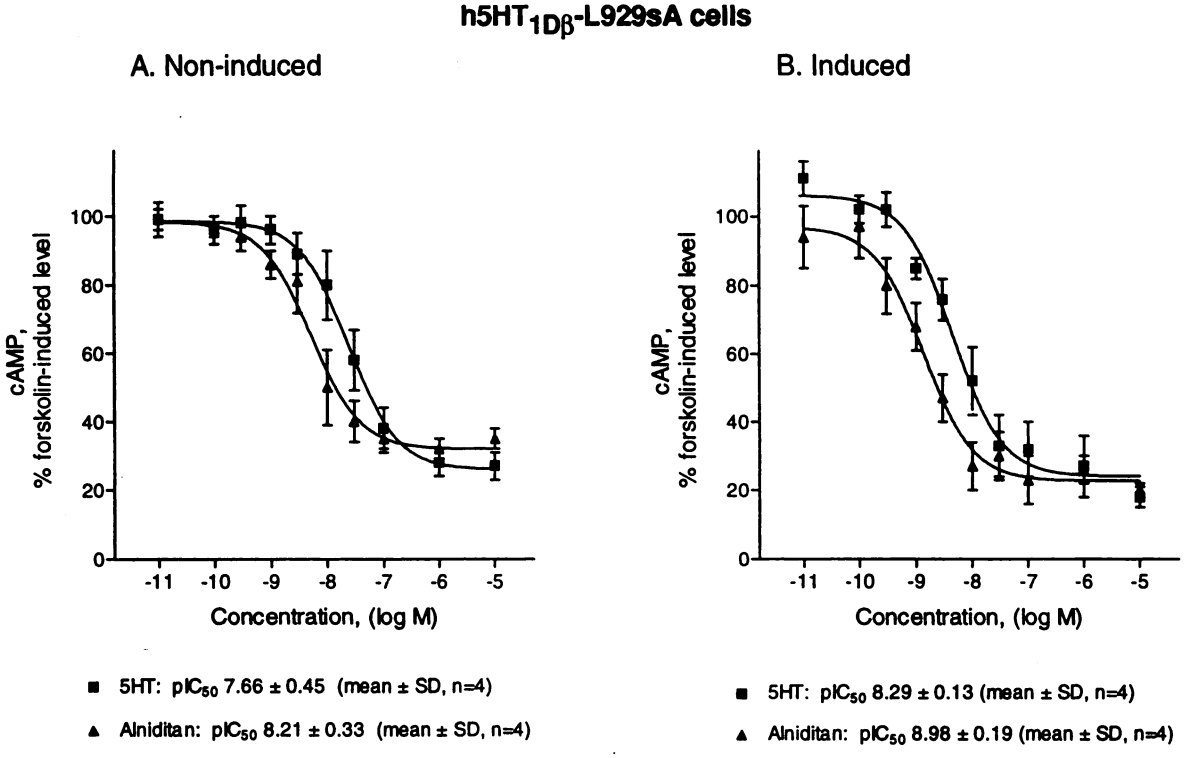


Fig. 6. Signal transduction mediated by h5-HT$_{1D\beta}$ receptors expressed in L929sA cells. A, Measurements in cells that were not induced by murine IFNβ, showing a low expression of h5-HT$_{1D\beta}$ receptors of 200–300 fmol/mg of protein. B, Measurements in cells induced with murine IFNβ expressing the h5-HT$_{1D\beta}$ receptor up to levels of 1800–2000 fmol/mg of protein. Cells were grown onto 24-well plates. Adenylyl cyclase was activated by the addition of forskolin (100 μM), and cells were incubated for 20 min in the presence of 3-isobutyl-1-methylxanthine (1 mM); cAMP (per well) was measured in extracts of cells and was taken as 100% (for absolute values, see text). The simultaneous addition of 5-HT or alniditan caused a concentration-dependent inhibition of the stimulated adenylyl cyclase. cAMP (per well) in extracts of cells that were incubated in the presence of agonists was expressed as percentage of cAMP (per well) obtained in the presence of forskolin only. Sigmoidal curves were fitted by nonlinear regression analysis, and pIC$_{50}$ values were derived.

involved in the antimigraine action of sumatriptan and dihydroergotamine. This was inferred from the observation that 5-HT$_{1D}$-type receptors probably have an inhibitory action on the release of inflammatory peptides, such as substance P and calcitonin gene-related peptide from the trigeminal nerve. Because in the trigeminal ganglia only 5-HT$_{1D\alpha}$ receptor mRNA could be detected and not 5-HT$_{1D\beta}$ receptor mRNA, a specific role of 5-HT$_{1D\alpha}$ receptors in the regulation of inflammatory peptide release can be proposed (43). Experimental data on the action of alniditan in this respect are not yet available.

Alniditan is also an agonist at 5-HT$_{1A}$ receptors (Table 1 and Fig. 7). In signal transduction assays, in which effects on second messenger formation were assayed in intact cells, alniditan was a 70-fold weaker agonist at 5-HT$_{1A}$ than at 5-HT$_{1D}$ receptors (Figs. 4, 6, and 7). In these functional assays, the potency difference was quite larger than in receptor-binding assays using cell membrane preparations, in which a difference in affinity of 4–10-fold was observed (Table 1). So far, 5-HT$_{1A}$ receptor-mediated effects were not apparent in studies using isolated vascular tissues[1] or in *in vivo* studies.[2]

We further found that alniditan and dihydroergotamine have a quite low affinity for h5-HT$_{1E}$ and h5-HT$_{1F}$ receptors (Table 1); the role of these receptor subtypes is still very speculative. Our findings suggest that they are probably not involved in the antimigraine action of alniditan or dihydroergotamine. It is questionable whether the relatively high affinity of sumatriptan for the 5-HT$_{1F}$ receptors contributes to the therapeutic action of this compound. Alniditan has a 50-fold lower binding affinity for rat 5-HT$_{1B}$ than for h5-HT$_{1D\beta}$ or c5-HT$_{1D}$ receptors (Table 1). It showed a much higher species preference than sumatriptan, which in our assays barely distinguished by 2-fold the human and rat receptors. Alniditan revealed moderate affinity for human α_2 receptor subtypes, for haloperidol-sensitive σ sites, and for human D$_4$ receptors. However, its binding affinities for these sites are 20–75-fold lower than those for the 5-HT$_{1D}$ receptors. Therefore, these interactions may not be of relevance for the *in vivo* actions of the compound. The weak interaction of alniditan with the vesicular monoamine transporter (400-fold potency difference with 5-HT$_{1D}$ receptor interaction) also will not be of importance for the *in vivo* action of the compound. However, when investigating the effect of alniditan on regulation of neurotransmitter release *in vitro*, one has to carefully consider the applied concentrations. Indeed, there is a theoretical possibility that at micromolar concentrations, effects on vesicular monoamine stores could occur (44). Such an effect could confound the probable 5-HT$_{1D}$ receptor-mediated inhibitory action on 5-HT and norepinephrine release from nerve terminals.[3]

Dihydroergotamine shows a much broader receptor inter-

[2] F. De Clerck, unpublished observations.

[3] M. Bakker, manuscript in preparation.

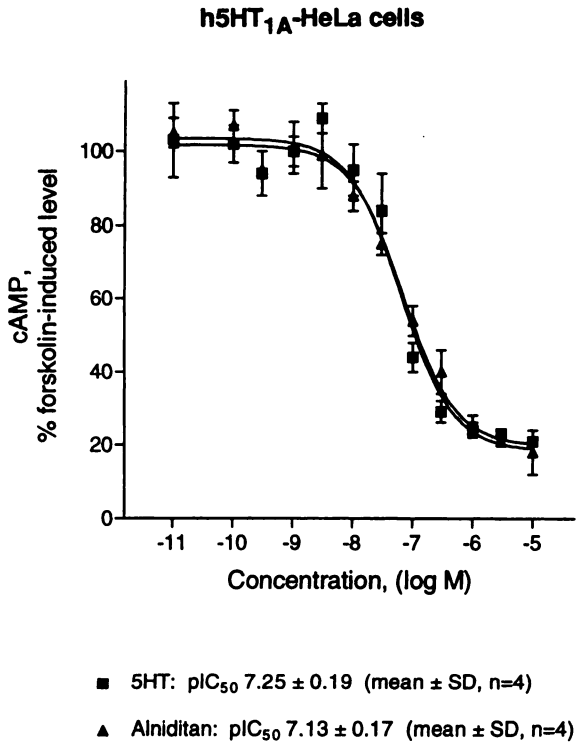


Fig. 7. Signal transduction mediated by h5-HT_{1A} receptors expressed in HeLa cells (Ha6clone). Cells were grown onto 24-well plates. Adenylyl cyclase was activated by the addition of forskolin (100 μM), and cells were incubated for 20 min in the presence of 3-isobutyl-1-methylxanthine (1 mM); cAMP (per well) measured in the extracts of these cells was taken as 100% (for absolute values, see text). The simultaneous addition of 5-HT or alniditan caused a concentration-dependent inhibition of the stimulated adenylyl cyclase. cAMP (per well) in extracts of cells that were incubated in the presence of agonist was expressed as percentage of the cAMP amounts (per well) obtained in the presence of forskolin only. Sigmoidal curves were fitted by nonlinear regression analysis, and pIC_{50} values were derived.

action profile than alniditan and sumatriptan (Table 1). In addition to potently interacting with several 5-HT receptor subtypes, it shows equally high binding affinity for α_1- and α_2-adrenergic receptor subtypes and for D_2, D_3, and D_4 receptors. These multiple receptor interactions of dihydroergotamine, in addition to the central activity of the compound, probably account for various side effects and may add to the dependence liability of the drug.

[^{3}H]Alniditan, a new radioligand for investigating 5-HT_{1D}-type receptors. [^{3}H]Alniditan proved to be an advantageous radioligand for the study of 5-HT_{1D} receptor subtypes in brain tissue (when 8-OH-DPAT was added to occlude 5-HT_{1A} receptors) and in cells expressing h5-$HT_{1D\alpha}$ and h5-$HT_{1D\beta}$ receptors. By virtue of its rapid association and dissociation kinetics, which were observed in three receptor preparations (Fig. 2), binding equilibrium is rapidly reached. Its nanomolar affinity for the 5-HT_{1D}-type receptors (Table 2) permits the use of very low concentrations, so nonspecific binding is kept to a minimum, and an advantageous ratio of specific versus nonspecific binding is obtained, even in calf brain tissue (Fig. 3). However, carrier-free concentrations of [^{3}H]alniditan of ≤10 nM (i.e., 10 × the K_D value) can be used, at which specific binding (defined with a structurally distinct compound; see below) is still clearly detectable, allowing labeling of the receptors at full saturation. The chemical structure of [^{3}H]alniditan is distinct from that of other radioligands that have been used for labeling 5-HT_{1D} receptors;[^{3}H]5-HT, [^{3}H]5-carboxamidotryptamine (45, 46), [^{3}H]sumatriptan (47), [^{3}H]L-694,247 (48), and [^{125}I]GTI (49) are all indole derivatives. Alniditan seems to be a chemically and metabolically stable compound. For defining the specific receptor binding of [^{3}H]alniditan, a structurally different inhibitor (sumatriptan) was used; this provides a better guarantee that the inhibited binding is purely receptor specific and not related to a structural moiety of the radioligand. The receptor-binding profile of alniditan, which was extensively investigated (see Table 1), suggests that [^{3}H]alniditan, when used at nanomolar concentrations will label only 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, and 5-HT_{1A} receptor sites. Because several selective ligands for 5-HT_{1A} receptors exist, these can be easily occluded (e.g., by 8-OH-DPAT, as was used here for binding studies in brain tissue). Under the conditions used, [^{3}H]alniditan apparently labeled a single specific binding site in the membrane preparations as revealed by the regular concentration binding curves and the rigorously linear Scatchard plots (Fig. 3). Most of the indole radioligands have the disadvantage of showing high affinity binding for several 5-HT receptors, among which the 5-HT_{1E} and/or 5-HT_{1F} receptor sites ([^{3}H]5-HT, [^{3}H]5-carboxamidotryptamine, [^{3}H]sumatriptan) or even 5-HT_7 receptors {e.g., [^{3}H]5-carboxamidotryptamine (50)}. For these 5-HT receptor subtypes, no selective agents exist, and therefore they cannot be easily occluded. Therefore, the labeling obtained with the above-mentioned radioligands may be less selective in certain brain regions. [^{125}I]GTI was reported to be a specific ligand for 5-$HT_{1B/1D}$ receptors in various species (51), but unfortunately information on the receptor-binding profile of the nonlabeled compound is missing, particularly with regard to recently cloned and identified 5-HT receptor subtypes and with regard to various neurotransmitter and neuropeptide receptors. Furthermore, the scarce pharmacological and functional information that is available was obtained with serotonin-*O*-carboxymethylglycyl tyrosinamide (i.e., the noniodinated precursor molecule of [^{125}I]GTI) (52). The reported K_D values of [^{125}I]GTI and serotonin-*O*-carboxymethylglycyl tyrosinamide were 1.3 and 28 nM for rat brain 5-HT_{1B} receptors and 6.4 and 67 nM for guinea pig brain 5-HT_{1D} receptors, respectively. This is a relatively low affinity for a ^{125}I-labeled ligand, which is mostly used at subnanomolar concentrations (i.e., far below the K_D value). Receptor saturation is hardly ever shown with this ligand (49, 51, 52).

Ligand-binding pharmacology of 5-HT_{1D} receptor subtypes. We studied 25 compounds for inhibition of [^{3}H]alniditan and [^{3}H]5-HT binding to 5-HT_{1D} receptors in calf substantia nigra and to h5-$HT_{1D\alpha}$ and h5-$HT_{1D\beta}$ receptors in membrane preparations of transfected cells. Putative agonists, except alniditan, were indole or ergoline derivatives. We included also the potent 5-HT_{1A} agonist 8-OH-DPAT, and we tested further putative antagonist with different chemical structures (only GR127935 is an indole). None of the compounds in the list structurally resembled alniditan.

In each of the membrane preparations, the K_i values of the compounds for inhibiting [^{3}H]alniditan and [^{3}H]5-HT binding were in rather good agreement (Table 3). However, some differences are noted between the various groups of compounds. The indole derivatives show 2–4-fold lower apparent K_i values when assayed with [^{3}H]5-HT compared with [^{3}H]alniditan. The ergoline derivatives and putative antago-

nists showed nearly equal apparent K_i values versus both radioligands.

These findings, together with the observation that [^{3}H]alniditan and [^{3}H]5-HT labeled the same number of binding sites in the membrane preparations for c5-HT$_{1D}$, h5-HT$_{1D\alpha}$, and h5-HT$_{1D\beta}$ receptors, respectively, indicate that the two radioligands label the same receptors. However, each of the radioligands, which are of different structural and physicochemical natures, probably show a different competition with compounds of different structural classes and different physicochemical properties. This may be due to differences in surface effects, or it could be that the radioligands and the different classes of compounds interact with partially different areas of the receptors.

The majority of the investigated compounds did not differentiate between h5-HT$_{1D\alpha}$ and h5-HT$_{1D\beta}$ receptors. This is apparent from Table 4, in which we show the ratios of apparent K_i values. Among the putative agonists, only methysergide revealed a 10-fold higher affinity for h5-HT$_{1D\alpha}$ than for h5-HT$_{1D\beta}$ receptors. Among the putative antagonists, ketanserin showed the widest dissociation, with a 60-fold higher affinity for h5-HT$_{1D\alpha}$ than for h5-HT$_{1D\beta}$ receptors, confirming the findings of Zgombick *et al.* (53). Ritanserin, risperidone, and ocaperidone (the latter was shown to be a full 5-HT$_{1D\alpha}$ antagonist in this study; Fig. 5) revealed a difference in affinity of 10–30-fold. However, the compounds listed above, which modestly distinguish between h5-HT$_{1D\alpha}$ and h5-HT$_{1D\beta}$ receptors, have broader pharmacological profiles. They are very potent 5-HT$_{2A}$ antagonists. Ritanserin, in addition, is a potent 5-HT$_{2C}$ antagonist, and risperidone and ocaperidone are potent dopamine D$_2$ antagonists (54). In contrast to the suggestion by Zgombick *et al.* (53), we believe that because of their broad pharmacological profile, these compounds may have only limited value for the study of differential functional effects of 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors *in vivo* or in tissues. Nevertheless, the use of these compounds in this study allowed us to conclude that 5-HT$_{1D}$ receptors labeled by [^{3}H]5-HT and [^{3}H]alniditan in calf substantia nigra are of the 5-HT$_{1D\beta}$-type (see the ratios for K_i values; Table 4). In a similar way, the lower affinity of ketanserin in assays using [^{125}I]GTI in human cortex (55) and in assays using [^{3}H]L-694,247 in pig caudate membranes (48) led to the suggestion that the sites labeled by the radioligands were of the 5-HT$_{1D\beta}$-type. The finding of predominantly 5-HT$_{1D\beta}$-type receptor-binding sites in brain tissue is in agreement with the demonstrated higher abundance of 5-HT$_{1D\beta}$ receptor mRNA in various mammalian brain regions in contrast to a limited occurrence of 5-HT$_{1D\alpha}$ receptor mRNA (56).[4]

In conclusion, this study shows that alniditan is a high affinity full agonist at 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors and a weaker agonist at 5-HT$_{1A}$ receptors. The cranial vasoconstrictive effect of alniditan is probably mediated by 5-HT$_{1D\beta}$ receptors. This property may contribute to the migraine-abortive action of alniditan.

[^{3}H]Alniditan proved to be an advantageous new agonist radioligand for the study of 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors *in vitro*. In calf brain substantia nigra membranes, the receptor sites labeled by [^{3}H]alniditan in the presence of 8-OH-DPAT seemed to be of the 5-HT$_{1D\beta}$-type.

[4] Bonaventure, manuscript in preparation.

Acknowledgments

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References

1. Van Lommen, G., M. De Bruyn, M. Schroven, W. Verschueren, W. Janssens, J. Verrelst, and J. Leysen. The discovery of a series of new non-indole 5HT$_{1D}$-agonists. *Bioorg. Med. Chem. Lett.* **5**:2649–2654 (1995).
2. Van de Water, A., J. D'Aubioul, W. Van Gerven, K. Van Ammel, and F. De Clerck. Selective vasoconstriction by alniditan in the carotid vascular bed of anaesthetized dogs. *Eur. J. Pharmacol.* **299**:127–137 (1996).
3. Goldstein, J., R. Schellens, H.-C. Diener, C. Dahlof, J. Olesen, J. M. Senard, T. Steiner, D. Simard, and I. Vingerhoets. Alniditan, a novel non-indole 5HT$_{1D}$-receptor agonist: a s.c. dose-finding trial, in *Proceedings of the 6th International Headache Research Seminar: Headache Treatment, Trial Methodology and New Drugs*. (1995).
4. Humphrey, P. P. A., and W. Feniuk. Mode of action of the anti-migraine drug sumatriptan. *Trends Pharmacol. Sci.* **12**:444–446 (1991).
5. Saxena, P. R., and M. O. Den Boer. Pharmacology of anti-migraine drugs. *J. Neurol.* **238**:S28–S35 (1991).
6. Heuring, R. E., and S. J. Peroutka. Characterization of a novel ^{3}H-5-hydroxytryptamine binding site subtype in bovine brain membranes. *J. Neurosci.* **7**:894–903 (1987).
7. Waeber, C., M. M. Dietl, D. Hoyer, and J. M. Palacios. 5HT$_1$ receptors in the vertebrate brain: regional distribution examined by autoradiography. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **340**:486–494 (1989).
8. Waeber, C., P. Schoeffter, D. Hoyer, and J. M. Palacios. The serotonin 5-HT$_{1D}$ receptor: a progress review. *Neurochem. Res.* **15**:567–582 (1990).
9. Bruinvels, A. T., B. Landwehrmeyer, A. Probst, J. M. Palacios, and D. Hoyer. A comparative autoradiographic study of 5-HT$_{1D}$ binding sites in human and guinea-pig brain using different radioligands. *Mol. Brain Res.* **21**:19–29 (1994).
10. Hoyer, D., and D. N. Middlemiss. Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol. Sci.* **10**:130–132 (1989).
11. Schoeffter, P., C. Waeber, J. M. Palacios, and D. Hoyer. The 5-hydroxytryptamine 5-HT$_{1D}$-receptor subtype is negatively coupled to adenylate cyclase in calf substantia nigra. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **337**:602–608 (1988).
12. Middlemiss, D. N. Blockade of the central 5-HT autoreceptor by β-adrenoceptor antagonists. *Eur. J. Pharmacol.* **120**:51–56 (1986).
13. Molderings, G. J., K. Werner, J. Likungu, and M. Göthert. Inhibition of noradrenaline release from the sympathetic nerves of the human saphenous vein via presynaptic 5-HT receptors similar to the 5-HT$_{1D}$ subtype. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **342**:371–377 (1990).
14. Perren, M. J., W. Feniuk, and P. P. A. Humphrey. Vascular 5-HT$_1$-like receptors that mediate contraction of the dog isolated saphenous vein and carotid arterial vasoconstriction in anaesthetized dogs are not of the 5-HT$_{1A}$ or 5-HT$_{1D}$ subtype. *Br. J. Pharmacol.* **102**:191–197 (1991).
15. Bax, W. A., D. Van Heuven-Nolsen, E. Bos, M. L. Simoons, and P. R. Saxena. 5-Hydroxytryptamine-induced contractions of the human isolated saphenous vein: involvement of 5-HT$_2$ and 5-HT$_{1D}$-like receptors, and a comparison with grafted veins. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **345**:500–508 (1992).
16. Hamel, E., L. Grégoire, and B. Lau. 5-HT$_1$ receptors mediating contraction in bovine cerebral arteries: a model for human cerebrovascular "5-HT$_{1D\beta}$" receptors. *Eur. J. Pharmacol.* **242**:75–82 (1993).
17. Buzzi, M. G., W. B. Carter, T. Shimizu, H. Heath III, and M. A. Moskowitz. Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. *Neuropharmacology* **30**:1193–1200 (1991).
18. Hartig, P. R., T. A. Branchek, and R. L. Weinshank. A subfamily of 5-HT$_{1D}$ receptor genes. *Trends Pharmacol. Sci.* **13**:152–159 (1992).
19. Hoyer, D., D. E. Clarke, J. R. Fozard, P. R. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena, and P. A. Humphrey. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.* **46**:157–203 (1994).
20. Hamblin, M. W., and M. A. Metcalf. Primary structure and functional characterization of a human 5-HT$_{1D}$-type serotonin receptor. *Mol. Pharmacol.* **40**:143–148 (1991).
21. Jin, H., D. Oksenberg, A. Ashkenazi, S. J. Peroutka, A. M. V. Duncan, R. Rozmahel, Y. Yang, G. Mengod, J. M. Palacios, and B. F. O'Dowd. Characterization of the human 5-hydroxytryptamine$_{1B}$ receptor. *J. Biol. Chem.* **267**:5735–5738 (1992).
22. Levy, F. O., T. Gudermann, E. Perez-Reyes, M. Birnbaumer, A. J. Kaumann, and L. Birnbaumer. Molecular cloning of a human serotonin receptor (S12) with a pharmacological profile resembling that of the 5-HT$_{1D}$ subtype. *J. Biol. Chem.* **267**:7553–7562 (1992).
23. Weinshank, R. L., J. M. Zgombick, M. J. Macchi, T. A. Branchek, and P. R.

Hartig. Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. *Proc. Natl. Acad. Sci. USA* **89**:3630–3634 (1992).
24. Maenhaut, C., J. Van Sande, C. Massart, C. Dinsart, F. Libert, E. Monferini, E. Giraldo, H. Ladinsky, G. Vassart, and J. E. Dumont. The orphan receptor cDNA RDC4 encodes a $5HT_{1D}$ serotonin receptor. *Biochem. Biophys. Res. Commun.* **180**:1460–1498 (1991).
25. Luyten, W. H. M. L., I. Van de Weyer, G. Nobels, P. Van Gompel, W. Gommeren, A. Lesage, and J. E. Leysen. Genomic cloning and heterologous expression of a recombinant guinea pig serotonin $5HT_{1D\alpha}$ receptor. *Abstr. Soc. Neurosci.* **21**:442.3 (1995).
26. Harwood, G., M. Lockyer, H. Giles, and N. Fairweather. Cloning and characterisation of the rabbit 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors. *FEBS Lett.* **377**:73–76 (1995).
27. Hamblin, M. W., R. W. McGuffin, M. A. Metcalf, D. M. Dorsa, and K. M. Merchant. Distinct 5-HT_{1B} and 5-HT_{1D} serotonin receptors in rat: structural and pharmacological comparison of the two cloned receptors. *Mol. Cell. Neurosci.* **3**:578–587 (1992).
28. Adham, N., P. Romanienko, P. Hartig, R. L. Weinshank, and T. Branchek. The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol. Pharmacol.* **41**:1–7 (1992).
29. Metcalf, M. A., R. W. McGuffin, and M. W. Hamblin. Conversion of the human 5-$HT_{1D\beta}$ serotonin receptor to the rat 5-HT_{1B} ligand-binding phenotype by Thr^{355}Asn site directed mutagenesis. *Biochem. Pharmacol.* **44**:1917–1920 (1992).
30. Oksenberg, D., S. A. Marsters, B. F. O'Dowd, H. Jin, S. Havlik, S. J. Peroutka, and A. Ashkenazi. A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT_{1B} receptors. *Nature (Lond.)* **360**:161–163 (1992).
31. Janssen, C. The synthesis of ^{3}H-labelled R 91274 at high specific activity, in *J. R. F. Non-clinical Pharmacokinetics Report*. Janssen Pharmaceutica, Beerse, Belgium, N99382 (1993).
32. Mertens, J., D. Terriere, V. Sipido, W. Gommeren, P. M. F. Janssen, and J. E. Leysen. Radiosynthesis of a new radioiodinated ligand for serotonin-$5HT_2$-receptors, a promising tracer for γ-emission tomography. *J. Labelled Compd. Radiopharm.* **34**:795–806 (1994).
33. Schotte, A., P. F. M. Janssen, W. Gommeren, W. H. M. L. Luyten, P. Van Gompel, A. S. Lesage, K. De Loore, and J. E. Leysen. Risperidone compared with new and reference antipsychotic drugs: *in vitro* and *in vivo* receptor binding. *Psychopharmacology* **124**:57–73 (1996).
34. Vanhoenacker, P., W. Gommeren, W. H. M. L. Luyten, J. E. Leysen, and G. Haegeman. Stable, high-level of human serotonin receptors in L929 cells using an inducible expression system. *Recept. Channels*, in press.
35. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
36. Hug, H., M. Costas, P. Staeheli, M. Aebi, and C. Weissmann. Organization of the murine *Mx* gene and characterization of its interferon- and virus-inducible promoter. *Mol. Cell. Biol.* **8**:3065–3079 (1988).
37. Lleonart, R., D. Näf, H. Browning, and C. Weissmann. A novel, quantitative bioassay for type I interferon using a recombinant indicator cell line. *Biotechnology (N. Y.)* **8**:1263–1267 (1990).
38. Oestreicher, E. G., and G. F. Pinto. A microcomputer program for fitting enzyme inhibition rate equations. *Comput. Biol. Med.* **17**:317–321 (1987).
39. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of the inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
40. Varrault, A., and J. Bockaert. Differential coupling of 5-HT_{1A} receptors occupied by 5HT or 8-OH-DPAT to adenylyl cyclase. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346**:367–374 (1992).
41. Hamel, E., E. Fan, D. Linville, V. Ting, J.-G. Villemure, and L.-S. Chia. Expression of mRNA for the serotonin 5-hydroxytryptamine$_{1D\beta}$ receptor subtype in human and bovine cerebral arteries. *Mol. Pharmacol.* **44**:242–246 (1993).
42. Moskowitz, M. A. Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol. Sci.* **13**:307–311 (1991).
43. Rebeck, W., K. Mayenard, B. Hyman, and M. A. Moskowitz. Selective $5HT_{1D\alpha}$ serotonin receptor gene expression in trigeminal ganglia: implication for antimigraine drug development. *Proc. Natl. Acad. Sci. USA* **91**:3666–3669 (1994).
44. Leysen, J. E., A. Eens, W. Gommeren, P. Van Gompel, J. Wynants, and P. A. J. Janssen. Identification of nonserotonergic [^{3}H]ketanserin binding sites associated with nerve terminals in rat brain and with platelets; relation with release of biogenic amine metabolites induced by ketanserin- and tetrabenazine-like drugs. *J. Pharmacol. Exp. Ther.* **244**:310–321 (1988).
45. Mahle, C. D., H. P. Norwak, R. J. Mattson, S. D. Hurt, and F. D. Yocca. [^{3}H]5-Carboxamidotryptamine labels multiple high affinity $5HT_{1D}$-like sites in guinea pig brain. *Eur. J. Pharmacol.* **205**:323–324 (1991).
46. Norwak, H. P., C. D. Mahle, and F. D. Yocca. [^{3}H]5-Carboxamidotryptamine labels $5HT_{1D}$ binding sites in bovine substantia nigra. *Br. J. Pharmacol.* **109**:1206–1211 (1993).
47. Waeber, C., and M. A. Moskowitz. [^{3}H]Sumatriptan labels both $5HT_{1D}$ and $5HT_{1F}$ receptor binding sites in the guinea pig brain: an autoradiographic study. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352**:263–275 (1995).
48. Heald, A., J. A. Stanton, S.-A. Osborne, D. N. Middlemiss, and M. S. Beer. [^{3}H]L-694,247 labels the $5HT_{1D\beta}$-receptor in pig caudate membranes. *Eur. J. Pharmacol.* **264**:213–216 (1994).
49. Boulenguez, P., L. Segu, J. Chauveau, A. Morel, J. Lanoir, and M. Delaage. Biochemical and pharmacological characterization of serotonin-*O*-carboxymethylglycyl[^{125}I]iodotyrosinamide, a new radioiodinated probe for $5HT_{1B}$ and $5HT_{1D}$ binding sites. *J. Neurochem.* **58**:951–959 (1992).
50. Gustafson, E. L., M. M. Durkin, J. A. Bard, J. Zgombick, and T. A. Branchek. A receptor autoradiographic and *in situ* hybridization analysis of the distribution of the 5-ht_7 receptor in rat brain. *Br. J. Pharmacol.* **117**:657–666 (1996).
51. Bruinvels, A. T., L. Hugues, J. Nozulak, J. Palacios, and D. Hoyer. 5-HT_{1D} binding sites in various species: similar pharmacological profile in dog, monkey, calf, guinea pig and human brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346**:243–248 (1992).
52. Boulenguez, P., J. Chauveau, L. Segu, A. Morel, M. Delaage, and J. Lanoir. Pharmacological characterization of serotonin-*O*-carboxymethyl-glycyl-tyrosinamide, a new selective indolic ligand for 5-hydroxytryptamine (5-HT) 1B and 5-HT_{1D} binding sites. *J. Pharmacol. Exp. Ther.* **259**:1360–1365 (1991).
53. Zgombick, J. M., L. E. Schechter, S. A. Kucharewicz, R. L. Weinshank, and T. A. Branchek. Ketanserin and ritanserin discriminate between recombinant human $5HT_{1D\alpha}$ and $5HT_{1D\beta}$ receptor subtypes. *Eur. J. Pharmacol.* **291**:9–15 (1995).
54. Leysen, J. E., P. M. F. Janssen, W. Gommeren, J. Wynants, P. J. Pauwels, and P. A. J. Janssen. *In vitro* and *in vivo* receptor binding and effect on monoamine turnover in rat brain regions of the novel antipsychotics risperidone and ocaperidone. *Mol. Pharmacol.* **41**:494–508 (1992).
55. Beer, M. S., and D. N. Middlemiss. Serotonin-5-O-carboxymethylglycyl[^{125}I]tyrosinamide labels the $5HT_{1D\beta}$-receptor subtype in human cortex. *Eur. J. Pharmacol.* **242**:195–198 (1993).
56. Bruinvels, A. T., B. Landwehrmeyer, E. L. Gustafson, M. M. Durkin, G. Mengod, T. A. Branchek, D. Hoyer, and J. M. Palacios. Localization of 5-HT_{1B}, 5-$HT_{1D\alpha}$, 5-HT_{1E} and 5-HT_{1F} receptor messenger RNA in rodent and primate brain. *Neuropharmacology* **33**:367–386 (1994).

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