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Development of serotonin transporter reuptake inhibition assays using JAR cells

Ann M. Decker* and Bruce E. Blough

Center for Drug Discovery, RTI International, Research Triangle Park, NC 27709, USA

Abstract

Introduction—The development and validation of serotonin transporter reuptake inhibition assays in 96-well format using commercially available human placental choriocarcinoma JAR cells is described.

Methods—JAR cells were first shown to uptake [³H]serotonin in a saturable fashion with a K_M value of 1 μ M as determined by a Michaelis-Menten kinetic analysis. The cells were then utilized to determine the reuptake inhibition potencies of known ligands and the results were compared with results previously generated in the two most commonly used transporter assays (rat brain synaptosomes and transfected HEK293 cells).

Results—Examination of a variety of ligands including selective serotonin reuptake inhibitors, tricyclic antidepressants, piperazine derivatives, and phenyltropane derivatives demonstrated that JAR cells are capable of detecting reuptake inhibition activity of a variety of ligands with potencies that correlate with one or both of the other assays.

Discussion—This study demonstrates a novel pharmacological method of assessing human serotonin transporter reuptake inhibition activity using commercially available JAR cells. Our results show that JAR cells provide an easily available and good alternative to using rat brain tissue and HEK293 cells, with the advantage of studying serotonin transporter reuptake inhibition in a human background.

Keywords

JAR cells; methods; reuptake inhibitors; serotonin transporter

*Corresponding author: Dr. Ann Decker, RTI International, Post Office Box 12194, Research Triangle Park, NC 27709, Telephone: 919-541-1246, Fax: 919-541-8868, adecker@rti.org.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. A.D. obtained funding to collect the data, conceived of and designed the analysis, conducted and verified the analysis, and drafted the manuscript and revisions of the manuscript. B.B. conceived of the analysis, verified the analysis, provided scientific feedback on the manuscript, and approved the final draft.

INTRODUCTION

Plasma membrane biogenic amine transporters (BATs) regulate monoaminergic signaling in the central nervous system (CNS) by transporting previously released monoamine neurotransmitters – dopamine, norepinephrine, and serotonin (DA, NE, and 5-HT transported via DAT, NET, and SERT, respectively) – from the synapse back to the neuronal cytoplasm (Amara & Sonders, 1998; Masson, Sagne, Hamon, & El Mestikawy, 1999). Ligands that interact with BATs are classified as either reuptake inhibitors or substrate-type releasers. Both types of ligands act to elevate extracellular neurotransmitter concentrations, but they act via different mechanisms (Amara & Kuhar, 1993). Reuptake inhibitors bind to transporters and block transporter-mediated reuptake of neurotransmitters, while substrate-type releasers bind to the substrate site on the transporter, are transported inside the neuron, and promote neurotransmitter efflux by transporter-mediated exchange (Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007; Rudnick & Clark, 1993; Sulzer, Sonders, Poulsen, & Galli, 2005). Because disruption of BAT function has been implicated in a number of neurological disease states including Parkinson's disease, schizophrenia, depression, anxiety, and psychostimulant addiction, understanding the mode of interaction between BATs and novel ligands could lead to the development of new therapeutics (Howell & Kimmel, 2008; Iversen, 2000; White, Walline, & Barker, 2005).

The two most widely used methods for measuring transporter activity utilize rat brain synaptosomes and transfected HEK293 cells. The rat brain synaptosome assay, developed by Rothman and colleagues at NIDA, uses functioning synaptosomes prepared from fresh rat brain homogenate; activity is assessed by incubating synaptosomes with compounds and [³H]tracers (Partilla, Baumann, Decker, Blough, & Rothman, 2016). The cell-based assay is performed similarly, but HEK293 cells with over-expressed human transporters are used instead of rat brain tissue (Eshleman, et al., 1999). Although the rat brain synaptosome assay produces reliable results and is widely used in the transporter field – in fact, our laboratory, in collaboration with Rothman and colleagues, has tested the transporter activity of over 1,500 novel compounds using this assay – the data obtained measure rat, not human, transporter activity and the assay requires animals. Conversely, human transporter activity can be measured using transfected HEK293 cells, but this cellular background does not resemble the native neuronal system as well as synaptosomes (Breukel, Besselsen, & Ghijsen, 1997; Thomas & Smart, 2005; Wilhelm, Johnson, Eshleman, & Janowsky, 2008). Recently, we questioned whether human-derived immortalized cell lines that endogenously express human transporter proteins could be used to characterize ligands, and if so, how well the potencies would compare with the other two established model systems. Herein we report the development and validation of SERT reuptake inhibition assays using commercially available human placental choriocarcinoma JAR cells.

Although the plasma membrane SERT endogenously expressed in JAR cells is placental in origin, a compelling body of literature has shown that this SERT is very similar to brain SERT and hence can be used to evaluate neuronal SERT activity. Several studies showed that SERT is expressed in high levels in JAR cells and displays sodium dependent 5-HT uptake that requires chloride ions; brain SERT has the same requirements (Cool, Leibach, Bhalla, Mahesh, & Ganapathy, 1991; Prasad, et al., 1996; S. Ramamoorthy, et al., 1993). Further

evaluation of SERT function in JAR cells showed that uptake of [³H]5-HT occurs exclusively through SERT and is inhibited by tricyclic (imipramine, desipramine) and non-tricyclic (paroxetine, fluoxetine) antidepressants (Cool, et al., 1991; Martel & Keating, 2003; Prasad, et al., 1996). Because of these properties, JAR cells have been used in multiple studies to characterize general hSERT function, including investigating the effects of organic cations on hSERT function and activity (Keating, Lemos, Monteiro, Azevedo, & Martel, 2004), examining transcriptional activation of hSERT (J. D. Ramamoorthy, et al., 1995), and determining how hSERT gene expression is regulated (Jayanthi, Ramamoorthy, Mahesh, Leibach, & Ganapathy, 1994; Kekuda, Leibach, Furesz, Smith, & Ganapathy, 2000). Collectively, these studies demonstrate that JAR cells could be a good model system for testing potencies of novel ligands at the human SERT.

METHODS

Materials

Cell culture reagents and consumables were purchased from Fisher Scientific. Fluoxetine, GBR12935, and GBR12909 were purchased from Tocris Bioscience. Citalopram, desipramine, and indatraline were purchased from Sigma Aldrich. RTI-55 and RTI-229 were gifts from Dr. F. Ivy Carroll at RTI International. [³H]5-HT was purchased from PerkinElmer and was diluted with 10 mM unlabeled 5-HT to reduce the specific activity. Immortalized human choriocarcinoma placental JAR cells were purchased from ATCC (ATCC HTB-144, Manassas VA) and were maintained at 37°C, 5% CO₂ in RPMI-1640 medium supplemented with 2.5 g/L glucose, 2.4 g/L HEPES, 0.11 g/L sodium pyruvate, 100 units penicillin/streptomycin, and 10% fetal bovine serum. All assays were conducted in KRH assay buffer containing 25 mM HEPES (pH 7.4 at 37°C), 125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 2 mM CaCl₂, 1.2 mM MgSO₄, 6 mM glucose, 0.1 mg/mL ascorbic acid, and 0.1 mg/mL pargyline. KRH wash buffer contained 9.6 mM HEPES (pH 7.4 at 4°C) and 154 mM NaCl. Abbreviations used within the methods are as follows: total binding (TB), non-specific binding (NSB), maximal binding (MB; MB = TB – NSB), specific binding (SB; SB = test compound cpm – NSB). All graphical analyses were performed in GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

Protein Determination

Protein concentration was determined using the Bradford assay (Pierce Coomassie Protein Assay Kit, ThermoFisher Scientific, 23200). Bovine Serum Albumin (BSA) was used as the standard, and lysed cells were prepared at three different dilutions (1:2, 1:5, 1:8) in KRH wash buffer. The assay was run in triplicate by following the manufacturer's instructions. Absorbance was measured on a CLARIOstar (BMG Biotech, Cary NC) multi-mode plate reader (595 nm). The blank measurement (KRH wash buffer) was subtracted from all measurements, and a standard curve was prepared by plotting the BSA standard absorbance against the concentration. The curve was fit to a second order polynomial quadratic curve and sample concentrations were interpolated. Experiments were repeated three times to obtain average protein concentrations for JAR cells (50,000 cells).

Uptake Kinetics

On day 1, JAR cells were plated at 50,000 cells/well in culture medium in PerkinElmer CulturPlate-96 white assay microplates pre-coated with PEI (25 µg/mL) and incubated overnight at 37°C, 5% CO₂. On day 2, the culture medium was removed, the cells were gently washed with 100 µL KRH assay buffer, and the wells were filled with 100 µL KRH assay buffer. Assay plates were kept at 37°C, 5% CO₂ for 15 minutes. Serial dilutions of the diluted [³H]5-HT tracer were prepared at 6× the final desired concentration in KRH assay buffer, and 25 µL was added to each well. 25 µL of 0.3% DMSO/KRH (TB) or 25 µL of 5 µM final citalopram (NSB) were added, and the cells were incubated for 60, 45, 30, 15, and 0 min at 37°C, 5% CO₂. At the end of each incubation, the assay buffer was removed and the cells were washed 2× with 200 µL cold KRH wash buffer. Cells were lysed in 25 µL 1% Triton x-100 in KRH wash buffer with shaking. Retained radioactivity was counted in 150 µL Microscint 20 using a TopCount NXT scintillation and luminescence counter. The counts (cpm) were converted to fmol radioactivity/mg protein and MB for each tracer concentration was calculated. The rate of reaction for each radioligand concentration was determined by plotting retained [³H]5-HT against each timepoint for each radioligand concentration. The kinetic constants K_M and V_{MAX} were generated by plotting each reaction rate against the corresponding radioligand concentrations using a Michaelis-Menten enzyme kinetic nonlinear regression.

Reuptake Inhibition Assay

On day 1, cells were plated as described in the uptake kinetics methods. On day 2, the culture medium was removed, the cells were gently washed with 100 µL KRH assay buffer, and the wells were filled with 100 µL KRH assay buffer. The assay plates were kept at 37°C, 5% CO₂ for 15 minutes. A working stock (1.0 µM final, K_M concentration) of the diluted [³H]5-HT tracer was prepared at 6× in KRH assay buffer. Serial dilutions of the test compounds were prepared at 6× the final desired concentration in 0.7% DMSO/KRH assay buffer. The assay was initiated by adding 25 µL of diluted [³H]5-HT tracer, 25 µL of the test compound dilutions, and 25 µL of 0.7% DMSO/KRH (TB) or 25 µL of 5 µM final citalopram (NSB). The assay was terminated after 60 min at 37°C, 5% CO₂ by removing the assay buffer and washing the cells 2× with 200 µL cold KRH wash buffer. Cells were lysed in 25 µL 1% Triton x-100 in KRH wash buffer with shaking. Retained radioactivity was counted in 150 µL Microscint 20 using a TopCount NXT scintillation and luminescence counter. The counts (cpm) were converted to fmol radioactivity/mg protein and MB and SB were calculated. SB was converted to % inhibition with the equation $SB = (1 - (SB/MB)) \times 100$. Percent inhibition values were plotted against the log of compound concentration. The data were fit to a three-parameter logistic curve to generate IC₅₀ values.

RESULTS

Uptake kinetics

Uptake kinetics were first evaluated in JAR cells to determine how well the cells could take up [³H]5-HT in a saturable fashion. This analysis was initially performed in 24-well format as a proof of concept but was then successfully transitioned to 96-well format to allow for a higher-throughput assay. Uptake kinetic experiments in 96-well format were performed by

incubating the cells (51.8 µg of protein/well) with a range of radioligand concentrations (5 µM – 10 nM) for multiple time points (60, 45, 30, 15, 0 min) at 37°C. A Michaelis-Menten kinetic analysis determined that [³H]5-HT uptake was saturable with $K_M = 1.0 \pm 0.2$ µM and $V_{MAX} = 943 \pm 72$ fmol radioactivity/mg protein/min (Figure 1). Experiments conducted at room temperature resulted in a linear uptake that was not saturable (data not shown).

Reuptake inhibition activity of previously characterized SERT ligands

Eight ligands including the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and citalopram, the tricyclic antidepressant desipramine, the piperazine derivatives GBR12935 and GBR12909, the phenyltropane derivatives RTI-55 and RTI-229, and the non-selective monoamine transporter inhibitor indatraline were evaluated for activity in the JAR cells (Table 1 and Figure 2). These compounds are well-characterized transporter ligands and have been tested in rat brain synaptosomes and hSERT-HEK293 cells, allowing for a loose comparison between species (rat and human) and assay system (native tissue, endogenous transporter, and over-expressed transporter).

The results with the SSRI fluoxetine compare well across all three assays, with IC_{50} values of 9.58 nM, 7.3 nM, and 15.8 nM in the rat brain synaptosomes, hSERT-HEK293 cells, and JAR cells, respectively. The SSRI citalopram results do not compare as well as fluoxetine but are still in close agreement. In rat brain synaptosomes and hSERT-HEK293 cells, the IC_{50} values are 2.68 nM and 3.5 nM, respectively, but the JAR cells produce an IC_{50} of 17.7 nM. Although this value is 7-fold and 5-fold less potent compared to the rat brain synaptosomes and hSERT-HEK293 cells, respectively, citalopram still registers as an active SERT reuptake inhibitor in the JAR cells. The tricyclic antidepressant desipramine had an IC_{50} value of 123 nM in JAR cells, which is 3-fold more potent than rat brain synaptosomes ($IC_{50} = 390$ nM) and 2-fold less potent compared to the hSERT-HEK293 cells ($IC_{50} = 64$ nM).

The piperazine derivative GBR12935 results compare well between the JAR cells ($IC_{50} > 10$ µM) and the hSERT-HEK293 cells ($IC_{50} = 6.8$ µM), but both do not compare well to the rat brain synaptosomes ($IC_{50} = 289$ nM). The GBR12909 results do not compare well between methods, as the compound is inactive in hSERT-HEK293 cells, moderately potent in JAR cells ($IC_{50} = 2.8$ µM), and highly potent in rat brain synaptosomes ($IC_{50} = 73.2$ nM).

The phenyltropane RTI-55 was very potent in the rat brain synaptosomes, hSERT-HEK293 cells, and JAR cells with IC_{50} values of 1.11 nM, 0.51 nM, and 1.31 nM, respectively. Interestingly, RTI-229 had similar potencies in the JAR cells ($IC_{50} = 589$ nM) and rat brain synaptosomes ($IC_{50} = 404$ nM) but was inactive (> 10 µM) in the hSERT-HEK293 cells. A similar type of activity profile was observed with the non-selective monoamine transporter inhibitor indatraline. In rat brain synaptosomes and JAR cells, this compound had IC_{50} values of 3.46 nM and 7.87 nM, respectively, while it was 87-fold less potent in hSERT-HEK293 cells ($IC_{50} = 648$ nM).

DISCUSSION

Our results show that JAR cells are capable of detecting the SERT reuptake inhibition activity of a variety of ligands with potencies that compare well with the other established transporter assays. Our results demonstrate that these cells can serve as an alternative method for detecting in vitro transporter activity, especially for those scientists in the transporter field who do not have access to fresh rat brain tissue or stable hSERT-HEK293 cells. A few previous reports in the literature have also used JAR cells to study reuptake inhibition activity, but none have examined the variety of ligands presented herein. Two of these reports used JAR cells to study SERT function and tested the reuptake inhibition activity of antidepressants, but at a single concentration, as a way to show the specificity of [³H]5-HT uptake through SERT (Cool, et al., 1991; Prasad, et al., 1996). One report determined reuptake inhibition potencies of desipramine (IC₅₀ = 169 nM) and fluoxetine (IC₅₀ = 46 nM) using JAR cells as part of a study examining uptake specificity of the SERT expressed in JAR cells (Martel & Keating, 2003). These results compare very well with the results obtained by our group, as desipramine had an IC₅₀ = 123 nM and fluoxetine had an IC₅₀ = 16 nM, 1.4-fold and 2.9-fold, respectively, more potent than the previously reported values, thus further validating the use of JAR cells as a model system to measure SERT reuptake inhibition.

While the current studies have focused on reuptake inhibition activity at SERT, these methods could be extended to cells endogenously expressing the DAT and NET proteins. For example, a number of studies have shown that human-derived immortalized neuroblastoma SH-SY5Y cells are a good human dopaminergic neuronal model system because they express functional hDAT proteins that uptake DA in a saturable, DAT-specific manner and have the ability to transport and synthesize DA (Jiang, Jiang, & Feng, 2004; Presgraves, Ahmed, Borwege, & Joyce, 2004; Watabe & Nakaki, 2008). Further, the SH-SY5Y cell line was determined to be a good noradrenergic model system as well due to the expression of hNET proteins that are capable of uptaking [¹²⁵I]mIBG, a compound known to be incorporated into neuroblastoma cells by NET, in a saturable hNET-specific fashion (Iavarone, Lasorella, Servidei, Riccardi, & Mastrangelo, 1993; Seitz, et al., 2000). These studies lend support to the notion of using human-derived immortalized cells to characterize DAT and NET reuptake inhibition activity.

In conclusion, this study demonstrates a novel pharmacological method of assessing hSERT reuptake inhibition activity using placental choriocarcinoma JAR cells that endogenously express high levels of hSERT. The data presented herein show that JAR cells are capable of detecting hSERT reuptake inhibition activity and thus can serve as a good alternative to using rat brain synaptosomes and hSERT-HEK293 cells. Further investigation of JAR cells and other human cells endogenously expressing hBATs will provide important information on additional applications of these unique model systems in the transporter field.

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Abbreviations

5-HT	serotonin
BAT	plasma membrane biogenic amine transporter
DA	dopamine
DAT	dopamine transporter
NE	norepinephrine
NET	norepinephrine transporter
SERT	serotonin transporter
SSRI	selective serotonin reuptake inhibitor

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Figure 1.

Michaelis-Menten kinetics of [³H]5-HT uptake by adherent JAR cells. Experiments were carried out at 37°C with multiple incubation times (60, 45, 30, 15, and 0 min) in 96-well plates (51.8 µg of protein/well) as described in the methods. All data shown are mean ± S.E.M. of N=3 conducted with duplicate determinations. JAR cells uptake [³H]5-HT with $K_M = 1.0 \pm 0.2 \mu\text{M}$ and $V_{MAX} = 943 \pm 72$ fmol radioactivity/mg protein/min. (A) Velocity of [³H]5-HT uptake as a function of [³H]5-HT concentration. (B) Plot of specific bound radioactivity (fmol radioactivity/mg protein) as a function of time to determine reaction rate for the Michaelis-Menten plot.

Figure 2.

Reuptake inhibition activity at the hSERT endogenously expressed in JAR cells.

Experiments were carried out with 1.0 μM final $[^3\text{H}]5\text{-HT}$ at 37°C for 60 minutes in 96-well plates (51.8 μg of protein/well) as described in the methods. All data shown are expressed as % inhibition and each data point is the mean $\pm$ S.E.M. of at least N=3 conducted with duplicate determinations. (A) Activity of the selective serotonin reuptake inhibitors fluoxetine (●) and citalopram (○). (B) Activity of the tricyclic antidepressant desipramine (■) and the non-selective monoamine transporter inhibitor indatraline (□). (C) Activity of the piperazine derivatives GBR12935 (◆) and GBR12909 (◇). (D) Activity of the phenyltropane derivatives RTI-55 (▲) and RTI-229 (▼).

Table 1

Comparison of reuptake inhibition activity (IC₅₀, nM) at SERT in JAR cells, rat brain synaptosomes, and transfected HEK293 cells.

Compound	hSERT, JAR cells (nM) ^a	rSERT, synaptosomes (nM)	hSERT-HEK293 cells (nM)
<i>Selective Serotonin Reuptake Inhibitors</i>			
Fluoxetine	15.8 ± 1.9	9.58 ± 0.88 ^b	7.3 ± 2.9 ^c
Citalopram	17.7 ± 4.2	2.40 ± 0.09 ^d (2.68)	3.5 ± 1.9 ^e
<i>Tricyclic antidepressant</i>			
Desipramine	123 ± 7	350 ± 13 ^d (390)	64 ± 17 ^c
<i>Piperazine derivatives</i>			
GBR12935	>10,000	289 ± 29 ^b	6,800 ± 3,400 ^c
GBR12909	2,770 ± 590	73.2 ± 1.5 ^b	>10,000 ^f
<i>Phenyltropane derivatives</i>			
RTI-55	1.31 ± 0.1	1.00 ± 0.03 ^d (1.11)	0.51 ± 0.14 ^c
RTI-229	589 ± 42	362 ± 13 ^d (404)	>10,000 ^f
<i>Non-selective monoamine transporter inhibitor</i>			
Indatraline	7.87 ± 1.3	3.10 ± 0.09 ^d (3.46)	648 ± 61 ^f

^aValues are mean ± S.E.M. of at least three independent experiments conducted in duplicate as described in the methods.

^bData reported as mean ± S.D. in (R. B. Rothman, et al., 1993).

^cData reported as mean ± S.E.M. in (Eshleman, et al., 1999).

^dData reported as mean ± S.D. in (Richard B. Rothman & Baumann, 2003) as K_i values which were converted to IC₅₀ values shown in parentheses using the Cheng-Prusoff equation $K_i = IC_{50} / (1 + L/K_M)$ (Cheng & Prusoff, 1973).

^eData reported as mean ± S.D. in (Jorgensen, Nielsen, Peters, & Dyhring, 2008).

^fUnpublished results generated by our group. Data are mean ± S.E.M. of at least three independent experiments conducted in duplicate.