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Original article

Influence of ligand binding kinetics on functional inhibition of human recombinant serotonin and norepinephrine transporters

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ABSTRACT

Introduction: Monoamine reuptake inhibitors treat a wide range of CNS disorders, including depression, obesity, and pain. The *in vitro* pharmacological properties of these inhibitors are determined routinely using radioligand binding and/or neurotransmitter uptake assays. Measurements from such studies can be influenced by assay design and ligand-specific characteristics, both of which may contribute to discrepancies in literature reports. **Methods:** We modified traditional methodologies to identify and account for factors that can confound *in vitro* potency determinations. Apparent equilibrium binding affinities (pK_i values) were determined in either HEK293 cells stably-transfected with human recombinant serotonin (SERT) or norepinephrine (NET) transporters, or membranes prepared from these cell lines. Care was taken to ensure that apparent affinities were measured under conditions that minimized ligand depletion and established equilibrium for both the radioligand and the compound of interest. An unlabelled ligand kinetic method was used to approximate inhibitor binding kinetic constants and corresponding dissociation half lives. To measure inhibitory effects on substrate uptake, both radiolabeled neurotransmitter ([^{3}H]-5-HT or [^{3}H]-NE) and fluorescence-based assays were used. The time-dependent nature of functional inhibition was examined using a fluorescent substrate uptake assay which provided real-time measurements of NET and SERT function. **Results:** SERT and NET inhibitors displayed a range of affinities, potencies, and inhibition modes by binding and functional uptake assays. Binding kinetic profiles for this panel of inhibitors were diverse, and affected *in vitro* measures using the former techniques. **Discussion:** In the present study we describe key features of *in vitro* assay methodology that can influence the apparent pharmacological profiles of standard SERT and/or NET inhibitors. Such information can serve as a foundation for understanding the *in vitro* profiles of monoamine reuptake inhibitors in the context of their clinical efficacy and tolerability.

1. Introduction

The neurotransmitters serotonin (5-HT) and norepinephrine (NE), the endogenous substrates of the serotonin (SERT) and norepinephrine (NET) transporters, regulate many neurological processes (Torres, Gainetdinov, & Caron, 2003). SERT and NET act as the primary mechanisms for clearance of synaptic 5-HT and NE, respectively, and their modulation yields clinical benefit in a variety of psychiatric disorders and pain syndromes (Stahl, Grady, Moret, & Briley, 2005). This broad clinical utility in several therapeutic areas has stimulated continued interest in the development of new monoamine transporter inhibitors (Whitlock et al., 2009).

Identification of novel SERT and/or NET ligands requires a number of different *in vitro* methodologies. Traditionally, this has included the determination of apparent binding affinities (pK_i values) by radioligand binding, as well as functional measures of potency (e.g. pIC_{50} values) in neurotransmitter uptake assays. The published literature indicates a range of affinities and potencies for well-characterized inhibitors and a poor correlation between the apparent binding affinity and inhibitory potency, particularly at SERT (Andersen et al., 2009; Bingham & Napier, 2009; Vaishnavi et al., 2004). Functional SERT potencies are generally more than 10-fold lower than the respective binding affinities, and shifts of nearly 200-fold were reported recently (Andersen et al., 2009). Such differences make it challenging to draw conclusions regarding the transporter selectivity profile and therefore confound estimates of target occupancies at a given inhibitor concentration. In the absence of either objective measures of occupancy at both targets, or reliable measures of transporter selectivity, the relationship between SERT and NET inhibition and clinical efficacy and tolerability profiles remains uncertain. Such information is critical to the directed design of new monoamine reuptake inhibitors and underscores the importance of assay methodology to evaluating novel monoamine transporter inhibitors *in vitro*.

In the present study we sought to address the discrepancy between apparent affinity and functional potency, with a focus on

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experimental factors contributing to these observations. Specifically, pK_i and pIC_{50} values were determined for a panel of clinically-relevant SERT and NET inhibitors, using conventional binding and uptake inhibition assays. We describe the influence of ligand depletion, membrane environment, temperature, binding kinetics, and transporter binding sites on the experimental design and analysis.

2. Methods

2.1. Materials

The following compounds were purchased from the organization indicated in parentheses: duloxetine (#7180, ChemPacific Corporation, Baltimore, MD); desipramine (D-3900, Sigma-Aldrich, St. Louis, MO); escitalopram (C505010, Toronto Research Chemicals (TRC), North York, ON); paroxetine (P-1372, Sigma-Aldrich, St. Louis, MO); clomipramine (C-7291, Sigma-Aldrich, St. Louis, MO); McN 5652 (#2148, Tocris, Ellisville, MO); venlafaxine (V120000, TRC, North York, ON); milnacipran (M-1318, Sigma-Aldrich, St. Louis, MO); and atomoxetine (T7947, Sigma-Aldrich, St. Louis, MO). Esreboxetine was isolated from reboxetine (40875, Waterstone Technology LLC, Carmel, IN). [^{3}H]-citalopram (NET1039) and [^{3}H]-5-HT (NET498) were purchased from Perkin Elmer Life Sciences (Boston, MA), [^{3}H]-NE (TRK584) from GE Healthcare Life Sciences (Piscataway, NJ), and [^{3}H]-nisoxetine from either Perkin Elmer (NET1084) or GE Healthcare (TRK942). The fluorescence-based Neurotransmitter Transporter Uptake Assay dye was obtained from Molecular Devices (Sunnyvale, CA).

2.2. Methods

2.2.1. Cell culture

HEK293 cells stably-transfected with human recombinant SERT (HEK293-hSERT) or human recombinant NET (HEK293-hNET) were obtained from Dr. Randy Blakely (Vanderbilt University, Tennessee). Cells were grown in DMEM medium (Gibco #11995-140) supplemented with fetal bovine serum (FBS; 10%) (F0926, Sigma-Aldrich, St. Louis, MO), or dialyzed FBS (10%) for SERT (#26400, GIBCO-Invitrogen Corp, Carlsbad, CA), penicillin (100 U/mL), streptomycin (100 μg/mL), L-GlutaMax (2 mM) (35050079, GIBCO-Invitrogen Corp., Carlsbad, CA), and G418 (250 μg/mL). Cells were incubated in a 5% CO_2, humidified incubator at 37 °C.

2.2.2. Equilibrium radioligand binding

Equilibrium binding assays were performed using P2 membrane preparations generated from HEK293-hSERT and HEK293-hNET cells. Frozen cell pellets were resuspended in ice-cold lysis buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and homogenized using a polytron PT2100 homogenizer (setting 19, 2 × 10 s). The resulting homogenates were centrifuged at 3400 × g (5 min at 4 °C). The pellet was discarded and the supernatant was centrifuged at 40,000 × g (30 min at 4 °C). The supernatant was discarded and the final pellet was resuspended in ice-cold membrane preparation buffer (50 mM Tris–HCl, 10% sucrose, pH 7.5).

Exploratory studies indicated that ligand depletion and/or binding kinetics could affect the apparent binding affinities. The high expression levels (B_{max} ~ 20–30 pmol/mg protein) of SERT and NET in the above cell lines increased the probability that specific ligand depletion (i.e. to the respective transporter target) would occur, particularly with high affinity ligands. To minimize both depletion and non-equilibrium conditions the following protocol was used with minor modifications, to account for ligand-specific characteristics (e.g. depletion, binding kinetics), as noted. Binding pK_i values were determined after an extended incubation period (e.g. 24 h) for compounds with slow binding kinetics. In general, no significant differences in pK_i values were observed after 1 h and 24 h incubation times for 'fast' compounds examined in parallel. We used theoretical calculations to estimate the potential for specific ligand depletion under the chosen assay conditions. Absence of ligand depletion was confirmed experimentally by demonstration that the pK_i values did not change when protein concentration was decreased and/or assay volume was increased.

Unless indicated otherwise, membrane radioligand binding assays were performed at room temperature in 96-well polypropylene plates in a total volume of 200 μL containing, respectively, SERT (0.5 μg) or NET (1–2 μg) membrane protein; [^{3}H]-citalopram (0.5–1 nM) or [^{3}H]-nisoxetine (1–2 nM), and eleven different concentrations of test compound ranging from 10 pM to 100 μM. For McN 5652 binding, a 1 mL assay volume was used due to previous reports of high non-specific ligand binding (Huang et al., 2002). Non-specific binding for all radioligand binding studies using [^{3}H]-citalopram or [^{3}H]-nisoxetine was determined in the presence of duloxetine (1 μM) or desipramine (1 μM), respectively. In all cases the final assay buffer was 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 0.1 mM ascorbic acid, 0.025% BSA, pH 7.4. Assays were incubated for 60 min (with the exception of McN 5652 and esreboxetine, for which competition assays at SERT and NET, respectively, were incubated for 24 h) at room temperature, and terminated by rapid filtration over 96-well GF/B glass fiber filter plates (Packard BioScience Co., Meriden, CT) presoaked in 0.3% polyethyleneimine. Filter plates were washed six times with wash buffer (ice-cold 50 mM Tris–HCl, 0.9% NaCl, at 4 °C) to remove unbound radioactivity. Plates were dried, 35–45 μL Microscint 20 liquid scintillation fluid (Packard BioScience Co., Meriden, CT) was added to each well, and the plates were counted in a Packard Topcount liquid scintillation counter (Packard BioScience Co., Meriden, CT).

Equilibrium competition studies at SERT also were conducted at 37 °C using membrane (1 μg protein) or whole-cell (10,000 cells/well) preparations of HEK293-hSERT cells, eleven concentrations of test compound (see above), and [^{3}H]-citalopram (~1.4 nM) in a total assay volume of 600 μL. The assay buffer was equivalent to that used for [^{3}H]-neurotransmitter uptake, i.e. final buffer composition was 7.5 mM HEPES, 12.5 mM Tris–HCl, 2.2 mM Na-Phosphate, 120 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 7.5 mM glucose, 1.7 mM $CaCl_2$, 250 μM ascorbic acid, 150 μM pargyline, 0.025% BSA, pH 7.4. Non-specific binding was determined in the presence of duloxetine (1 μM). Reactions were incubated at 37 °C for 60 min prior to termination by rapid filtration (as above).

Binding data were normalized using total (in the absence of test compound) and non-specific (determined as described above) binding from the same plate as follows: % specific binding = 100 × (value − non-specific binding) / (total − non-specific binding). Data then were analyzed by non-linear regression analysis with the GraphPad Prism Software package (GraphPad Software, Inc., San Diego, CA) using the 4 parameter sigmoidal dose–response algorithm. The bottom (curve minimum) was fixed to zero while the top (curve maximum) was a fitted parameter which was shared across all curves on a given plate. Hill slopes were near unity (95% confidence intervals from 0.7 to 1.0) and are not reported. Apparent K_i values for test compounds were calculated, in Microsoft Excel (Microsoft Corp., Redmond, WA), from the best-fit IC_{50} values, and the radioligand K_d values using the Cheng–Prusoff equation (Cheng & Prusoff, 1973): $K_i = IC_{50} / (1 + [L]/K_d)$ where $[L]$ = concentration of [^{3}H]-radioligand and K_d = equilibrium dissociation binding constant. For [^{3}H]-citalopram, the pK_d values for binding to SERT in membrane (room temperature and 37 °C) or whole-cell preparations (37 °C) of HEK293-hSERT cells were, respectively, 9.05 ± 0.02 (n = 3), 8.43 ± 0.03 (n = 4) and 8.38 ± 0.03 (n = 4). For [^{3}H]-nisoxetine the pK_d value for binding to NET in membrane preparations (room temperature) of HEK293-hNET cells was 8.45 ± 0.02 (n = 3). pK_i values are calculated by: $-\log[K_i\ (M)]$, and are reported to one decimal point. NET selectivity was calculated by: $10^{(\text{NET p}Ki - \text{SERT p}Ki)}$.