Biochemical Pharmacology, Vol. 51, pp. 1145–1151, 1996.
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ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00028-7

Pharmacological Characterization of the Cloned Human 5-Hydroxytryptamine Transporter

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ABSTRACT. We performed an extensive pharmacological study of the 5-hydroxytryptamine (5-HT) transporter polypeptide cloned from human placenta. Transient expression of this 630 amino acid polypeptide in HeLa cells led to saturable 5-HT uptake activity (K_m = 858 nM). This 5-HT uptake was blocked by selective 5-HT inhibitors, such as citalopram, litoxetine, sertraline, and indalpine, with K_i values in the low nanomolar range, and it exhibited a pharmacological profile similar to that found in rat brain. [^{3}H]Citalopram binding to membrane preparations of the transfected cells occurred to a single class of high-affinity binding sites (K_d = 5.3 nM) and was potently inhibited by selective 5-HT uptake inhibitors. The pharmacological profile of [^{3}H]citalopram binding to these transfected cells showed a good correlation with that of [^{3}H]paroxetine binding to the rat cerebral cortical 5-HT transporter (r = 0.79). These data confirm that the full pharmacological characteristics of the 5-HT transport system are conferred by the expression of the 630 amino acid human placental 5-HT transporter polypeptide. [^{3}H]Citalopram should, therefore, provide a useful probe for more insights at a molecular level into this cloned 5-HT transport system. BIOCHEM PHARMACOL 51;9:1145–1151, 1996.

KEY WORDS. 5-hydroxytryptamine transporter; human placenta; [^{3}H]5-HT uptake; [^{3}H]citalopram binding; fluoxetine; transfection

The Na^+/Cl^- ion-coupled 5-HT† transport system (or 5-HT transporter) mediates the uptake of 5-HT into a variety of cell types (e.g. serotonergic neurones, platelets, mast cells, and endothelial cells), thereby reducing extracellular concentrations of this biogenic amine. Over the past 15 years, a variety of compounds that selectively inhibit this 5-HT transport system (e.g. paroxetine, fluoxetine, citalopram, and litoxetine) have been described [1]. Moreover, clinical studies have revealed the therapeutic efficacy of this class of compounds in the management of depression, and have suggested their potential use as agents for treating eating and obsessive-compulsive disorders [2].

A 5-HT transporter polypeptide cDNA has been isolated from rats [3, 4] and from humans [5]. The deduced amino acid sequences of these 5-HT transporters encode 630 amino acid polypeptides with 12 putative transmembrane spanning domains. More recently, side-by-side comparisons of the pharmacologies of these recombinantly expressed rat and human 5-HT transporters have suggested species differences, notably for many tricyclic antidepressants [6]. Moreover, by using cross-species chimeras of these two cloned transporters, a tricyclic binding site has been located at or near the putative transmembrane domain 12 of these transporters [6].

In the past, [^{3}H]5-HT uptake assays and binding studies with selective radiolabelled 5-HT uptake inhibitors were used to characterize the 5-HT transporter in a variety of tissues. For the present study, we have used these assay systems to perform an extensive pharmacological study of the cloned human placental 5-HT transporter polypeptide transiently expressed in a surrogate HeLa cell line. The properties of the recombinantly expressed transporter polypeptide compared to those of the native 5-HT transporter protein are discussed.

MATERIAL AND METHODS

Material

Plasmid pRcCMV-hSERT containing the human placental 5-HT transporter cDNA was obtained from Dr. R. Blakely, Emory University (Atlanta, GA, U.S.A.). HeLa cells were obtained from ATCC, and [1,2-^{3}H(N)]5-HT and [N-methyl-^{3}H]citalopram from Dupont NEN. Cell culture reagents were from Gibco BRL.

Other compounds and sources are as follows: litoxetine benzoate, imipramine HCl, fluoxetine HCl, citalopram oxalate, nomifensine HCl, desmethylchlorimipramine HCl, fluvoxamine maleate, ondansetron from Synthelabo Recherche (Bagneux, France); sertraline-HCl from Pfizer (Sandwich, U.K.); desipramine HCl and maprotiline HCl

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† *Abbreviations:* 5-HT, 5-hydroxytryptamine; GBR 12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl] piperazine; 8OH-DPAT, 8-hydroxy-dipropylaminotetralin; DMEM, Dulbeco's modified Eagle medium.

Received 20 February 1995; accepted 11 December 1995.

from Ciba-Geigy (Basel, Switzerland); 5-HT creatinine sulphate from Sigma (St. Louis, MO, U.S.A.); cocaine HCl from La Coopération Pharmaceutique Française; indalpine HCl from Pharmuka (Paris); 8-OH-DPAT-HBr and GBR 12909-HCl from Research Biochemicals Inc. (Natick, MA); and ketanserin from Janssen (Beerse, Belgium).

Cell Culture

HeLa cells were grown at 37°C under 5% CO_2 in DMEM containing 5% fetal calf serum and 50 U/mL penicillin-streptomycin.

Transfection

Plasmid DNA was purified using the Qiagen kit. HeLa cells (5.10^4/well in 24-well plates or 2.10^6/10 mm diameter plates) were transfected with the human 5-HT transporter containing plasmid (1 μg/well and 20 μg/plate) by the calcium phosphate method [7].

[^{3}H]5-HT Transport Assay

The transport assays were performed 24 to 72 hr after transfection of cells cultured in 24-well plates using a previously described procedure [8]. Typically, cells were incubated for 15 min at 37°C in the presence of 20 nM [^{3}H]5-HT with or without various concentrations of unlabelled test compounds. Nonspecific [^{3}H]5-HT accumulation was assessed in parallel incubations containing 10 μM fluoxetine.

[^{3}H]Citalopram Binding

The transfected cells were collected 48–72 hr after transfection, pelleted, and either used immediately or stored at −80°C. Cell pellets were resuspended in buffer I (5 mM Tris-HCl pH 7.4, containing 5 mM EDTA), homogenized with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) and centrifuged at 4°C for 20 min at 46,000 g. The supernatant was discarded and the whole operation repeated once, using buffer II (50 mM Tris-HCl pH 7.4, containing 120 mM NaCl and 5 mM KCl). The final membrane pellet was resuspended in buffer II and the protein content determined by the Bradford method [9] using a BioRad (Ivry sur Seine, France) kit.

Membranes (40 μg) were incubated for 1 hr at 25°C in buffer II in the presence of [^{3}H]citalopram (0.8 nM unless otherwise specified), with or without various concentrations of unlabelled inhibitors. Nonspecific binding was assessed in the presence of 10 μM fluoxetine. Free ligand was eliminated by filtration on 0.05% polyethylenimine pretreated GF/B filters. The heat-dried filters were counted in a Packard scintillation counter in the presence of 3 mL of a toluene-based scintillator Packard (Groningen, The Netherlands). Each assay point represents the mean of triplicate determinations. Equilibrium saturation binding data were analysed with the LIGAND program [10] and competition binding data with the ALLFIT program [11] according to one site fit.

RESULTS

[^{3}H]5-HT Transport

Incubation of human 5-HT transporter cDNA-transfected HeLa cells with 20 nM [^{3}H]5-HT produced a time-dependent intracellular accumulation of [^{3}H]5-HT (Fig. 1). The accumulation of this radiolabelled biogenic amine was totally inhibited in the presence of 10 μM fluoxetine (Fig. 1) and did not occur in nontransfected HeLa cells (data not shown). [^{3}H]5-HT uptake into the transfected HeLa cells was concentration-dependent and saturable with respect to this substrate (Fig. 2), yielding the following transport parameters: K_m = 858 ± 173 nM, V_{max} = 37.1 × 10^{-18} ± 14.9 × 10^{-18} mol/cell/min (mean ± SEM of 4 experiments).

Competitive inhibition experiments of [^{3}H]5-HT uptake into the human 5-HT transporter cDNA-transfected HeLa cells were performed using a variety of compounds (Table 1). Compounds that inhibited [^{3}H]5-HT uptake produced monophasic inhibition curves with Hill coefficients close to unity (data not shown). As such, data have been transformed to K_i values to permit affinity comparisons. The selective 5-HT uptake inhibitors (i.e. citalopram, litoxetine, sertraline, and indalpine) potently inhibited [^{3}H]5-HT uptake with K_i values in the low nanomolar range. In contrast, maprotiline (a noradrenaline uptake inhibitor), ketanserin (a 5-HT_2 receptor antagonist), and ondansetron (a 5-HT_3 receptor antagonist) did not inhibit [^{3}H]5-HT uptake (K_i values > 10 μM). In addition, GBR-12909 (a dopamine uptake inhibitor) and 8-OH-DPAT (a 5-HT_{1A} receptor agonist) were at least 100-fold less potent than citalopram or indalpine at inhibiting [^{3}H]5-HT uptake. Cocaine, a nonselective and low-potency monoamine uptake inhibitor, exhibited a K_i value in the low micromolar range.

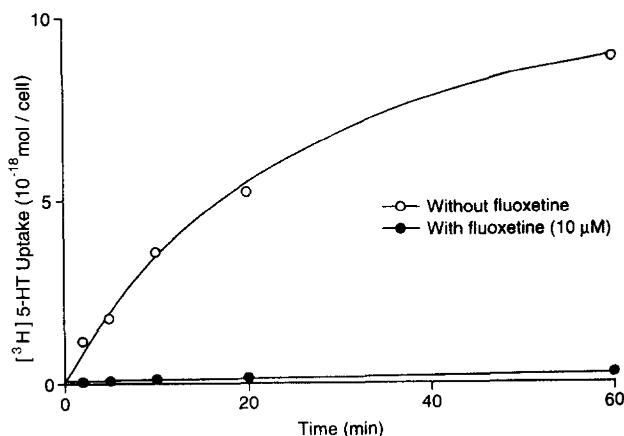

FIG. 1. Time-course of [^{3}H]5-HT accumulation in human 5-HT transporter cDNA-transfected HeLa cells. Transfected cells were assayed for 5-HT uptake using 100 nM [^{3}H]5-HT in the presence and absence of 10 μM fluoxetine. Assays were performed for the indicated time-points and each value represents the mean of triplicate determinations.

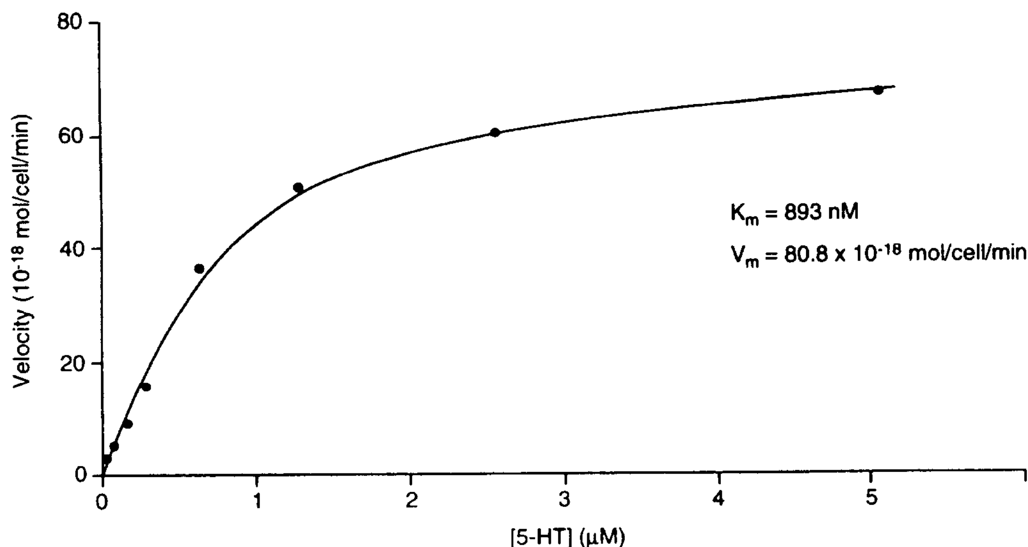

FIG. 2. Kinetics of [^{3}H]5-HT transport into human 5-HT transporter cDNA-transfected HeLa cells. Transfected cells were incubated with various concentrations (35 nM–4.5 μM) of [^{3}H]5-HT and 5-HT transport assays performed as described in Material and Methods. V_{max} and K_m values were determined using the Enzfitter nonlinear regression analysis program. A saturation isotherm of a typical experiment is shown where each value represents the mean of triplicate determinations.

Correlation analysis of the potencies of selected compounds to inhibit [^{3}H]5-HT uptake in human 5-HT transporter cDNA-transfected HeLa cells and rat brain synaptosomes gave a highly significant Spearman's rho correlation coefficient of 0.786 ($P < 0.05$, Fig. 3).

[^{3}H]Citalopram Binding

The binding of [^{3}H]citalopram, a highly selective 5-HT uptake inhibitor, to crude membrane preparations of human 5-HT transporter cDNA-transfected HeLa cells was characterized. [^{3}H]Citalopram binding to these membranes was saturable (Fig. 4) and occurred to a single class of high-affinity binding sites (K_d = 5.3 ± 1.25 nM; B_{max} = 7.33 ± 0.59 pmol/mg prot; mean ± SEM of 3 experiments).

The potencies of a number of selected compounds to inhibit [^{3}H]citalopram binding to membrane preparations of human 5-HT transporter cDNA-transfected HeLa cells were determined under competitive equilibrium conditions

TABLE 1. Inhibition by various compounds of [^{3}H]5-HT uptake into human 5-HT transporter cDNA-transfected HeLa cells

	K_i (nM)	
Compound	**Human clone**	**Rat brain**
Indalpine	2.7 ± 0.3	2.0*
Litoxetine	7.6 ± 1.4	7.0†
Sertraline	8.8 ± 1.9	13.0‡
Citalopram	10.1 ± 2.6	1.5*
Fluoxetine	51.1 ± 1.4	25§
Desipramine	240 ± 64	175*
Cocaine	1446 ± 443	217*
8-OH-DPAT	>1000	–
GBR 12909	>1000	–
Maprotiline	>10,000	–
Ondansetron	>10,000	–
Ketanserin	>10,000	–

Transfected cells were incubated with 20 nM [^{3}H]5-HT in the presence and absence of test compound as described in Materials and Methods. Data represent the mean ± SEM of at least 3 different experiments and are expressed as K_i values [$K_i = IC_{50}/(1 + c/K_m)$].

* [12]; †[13]; ‡[14]; §[15].

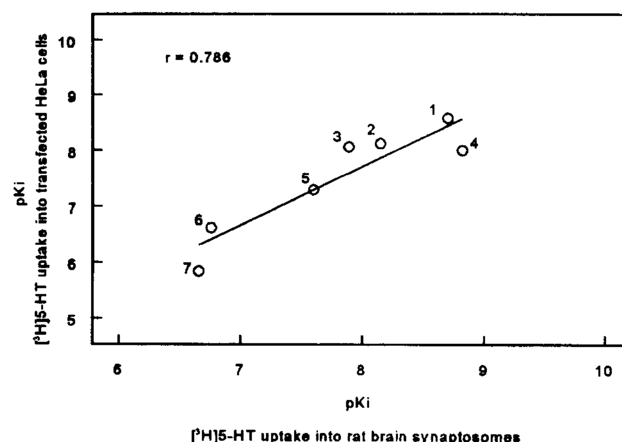

FIG. 3. Correlation analysis of potencies of selected compounds to inhibit [^{3}H]5-HT transport into human 5-HT transporter cDNA-transfected HeLa cells and rat brain synaptosomes. The pK_i values of indalpine (1), litoxetine (2), sertraline (3), citalopram (4), fluoxetine (5), desipramine (6) and cocaine (7) to inhibit [^{3}H]5-HT uptake into the transfected HeLa cells (data from Table 1) and rat brain [12–14] or hypothalamic [15] synaptosomal preparations were compared by the Spearman-rho's rank correlation analysis method.

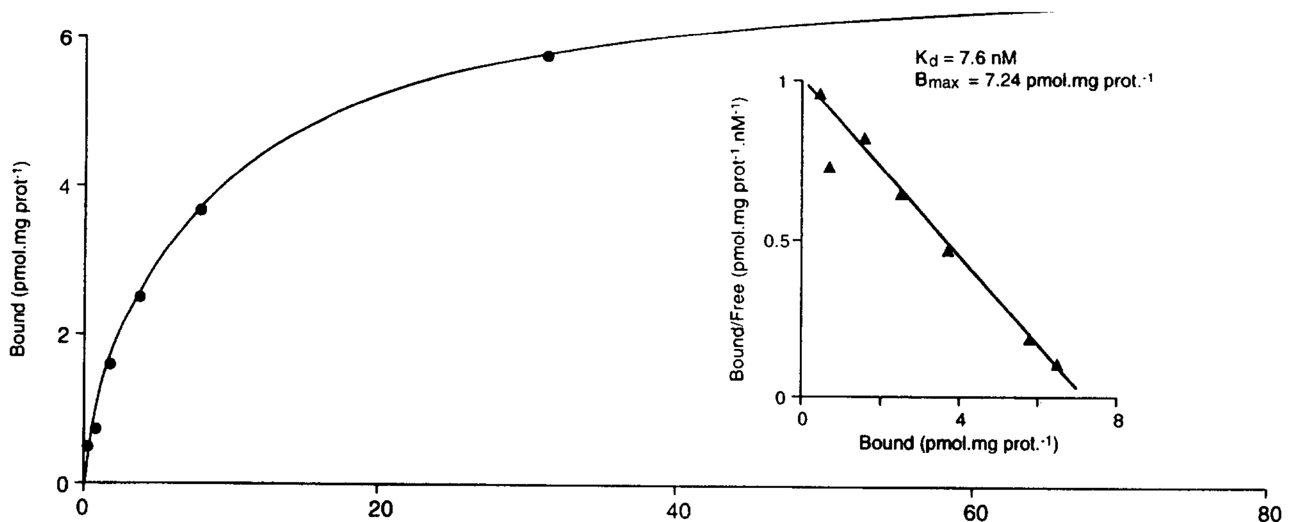

FIG. 4. Equilibrium saturation binding of [^{3}H]citalopram to membrane preparations of human 5-HT transporter cDNA-transfected cells. Membrane preparations of the transfected cells were incubated with 7 concentrations of [^{3}H]citalopram between 0.5 and 65 nM. A saturation isotherm of a typical experiment is shown where each point represents the mean of duplicate determinations. Specific binding was defined using 10 μM fluoxetine. Inset: transformation of specific binding data, according to Scatchard.

(Table 2). Compounds that inhibited [^{3}H]citalopram binding produced monophasic inhibition curves with Hill coefficients close to unity (data not shown). The tricyclic antidepressants (imipramine and desmethylchlorimipramine) as well as the nontricyclic 5-HT uptake inhibitors (indalpine, sertraline, litoxetine, citalopram, fluoxetine, and fluvoxamine) were very potent inhibitors of [^{3}H]citalopram binding to membrane preparations of these transfected cells (Table 2). Other compounds tested, belonging to a variety of different pharmacological classes, either showed much lower potencies or did not inhibit [^{3}H]citalopram binding even at high (10 μM) concentrations (Table 2).

Correlation analysis of the potencies of a number of selected compounds to inhibit [^{3}H]citalopram binding to membrane preparations of human 5-HT transporter cDNA-transfected HeLa cells and [^{3}H]paroxetine binding to rat cerebral cortical membranes produced a highly significant correlation coefficient of 0.79 ($P < 0.01$; Fig. 5).

TABLE 2. Inhibition by various compounds of [^{3}H]citalopram binding to human 5-HT transporter cDNA-transfected HeLa cells

	K_i (nM)	
Compound	**[^{3}H]citalopram binding Human clone**	**[^{3}H]paroxetine binding Rat brain**
Indalpine	3.9 ± 1.1	1.7 ± 0.9*
Sertraline	3.0 ± 0.7	1.24†
Litoxetine	5.8 ± 1.6	3.2‡
Fluoxetine	8.4 ± 2.0	13.5 ± 4.0*
Citalopram	9.6 ± 1.2	1.0 ± 0.1*
Imipramine	10.6 ± 2.1	41.0 ± 8.0*
Desmethylchlorimipramine	10.7 ± 1.4	29.5 ± 3.0*
Fluvoxamine	14.0 ± 0.3	10.0 ± 4.0*
Desipramine	196 ± 30	834.5 ± 40.5*
Cocaine	573 ± 100	>1000*
8-OH-DPAT	1000 ± 210	>1000‡
GBR 12909	2000 ± 240	
Nomifensine	5000 ± 290	2620 ± 560*
Ondansetron	>10,000	>10,000†
Ketanserin	>10,000	
Maprotiline	>10,000	

Crude membrane preparations of transfected cells were incubated with 0.8 nM [^{3}H]citalopram in the presence and absence of test compound as described in Materials and Methods. Data represent the mean ± SEM of 3 different experiments and are expressed as K_i values [$K_i = IC_{50}/(1 + c/K_d)$].

* [18]; †D. Graham and S. Z. Langer, unpublished observations; ‡[13].

DISCUSSION

Uptake assays using radiolabelled 5-HT or binding assays using selective radiolabelled 5-HT uptake inhibitors, such as [^{3}H]paroxetine or [^{3}H]citalopram, have been utilized extensively *in vitro* to characterize the sodium-ion coupled 5-HT transporter in a variety of mammalian cells or tissues (for review, see [1]). Recently, molecular cloning techniques have identified cDNAs from rats and humans that encode polypeptides of 630 amino acids (92% amino acid identity between the rat and human polypeptide) which, upon expression, have been suggested to elicit the pharmacological properties of the 5-HT transporter [3–6]. In the present report, we have chosen to perform a comprehensive pharmacological study of the expression product of one of these 5-HT transporter encoding cDNAs (that isolated from human placenta, [5]). For this purpose, we have carried out [^{3}H]5-HT uptake and [^{3}H]citalopram binding assays on HeLa cells transiently transfected with this 5-HT transporter cDNA and compared the pharmacological

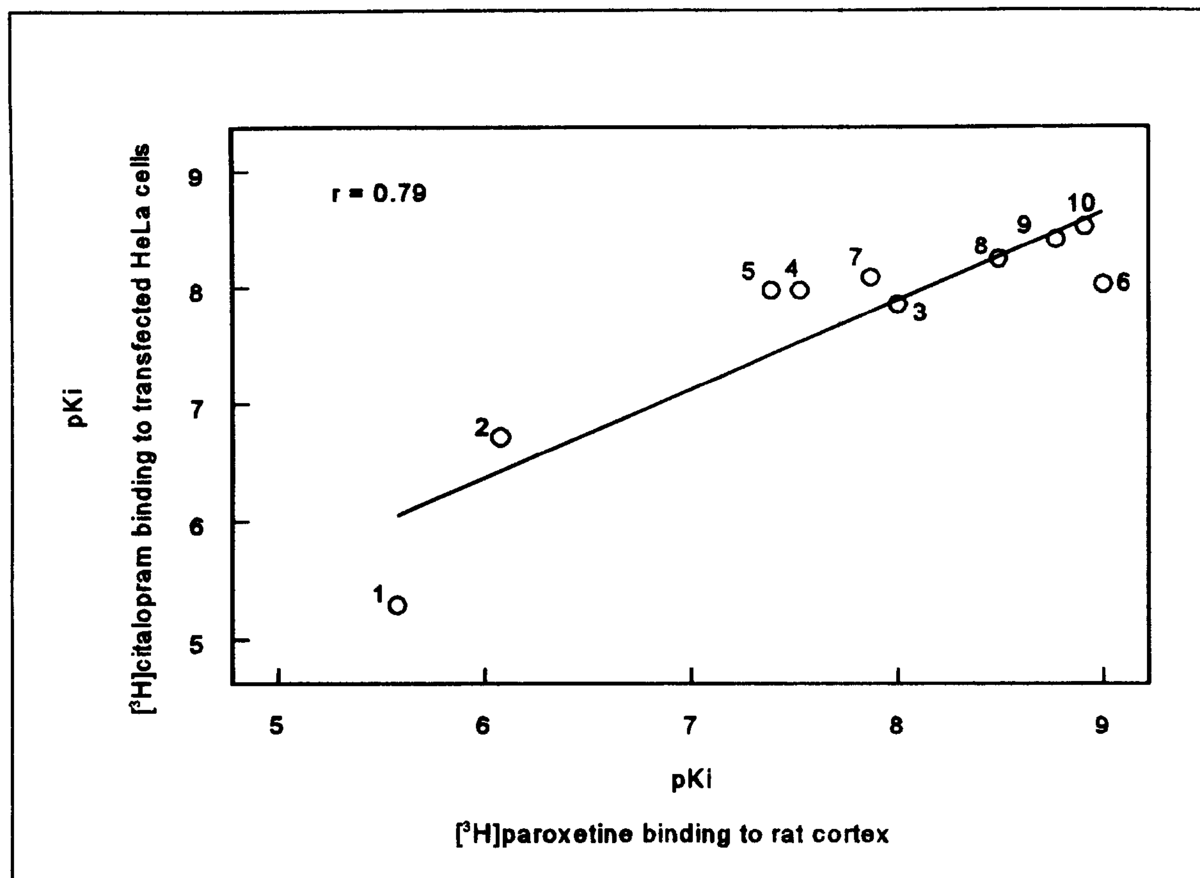

FIG. 5. Correlation between potencies of selected compounds to inhibit [^{3}H]citalopram binding to human 5-HT transporter cDNA-transfected cells and [^{3}H]paroxetine binding to rat cerebral cortex. The pK_i values of compounds to inhibit [^{3}H]citalopram binding to transfected cell membranes (data from Table 2) were compared using the Spearman's rho's rank correlation analysis method to their pK_i values to inhibit [^{3}H]paroxetine binding to rat cerebral cortical membranes [13, 18] and unpublished data from our laboratory 1, nomifensine; 2, desipramine; 3, fluvoxamine; 4, desmethylchlorimipramine; 5, imipramine; 6, citalopram; 7, fluoxetine; 8, litoxetine; 9, indalpine; 10, sertraline.

properties of the resulting expressed polypeptide to that obtained using similar assays performed on native 5-HT transporters.

Transfection of HeLa cells with this human placental 5-HT transporter cDNA conferred the property of 5-HT uptake upon these cells. This transport of 5-HT could be blocked by the addition of the selective 5-HT uptake inhibitor fluoxetine; the fluoxetine-sensitive transport process was saturable with respect to biogenic amine substrate. The K_m value (858 nM) of 5-HT transport was similar not only to that previously reported for this recombinantly expressed transport system [5] but also for sodium-ion coupled 5-HT uptake in human platelets [16] and rat brain slices [17]. The uptake of 5-HT by this recombinantly expressed 5-HT transporter was potently inhibited at low nanomolar concentrations by a number of selective 5-HT uptake inhibitors, including indalpine, sertraline, litoxetine, fluoxetine, and citalopram. In contrast, inhibitors of other monoamine uptake systems (e.g. the noradrenaline uptake inhibitors, desipramine and maprotiline) or of compounds that interact with different 5-HT receptor systems had little or no effect upon 5-HT uptake into the transfected cells. As such, the kinetic and pharmacological properties of this recombinantly expressed 5-HT uptake system indicate that it, indeed, corresponds to those expected of a sodium-ion coupled 5-HT transporter.

With the development of selective serotonin uptake inhibitors, radiolabelled forms of these compounds, such as [^{3}H]paroxetine [18] or [^{3}H]citalopram [19], have been utilized both *in vitro* and *in vivo* to characterize the 5-HT transporter in a number of different mammalian tissues. For example, [^{3}H]paroxetine and [^{3}H]citalopram have recently been used [20] to study the 5-HT transporter in 3 human brain regions (hypothalamus, frontal cortex, and caudate nucleus). In addition, [^{3}H]paroxetine has been used as a molecular probe to show that the site of interaction of the selective 5-HT uptake inhibitors (including citalopram) occur at mutually exclusive binding domains on the 5-HT transporter macromolecule [21]. Given this background, expression of the human placental 5-HT transporter in HeLa cells was also characterized by *in vitro* radioligand binding techniques. We opted to utilize [^{3}H]citalopram as

the preferential ligand for the present study because of the commercial availability of [^{3}H]citalopram at a specific activity nearly three-fold higher than that of [^{3}H]-paroxetine.

In addition to sodium-ion coupled 5-HT uptake, transfection of the HeLa cells with the human placental 5-HT transporter cDNA conferred the property to specifically bind [^{3}H]citalopram upon these cells. This binding of [^{3}H]citalopram occurred to a single class of noninteracting binding sites with a K_d value close to those reported for [^{3}H]citalopram binding to rat cerebral cortical [19] and human brain membrane preparations [20]. The pharmacological profile of [^{3}H]citalopram binding to membrane preparations of human placental 5-HT transporter cDNA transfected HeLa cells revealed that only the selective 5-HT uptake inhibitors potently inhibited the binding of this radioligand at low nanomolar concentrations. In general, the potencies of compounds to inhibit [^{3}H]paroxetine or [^{3}H]citalopram binding to native human serotonin transporter preparations [20,22] were in good agreement with the values that we now report using [^{3}H]citalopram as a probe for these compounds with the cloned human placental 5-HT transporter.

The potencies (K_i values) of drugs to inhibit [^{3}H]citalopram binding to transfected HeLa cell membrane preparations also correlated, in general, with their potencies to inhibit [^{3}H]paroxetine binding to rat cerebral cortical membrane preparations. It should be noted, however, that some tricyclic antidepressants, such as imipramine and chlorimipramine, have been suggested [6, 16] to be more effective inhibitors of the human 5-HT uptake system compared to that of the rat. Comparison of [^{3}H]radioligand binding data revealed that the tricyclics, imipramine, chlorimipramine, and desipramine, exhibited 4-fold, 3-fold, and 4-fold higher potencies, respectively, in inhibiting [^{3}H]citalopram binding to the cloned human 5-HT transporter [our present study], compared to [^{3}H]paroxetine binding to the native 5-HT transporter of rat cerebral cortex [18]. Similarly, a previous study characterizing the native 5-HT transporter of human placental membrane preparations, using [^{3}H]paroxetine as a probe, reported a K_i value for imipramine [22] 10-fold lower than that previously reported for this compound to inhibit [^{3}H]paroxetine binding to the rat brain 5-HT transporter [18]. These radioligand binding data, thus, support previous suggestions [6, 16] of differential affinity of imipramine for the human compared to the rat 5-HT transporter.

Together with [^{3}H]5-HT uptake data ([5] and this study), the properties of the binding of the selective 5-HT uptake inhibitor, [^{3}H]citalopram, to transfected HeLa cell membranes illustrate that the full pharmacological characteristics of the 5-HT transport system are conferred by the expression of the 630 amino acid human placental 5-HT transporter polypeptide. That these pharmacological properties are a feature of the intrinsic properties of the transporter macromolecule and are independent of the expression method, are supported by the findings that two different transfection protocols (calcium phosphate, present study; vaccinia virus [5]) utilized in the same host cell type produced similar findings.

In previous reports, the nonselective cocaine analogue [^{125}I]RTI-55 has been the ligand of choice to detect the cloned 5-HT transporter [8]. Nonetheless, although this radioligand permits the detection of very low amounts of transporter protein because of its higher specific activity, it also has the disadvantage of limited half-life and increased biohazard risk compared to [^{3}H] radioligands. The utilization of [^{3}H]citalopram in this study, therefore, not only provided a more selective but also a more convenient probe to label the cloned 5-HT transporter. Given that [^{3}H]citalopram and 5-HT bind to mutually exclusive domains on this transporter [21], the utilization of this radioligand in future site-directed mutagenesis studies will hopefully lead to more detailed insights into the structure of its substrate binding site, as well as associated co-transport sites for Na^+ and Cl^- ions.

The 5-HT transport system has been reported to be present in a number of different cell types including neurones [23], glial cells [24], platelets [25], mast cells [26], endothelial cells [27], and lymphocytes [28]. This widespread localization could suggest the existence of different 5-HT transporter subtypes, as has been noted for other receptor and transport systems. Nonetheless, molecular cloning data, to date, indicate the existence of only one 5-HT transporter gene [5]. Thus, the cDNAs isolated from rat brain and basophil leukemic cells that encode 630 amino acid 5-HT transporter polypeptides are identical in sequence [3–5]. Similarly, the deduced amino acid sequences from cDNAs isolated from human placenta [5], brain [29], and platelets [30] encode 630 amino acid 5-HT transporter polypeptides with 100% amino acid identity. Recently, however, the presence of different 5-HT transporter mRNA transcripts has been reported both in the brain [31] and in peripheral tissues, such as the placenta and lung [5]. These could be generated by differential initiation or by differential termination and, thereby, represent noncoding variations. Alternatively, these transcripts might result from differential maturation leading to different splice variant coding forms of the 5-HT transporter. Such splice variants if, indeed, present, could have considerable functional significance in certain disease states, given past suggestions of altered serotonergic neurotransmission in affective disorders [32]. One important perspective will, therefore, be to examine these different mRNA transcripts in greater depth. Investigation of the pharmacological properties of any resulting splice variants, with respect to the selective 5-HT uptake inhibitors, might turn out to be important because any differences noted could have considerable therapeutic connotations.

The authors thank Chantal Drouet-Pétré for excellent technical assistance and Sylvianne Jeanpetit for secretarial help during the preparation of this manuscript.

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