Radioligand binding Assay - Protocol 1:

Competition binding experiments were utilized to determine the *in vitro* binding potencies (IC_{50} values) of compounds for the serotonin transporter (SERT), the dopamine transporter (DAT), and the norepinephrine transporter (NET). Crude membranes prepared from recombinant, overexpressing HEK cell lines transfected with cDNA encoding each transporter served as the source of the transporters. Radioligands were chosen and saturation binding analysis was performed to determine the Kd for the radioligand selected for each transporter. All competition binding assays were performed at the Kd concentration to enable direct comparisons of the IC_{50} values between assays. The designated radioligands for each assay and their Kd concentrations are as follows: the SERT assay used [^{125}I]-RTI-55 at 260 pM, the DAT assay used [^{125}I]-RTI-55 at 125 pM, and the NET assay used [^{125}I]-2-iodo-nisoxetine at 550 pM. Each individual IC_{50} value was determined from a 20-point concentration response curve run in either duplicate or triplicate.

Radioligand binding assay - Protocol 2:

In order to evaluate the relative affinity of the various compounds at serotonin transporter (SERT), the dopamine transporter (DAT), and the norepinephrine transporter (NET), HEK293E cell lines were developed to express each of the three human transporters. cDNAs containing the complete coding regions of each transporter were amplified by PCR from human brain libraries. The cDNAs contained in pCRII vectors were sequenced to verify their identity and then subcloned into an Epstein-Barr virus based expression plasmid (E. Shen, GM Cooke, RA Horlick, Gene 156:235-239, 1995). This plasmid containing the coding sequence for one of the human transporters was transfected into HEK93E cells. Successful transfection was verified by the ability of known reuptake blockers to inhibit the uptake of tritiated NE, DA or 5HT.

For binding, cells were homogenized, centrifuged, and then resuspended in incubation buffer (5 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4). Then, the appropriate radioligand was added. For NET binding, [^{3}H] Nisoxetine (86.0 Ci/mmol, NEN/DuPont) was added to a final concentration of approximately 5 nM. For DAT binding, [^{3}H] WIN 35,428 (84.5 Ci/mmol) at 15 nM was added. For 5HTT binding, [^{3}H] Citolapram (85.0 Ci/mmol) at 1 nM was added. Then, various concentrations (10^{-5} to 10^{-11} M) of the compound of interest were added to displace the radioligand. Incubation was carried out at room temperature for 1 hour in a 96 well plate. Following incubation, the plates were placed on a harvester and washed quickly 4 times with (50 mM tris, 0.9% NaCl, pH 7.4) where the cell membranes containing the bound radioactive label were trapped on Whatman GF/B filters. Scintillation cocktail was added to the filters which were then counted in a Packard TopCount. Binding affinities of the compounds of interest were determined by non-linear curve regression using GraphPad Prism 2.01 software. Non-specific binding was determined by displacement with 10 micromolar mazindol.

Protocol for the measurement of *Ex vivo* occupancy:

Male Sprague Dawley rats (250-350 g; Charles River, Margate, Kent UK) were orally dosed with vehicle (0.25% carboxymethylcellulose) or **10i** at the doses specified. Four hours post-dosing, animals were sacrificed, and the forebrain was collected to assess occupancy at the serotonin transporter (SERT), norepinephrine transporter (NET) and dopamine transporter (DAT). One forebrain hemisphere from each animal was thawed and homogenized in assay buffer containing 50 mM Tris, 120 mM NaCl and 5 mM KCl (pH 7.6) with the ratio of tissue weight of 0.69g/6 ml and homogenized using a polytron homogenizer. Sample aliquots were frozen immediately and stored at -80^{o}C. On the day of ex vivo binding, frozen sample aliquots were thawed and needle homogenized and 1 mg of tissue incubated for SERT, NET and DAT binding using the assay conditions summarized in Table 1. After incubation, the reactions were terminated by the addition of ice cold assay buffer and rapid filtration through a Brandel Cell Harvester using FPXLR-196 filters. The filters were washed twice with ice-cold incubation buffer, punched into a clear plate prior to the addition of 200 ml scintillation fluid per well. Bound radioligand was measured using a Wallac Microbeta liquid scintillation counter. Specific binding was calculated by subtracting the value of the non-specific binding from that of the total binding in each sample. The percent occupancy was calculated as (1-specific binding in drug treated/specific binding in vehicle treated) x 100% for each subject and results expressed as the mean ± SEM % occupancy for each treatment group.

Table 1: Ex Vivo Binding Assay Conditions Used to Determine Serotonin, Norepinephrine and Dopamine Transporter Occupancy

Transporter	Radioligand	Agent to Define Non-Specific Binding	Assay Buffer	Incubation Time and Temperature
SERT	2 nM [^{3}H]Citalopram	Fluoxetine (10 mM)	Tris 50 mM, NaCl 120 mM, KCl 5 mM	10 minutes, 4^{0}C
DAT	0.1 nM [^{125}I]RTI-55 (in the presence of 0.5 mM citalopram)	GBR-12935 (10 mM)	Sodium phosphate buffer 30 mM	10 minutes, 4^{o}C
NET	5 nM [^{3}H]Nisoxetine	Reboxetine (10 mM)	Tris 50 mM, NaCl 300 mM, KCl 5 mM	20 minutes, 4^{o}C

Protocol for rat forced-swim test

On day 1 of each study, rats were placed individually into a plexiglass cylinder (65 cm height, 29 cm diameter) filled with at least 30 cm of water, ranging in temperature between 24 and 26˚C, for 15 minutes. Following this pre-swim, animals were dried and replaced in their home cage. On the following day, 24 hours after the initial pre-swim, animals were returned to the tank for another 5-minute swim. Their behavior during this session was videotaped for later scoring. The water in the tank was replaced and its temperature checked for each animal. A time sampling technique was used to score the behaviors during review of the videotapes. At the end of each 5-second period during the 5-minute test session, the animal's immobility was defined as the rat floating in the water without struggling and making only those movements necessary to keep its head above water. AMR-2 was dosed three times prior to testing on day 2: 23.5 (immediately after the pre-swim on day 1), 5 and 1 hour before the test.

Protocol for mouse tail suspension model

On the day of testing, mice were transported in their home cages to the testing room and allowed to acclimate to the room for one hour. The test compound was then administered PO gavage 60 minutes before behavioral testing. To initiate testing, each animal was suspended by its tail from a hook attached to the ceiling of the tail suspension cubicle for a 6min test. The tail was secured with adhesive tape placed about 2 cm from the tip of the tail. Immobility is measured using the Med Associates (St. Albans, Vt.) software, using threshold 2, gain 4 settings. The chambers were cleaned with 70% ethanol between animals. Following behavioral testing, animals were either returned to their home cage or sacrificed, and plasma samples and brains were taken for analysis of plasma exposure and transporter occupancy.