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Development of norepinephrine transporter reuptake inhibition assays using SK-N-BE(2)C cells

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Abstract

This report describes efforts to develop and validate novel norepinephrine transporter reuptake inhibition assays using human neuroblastoma SK-N-BE(2)C cells in 24-well format. Before conducting the assays, the SK-N-BE(2)C cells were first evaluated for their ability to uptake [^{3}H]norepinephrine and were shown to have a saturable uptake with a K_M value of 416 nM. Using this determined K_M value, reuptake inhibition assays were then conducted with a variety of ligands including antidepressants, as well as piperazine and phenyltropane derivatives. The results obtained with the SK-N-BE(2)C cells indicate that this model system can detect a range of ligand potencies, which compare well with other established transporter assays. Our data suggest that SK-N-BE(2)C cells have potential utility to serve as another model system to detect norepinephrine reuptake inhibition activity.

Keyword: Neuroscience

1. Introduction

Plasma membrane biogenic amine transporters (BATs) regulate monoamine neurotransmitter concentrations in the Central Nervous System (CNS) by transporting previously released monoamine neurotransmitters – dopamine, norepinephrine, and

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serotonin (DA, NE, and 5-HT, respectively) – from the synaptic cleft to the neuronal cytoplasm [1]. BATs play a major role in a plethora of physiological conditions including addiction, depression, anxiety, schizophrenia, and Parkinson's disease, and thus are the targets of many therapeutics and synthetic ligands [2]. Both types of transporter ligands (reuptake inhibitors and substrate-type releasers) elevate extracellular neurotransmitter concentrations, but they act by different mechanisms [3]. 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 [4, 5, 6].

Currently, two in vitro assays are widely used to measure transporter ligand activity using either rat brain synaptosomes or transfected HEK293 cells. In the rat brain synaptosome assay, freshly prepared functioning synaptosomes are incubated with test ligands and [^{3}H]tracers to measure activity at rodent transporters [7]. The cell-based assay is performed similarly, except that HEK293 cells with over-expressed human transporters are used instead of synaptosomes [8]. Recently, we questioned whether it would be possible to develop a new cell-based in vitro assay that measured endogenous human transporter activity in a human derived cellular background, similar to the endogenous rodent transporter activity measured in synaptosomes. In this regard, we developed and validated an assay that measures endogenous serotonin transporter (SERT) reuptake inhibition activity in human derived choriocarcinoma JAR cells [9]. In this study, we showed that JAR cells were capable of detecting reuptake inhibition activity of known ligands with potencies that compared well with the rat brain synaptosomes and hSERT-HEK293 cells. Because of the positive results we obtained with JAR cells, we set out to develop a similar assay to measure norepinephrine transporter (NET) reuptake inhibition activity using human neuroblastoma SK-N-BE(2)C cells.

SK-N-BE(2)C cells were chosen to test NET activity because they have essential noradrenergic phenotypes. These cells synthesize norepinephrine, uptake norepinephrine in a specific manner, contain neurofilament proteins, and express biosynthetic enzymes such as dopamine β-hydroxylase [10, 11]. Because of these noradrenergic phenotypes, multiple studies have been conducted that characterize hNET function and regulation using these cells. For example, SK-N-BE(2)C cells have been used to examine the relationship between stress and hNET up-regulation [12], the role of transcription factors in the noradrenergic system (protein levels, NE uptake), [13] and the regulation of hNET gene expression [14]. Due to their high level of hNET expression, SK-N-BE(2)C cells have also been used to evaluate analogues of [^{125}I]MIBG, a radiolabeled norepinephrine analog and NET substrate used clinically to image neuroendocrine tumors [15, 16]. All of these published reports collectively indicate that SK-N-BE(2)C cells could serve as a good model system to test reuptake inhibitors.

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2. Materials and methods

2.1. 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. [^{3}H]NE was purchased from PerkinElmer and was diluted with 10 mM unlabeled NE to reduce the specific activity. Immortalized human neuroblastoma SK-N-BE(2)C cells were purchased from ATCC (CRL-2268, Manassas VA) and were maintained at 37 °C, 5% CO_2 in 1:1 Ham's F12:MEM (containing 1 mM sodium pyruvate and 1X non-essential amino acids) supplemented with 100 units each of Penicillin/Streptomycin (P/S) and 10% fetal bovine serum (FBS). All assays were conducted in KRH assay buffer containing 25 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 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: 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).

2.2. 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 SK-N-BE(2)C cells (200,000 cells).

2.3. Uptake kinetics

On day 1, SK-N-BE(2)C cells were plated at 200,000 cells/well in 24-well polystyrene clear microplates pre-coated with rat tail type I collagen (86.9 μg/mL) and incubated overnight at 37 °C, 5% CO_2. On day 2, culture medium was removed, the cells were gently washed with 200 μL KRH assay buffer, and wells were filled with 150 μL KRH assay buffer. Assay plates were kept at RT for 15 minutes. Serial dilutions

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of the diluted [^{3}H]NE tracer were prepared at 8x the final desired concentration in KRH assay buffer and 25 μL was added to each well. 25 μL of 0.4% DMSO/KRH (TB) or 25 μL of 5 μM final desipramine (NSB) were added and the cells were incubated for 1 hr, 30 min, 15 min, and 0 min at RT. At the end of each incubation, the assay buffer was removed and the cells were washed 2 times with 300 μL cold KRH wash buffer. Cells were lysed in 300 μL 1% Triton x-100 in KRH wash buffer with shaking for 60 min. Contents of each well were transferred to 7 mL scintillation vials and 5 mL Ultima Gold scintillation cocktail was added. Retained radioactivity was counted by a Packard Tri-Carb liquid scintillation counter. The counts (cpm) were converted to fmol radioactivity/mg protein and MB for each tracer concentration was calculated. The data were analyzed using a Michaelis-Menten enzyme kinetic nonlinear regression to determine V_{MAX} and K_M.

2.4. Reuptake inhibition assay − SK-N-BE(2)C cells

On day 1, cells were plated as indicated in the uptake kinetics methods. A working stock (416 nM final, K_M concentration) of the diluted [^{3}H]NE tracer was prepared at 8x in KRH assay buffer. Serial dilutions of the test compounds were prepared at 8x the final desired concentration in 0.7% DMSO/KRH assay buffer. The assay was initiated by adding 25 μL of the working [^{3}H]NE tracer, 25 μL of the test compound dilutions, and 25 μL of 0.7% DMSO/KRH (TB) or 25 μL of 5 μM final desipramine (NSB). The assay was terminated after 105 min at RT by removing the assay buffer and washing the cells 2 times with 300 μL cold KRH wash buffer. Cells were lysed in 300 μL 1% Triton x-100 in KRH wash buffer with shaking for 60 min. Contents of each well were transferred to 7 mL scintillation vials and 5 mL Ultima Gold scintillation cocktail was added. Retained radioactivity was counted by a Packard Tri-Carb liquid scintillation 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)) x 100. Percent inhibition values were plotted against the log of compound concentration. Data were fit to a three-parameter logistic curve to generate IC_{50} values.

2.5. Reuptake inhibition assay − hNET-HEK293 cells

On day 1, cells were plated at 200,000 cells/well in 24-well polystyrene plates precoated with PEI (25 μg/mL) and incubated overnight at 37 °C, 5% CO_2. On day 2, culture medium was removed, the cells were gently washed with 200 μL KRH assay buffer, and wells were filled with 150 μL KRH assay buffer. A working stock (20 nM final) of [^{3}H]NE tracer was prepared at 8x the desired final concentration in KRH assay buffer. Serial dilutions of the test compounds were prepared at 8x the final desired concentration in 0.7% DMSO/KRH assay buffer. The cells were incubated with 25 μL of the test compound dilutions, 25 μL of 0.7% DMSO/KRH (TB), or

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25 μL of 5 μM final indatraline (NSB) for 10 min at RT. The assay was initiated by adding 25 μL of diluted [^{3}H]NE tracer and the cells were incubated for 10 min at RT. The assay was terminated by removing the assay buffer and washing the cells 2x with 300 μL cold KRH wash buffer. Cells were lysed in 300 μL 1% Triton x-100 in KRH wash buffer with shaking. Retained radioactivity was counted in 5 mL Ultima Gold scintillation cocktail using a Liquid Scintillation Counter and data were analyzed as described in the Methods 2.4 section.

3. Results and discussion

3.1. Uptake kinetics

Before conducting the reuptake inhibition assays, SK-N-BE(2)C cells were first examined for their ability to uptake [^{3}H]NE in a saturable fashion. Cells (163 μg of protein/well) were incubated with a range of radioligand concentrations (5 μM−10 nM) for multiple time points (60, 30, 15, 0 min) at RT in 24-well plates. A Michaelis-Menten kinetic analysis determined that [^{3}H]NE uptake was saturable with $K_M = 416 \pm 53$ nM and $V_{MAX} = 195 \pm 13$ fmol radioactivity/mg protein/min (Fig. 1). Efforts to transition the assay to 96-well format for a higher throughput assay resulted in no specific binding, likely due to the lower protein concentration in the wells. Further, experiments conducted at 37 °C resulted in a linear uptake that was not saturable (data not shown).

3.2. Reuptake inhibition activity of previously characterized ligands

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 reuptake inhibition activity in the SK-N-BE(2)C cells (Table 1 and Fig. 2).

The fluoxetine potency in SK-N-BE(2)C cells compares fairly well with the other two methods as the compound has an IC_{50} value of 5 μM in SK-N-BE(2)C cells, which is 6.8-fold and 4.9-fold less potent than rat brain synaptosomes (IC_{50} = 742 nM) and hNET-HEK293 cells (IC_{50} = 1,020 nM), respectively. The other SSRI included in this study, citalopram, has $IC_{50} > 10$ μM in both SK-N-BE(2)C and hNET-HEK293 cells, but has an IC_{50} value of 4,671 nM in rat brain synaptosomes. The tricyclic antidepressant desipramine has an IC_{50} value of 23.1 nM in SK-N-BE(2)C cells, which is 2.6-fold less potent than rat brain synaptosomes (IC_{50} = 8.97 nM) and 5.5-fold less potent than hNET-HEK293 cells (IC_{50} = 4.2 nM). Overall, for these three antidepressants, it appears that the SK-N-BE(2)C cells provide potencies that compare well with the other two established assays. Further,

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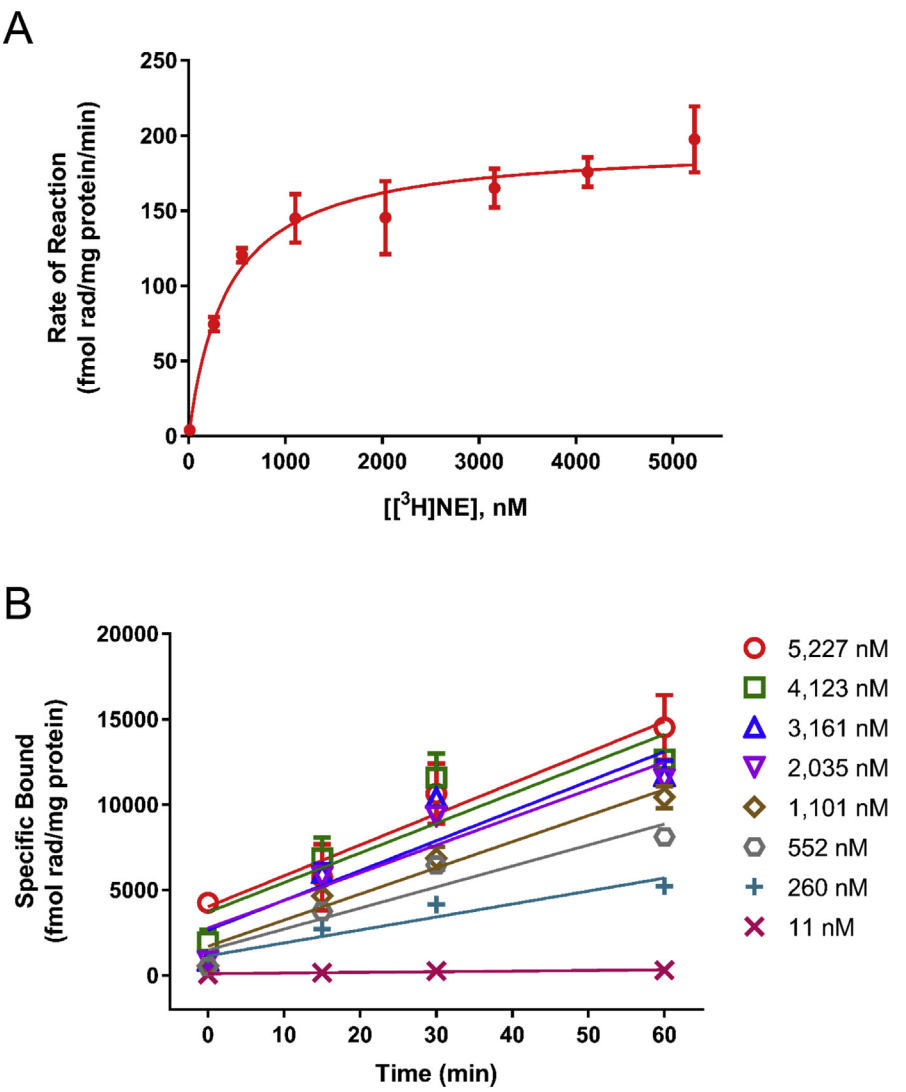

Fig. 1. Michaelis-Menten kinetics of [^{3}H]NE uptake by adherent SK-N-BE(2)C cells. Experiments were carried out at RT with multiple incubation times (60, 30, 15, 0 min) in 24-well plates (163 μg of protein/well). All data shown are mean ± S.E.M. of N = 3 conducted with duplicate determinations. SK-N-BE(2)C cells uptake [^{3}H]NE with K_M = 416 ± 53 nM and V_{MAX} = 195 ± 13 fmol radioactivity/mg protein/min. (A) Velocity of [^{3}H]NE uptake as a function of [^{3}H]NE 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.

the potency rank order remains the same between all three methods (desipramine > fluoxetine > citalopram).

The piperazine derivatives have mixed correlations between the three assay methods. GBR12935 has an IC_{50} value of 3,470 nM in SK-N-BE(2)C cells, which is 11.6-fold and 14.8-fold less potent compared to rat brain synaptosomes (IC_{50} = 299 nM) and hNET-HEK293 cells (IC_{50} = 235 nM), respectively. A similar difference is seen with GBR12909 as the compound is moderately potent in SK-N-BE(2)

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Table 1. Comparison of reuptake inhibition activity (IC_{50}, nM) at NET in SK-N-BE(2)C cells, rat brain synaptosomes, and transfected HEK293 cells.

Compound	hNET, SK-N-BE(2)C cells (nM)[a]	rNET, synaptosomes (nM)[b]	hNET-HEK293 cells (nM)
Selective Serotonin Reuptake Inhibitors			
Fluoxetine	5020 ± 370	688 ± 39 (742)	1020 ± 180[c]
Citalopram	>10,000	4,332 ± 295 (4,671)	>10,000[d]
Tricyclic antidepressants			
Desipramine	23.1 ± 3.1	8.32 ± 1.19 (8.97)	4.2 ± 1.1[c]
Piperazine derivatives			
GBR12935	3470 ± 150	277 ± 23 (299)	235 ± 53[c]
GBR12909	2400 ± 360	79.2 ± 4.9 (85.4)	>10,000[d]
Phenyltropane derivatives			
RTI-55	37.9 ± 3.5	5.89 ± 0.53 (6.35)	2.63 ± 0.76[c]
RTI-229	797 ± 78	19.5 ± 0.6 (21.0)	935 ± 140[d]
Nonselective monoamine transporter inhibitor			
Indatraline	66.7 ± 7.3	12.6 ± 0.5 (13.6)	645 ± 44[d]

[a] Values are mean ± S.E.M. of at least three independent experiments conducted in duplicate as described in the Methods.

[b] Data reported as mean ± S.D. in [17] as K_i values which were converted to IC_{50} values shown in parentheses using the Cheng-Prusoff equation $K_i = IC_{50}/(1 + L/K_M)$ [18].

[c] Data reported as mean ± S.E.M. in [8].

[d] Unpublished results generated by our group. Data are mean ± S.E.M. of at least three independent experiments conducted in duplicate as described in the Methods.

C cells (IC_{50} = 2,400 nM), very potent in rat brain synaptosomes (IC_{50} = 85.4 nM), and inactive in hNET-HEK293 cells. RTI-55 potencies compare fairly well as the compound has an IC_{50} value of 37.9 nM in SK-N-BE(2)C cells, which is 6-fold and 14.4-fold less potent than rat brain synaptosomes (IC_{50} = 6.35 nM) and hNET-HEK293 cells (IC_{50} = 2.63 nM), respectively. RTI-229 has similar potencies in SK-N-BE(2)C cells (IC_{50} = 797 nM) and hNET-HEK293 cells (IC_{50} = 935 nM), but the compound is 38-fold more potent in rat brain synaptosomes (IC_{50} = 21.0 nM). The results with the nonselective monoamine transporter inhibitor indatraline correlate fairly well as the compound is moderately potent in SK-N-BE(2)C cells with an IC_{50} value of 66.7 nM, which is 4.9-fold less potent than rat brain synaptosomes (IC_{50} = 13.6 nM), and 9.7-fold more potent than hNET-HEK293 cells (IC_{50} = 645 nM).

It is important to note a potential limitation of the data comparison between the SK-N-BE(2)C cells and hNET-HEK293 cells in the present study. Half of the compounds

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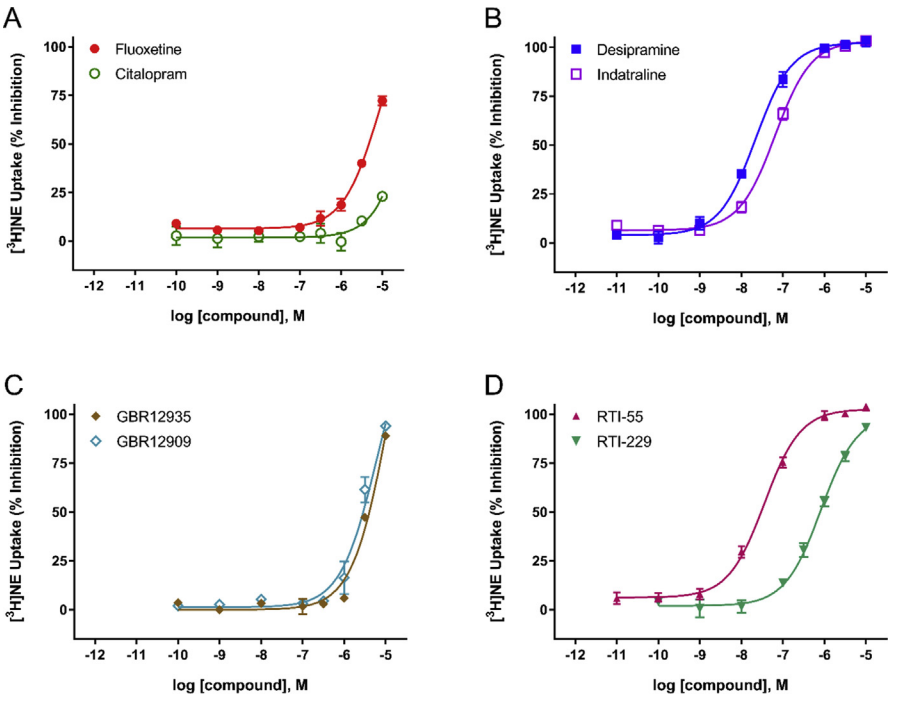

Fig. 2. Reuptake inhibition activity at the hNET endogenously expressed in SK-N-BE(2)C cells. Experiments were carried out with 416 nM final [^{3}H]NE at RT for 105 minutes in 24-well plates (163 μg of protein/well). All data shown are expressed as % inhibition and each data point is the mean ± S.E.M. of 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 (▼).

(fluoxetine, desipramine, GBR12935, RTI-55) were evaluated in hNET-HEK293 cells by Eshleman and colleagues [8], while the rest (citalopram, GBR12909, RTI-229, indatraline) were not included in the Eshleman study and hence were evaluated by our laboratory (unpublished results). Generally, the results from the Eshleman study compare favorably with the rat brain synaptosomes while the results from our study do not, likely due to differences in the assay protocols wherein our lab measured reuptake inhibition in plated cells, while the Eshleman lab used cell suspensions. Because the literature is replete with examples of differences in compound potencies between different research groups using the same type of assay, it would be more ideal to compare the SK-N-BE(2)C potencies with hNET-HEK293 potencies generated from one lab. Given the lack of hNET-HEK293 data generated by one lab, it may be more appropriate to compare the SK-N-BE(2)C results only with rat brain synaptosomes. In this regard, the reuptake inhibition potencies generated in SK-N-BE(2)C cells are all less potent compared to rat brain synaptosomes (2- to 38-fold), speaking to the sensitivities of the two assays, but the rank order remains very well conserved between the methods. In SK-N-BE(2)C cells, the reuptake

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inhibition rank order potency (from most potent to least potent) is desipramine > RTI-55 > indatraline > RTI-229 > GBR12909 > GBR12935 > fluoxetine > citalopram. In rat brain synaptosomes, the rank order is exactly the same, except RTI-55 and desipramine are switched. The direct correlation between potency rank order further substantiates that SK-N-BE(2)C cells can potentially serve as another type of in vitro transporter assay.

4. Conclusions

This study demonstrates a novel method of assessing hNET reuptake inhibition activity using human neuroblastoma SK-N-BE(2)C cells that endogenously express high levels of hNET. The data presented herein show that SK-N-BE(2)C cells are capable of detecting hNET reuptake inhibition activity and can potentially serve as another type of in vitro assay for researchers in the transporter field. Further investigation of other human cells endogenously expressing hBATs will provide important information on additional applications of these model systems in the transporter field.

Declarations

Author contribution statement

Ann M. Decker: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Bruce E. Blough: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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