Supplementary Material

Synthesis of chiral $GABA_A$ receptor subtype selective ligands as potential agents to treat schizophrenia as well as depression

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Table of Contents

1. Catalepsy assay

Animals

Animals Male Sprague Dawley rats (350-450 g) were singly housed and kept on a reverse 12 hr dark/light cycle (lights on 7:00 pm) with food and water provided ad libitum. Behavioral testing occurred between the hours of 9:00 am and 4:00 pm with all animals individually handled a minimum of five minutes each for two days prior to the testing days.

Drugs

Drugs SH-053-2'F-*R*-CH_3 (**1**), SH-053-2'F-*S*-CH_3 (**2**), and haloperidol (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide to a final concentration of 30 and 0.1 mg/mL, respectively. Dimethyl sulfoxide, while not an ideal vehicle for animal behavioral work, is an acceptable formulation for rats (Gad et al., 2006). Phencyclidine hydrochloride (NIDA Drug Supply Program, Research Triangle, NC) was dissolved in saline at a concentration of 1.5 mg/mL.

Catalepsy assay[1]

Catalepsy was tested as per the methods reviewed by Sanberg et al. (1988). In short, the subjects were placed in a test box that has an open top and a stainless steel floor and grey plastic walls (34.50 cm x 19.60 cm x 23.00 cm). The box contains a stainless steel bar 1.25 cm in diameter laterally mounted 12.50 cm above the floor and 8.50 cm from one end of the box. Haloperidol (0.1 mg/kg), SH-053-2'F-*S*-CH_3, SH-053-2'F-*R*-CH_3 (30 mg/kg), or vehicle was administered intraperitoneally and subjects were tested at 30, 60, and 120 min post-injection with a maximum of five trials per session. Time (seconds) was measured from the placement of the subject's front paws on the bar until one paw moves or slips from the bar. A maximum of five consecutive trials were attempted. Criteria time for a completed trial is between ten seconds and five minutes. After five trials, if the subject had not remained on the bar for a minimum of five seconds, the longest time of the five trials was recorded.

2. Prepulse Inhibition

Prepulse inhibition testing[2] was run according to Bakshi and Geyer (1995). Subjects were placed on a platform in a sound-attenuating chamber (10.875"x14"x19.5"; Hamilton-Kinder, CA) that rested on a motion sensing plate. A matching session was conducted to determine the magnitude of the startle response for each subject. This session consisted of a five minute habituation period followed by 20 trials; 17 trials involving the presentation of a single auditory stimulus (pulse stimulus; 50 dB above a 60 dB background noise) and three trials in which a prepulse stimulus (10 dB above background) was presented 100 milliseconds before the pulse auditory stimulus. Subjects were then assigned to the various treatment groups based on the magnitude of their startle response. At least one day later, an experimental session was conducted to assess sensorimotor gating.

Subjects were given an intraperitoneal injection of SH-053-2'F-*S*-CH_3, SH-053-2'F-*R*-CH_3 (30 mg/kg), or vehicle 60 min before testing, followed by subcutaneous injection of either phencyclidine hydrochloride (1.5 mg/kg) or saline ten minutes prior to testing. In the experimental session, after a five or ten minute habituation period, subjects received 58 discrete trials; 26 trials during which the pulse stimulus (50 dB above background) was presented alone, 8 trials each in which the pulse stimulus was preceded by a prepulse stimulus (5, 10, or 15 dB above background) and 8 background trials with no pulse and only background noise. The first 6 pulse alone trials were not included in the average startle stimulus to achieve a relatively stable level of startle reactivity. The percent of prepulse inhibition was determined as 100-(average prepulse startle response/average startle stimulus alone)*100.

Statistics Comparisons in each experiment were analyzed using a repeated measures analysis of variance (ANOVA) with drug treatment as between-subject variables and time of prepulse intensity as within-subjects variables for catalepsy and prepulse inhibition, respectively. Significant interactions and main effects were further analyzed using a Fisher LSD post hoc test ($p<0.05$).

3. Seizure protection in the 6 Hz electroshock assay

Adult male CF1 mice (18-25 g) were pretreated intraperitoneally (i.p.) with the test compound at either 100 or 150 mg/kg. Each treatment group (n = 4 mice/group) was examined for anticonvulsive effects at one of five time points (1/4, 1/2, 1, 2, and 4 hr) after treatment with the test compound. Following pretreatment, each mouse received a drop of 0.5% tetracaine hydrochloride applied to each eye. The mouse was then challenged with the low-frequency (6 Hz) stimulation for 3 sec delivered through corneal electrodes. The low-frequency (6 Hz), long-duration (3 sec) stimuli are initially delivered at 32 mA intensity. Animals are manually restrained and released immediately following the stimulation and observed for the presence or absence of seizure activity. If the test compound is effective in the 6 Hz screen, mice were assessed in a dose-response using 5, 15, 30 and 60 mg/kg i.p. doses to determine the ED_{50} value.

4. Ataxic assessment in the rotorod assay

Adult mice were dosed (i.p.) 30 minutes prior to being placed on a rotating rod at a speed of 6 rpm.[3] The ligand was considered toxic (sedating/ataxia) if the animal fell off this rotating rod three times during a 1-min period. In addition to minimal motor impairment, animals may exhibit a circular or zigzag gait, abnormal body posture and spread of the legs, tremors, hyperactivity, lack of exploratory behavior, somnolence, stupor, catalepsy, loss of placing response and changes in muscle tone and are noted accordingly by the observer. Toxicity equates to sedation or ataxia.

5. Microsomal stability assay

The 4 µL of 1 mM test compound at a final concentration of 10 µM dissolved in DMSO/ACN/ Methanol/ Ethanol was preincubated at 37 °C for 5 minutes on a digital heating shaking dry bath (Fischer scientific, Pittsburgh, PA) in a mixture containing 282 µL of water, 80 µL of phosphate buffer (0.5 M, pH 7.4), 20 µL of NADPH Regenerating System Solution A (BD Bioscience, San Jose, CA) and 4 µL of NADPH Regenerating System Solution B (BD Bioscience, San Jose, CA) in a total volume of 391.2 µL. Following preincubation, the reaction was initiated by addition of 8.8 µL of either human liver microsomes (BD Gentest, San Jose, CA), mouse liver microsomes (Life technologies, Rockford, IL), at a protein concentration of 0.5 mg/mL. Aliquots of 50 µL were taken at time intervals of 0 (without microsomes), 10, 20, 40, 60, 90 and 120 minutes. Each aliquot was added to 100 µL of cold acetonitrile solution containing 1 µM/2 µM internal standard (Verapamil or 4,5-diphenyl imidazole). This was followed by sonication for 10 seconds and centrifugation at 10,000 rpm for 5 minutes. The 100 µL of the supernatant was transferred into Spin-X HPLC filter tubes (Corning Incorporated, NY) and centrifuged at 13,000 rpm for 5 minutes. The filtrate was diluted 100 fold and subsequently analyzed by LC-MS/MS with a Shimadzu LCMS 8040, (Shimadzu Scientific Instruments, Columbia, MD). The ratio of the peak areas of the internal standard and test compound was calculated for every time point and the natural log of the ratio were plotted against time to determine the linear slope (k). The metabolic rate ($k*C_0/C$), half-life (0.693/k), and internal clearance (V*k) were calculated, where k is the slope, C_0 is the initial concentration of test compound, C is the concentration of microsomes, and V is the volume of incubation in µL per microsomal protein in mg. All experiments were repeated three times in duplicates.

6. Cytotoxicity assay

Human liver hepatocellular carcinoma (HEPG2) and human embryonic kidney 293T (HEK293T) cell lines were purchased (ATCC) and cultured in 75 cm^2 flasks (CellStar). Cells were grown in DMEM/High Glucose (Hyclone, #SH3024301) media to which non-essential amino acids (Hyclone, #SH30238.01), 10 mM HEPES (Hyclone, #SH302237.01), 5 x 106 units of penicillin and streptomycin (Hyclone, #SV30010), and 10% of heat inactivated fetal bovine serum (Gibco, #10082147) were added. Cells were harvested using 0.05% Trypsin (Hyclone, #SH3023601), washed with PBS, and dispensed into sterile white, optical bottom 384-well plates (NUNC, #142762). After two hours, small molecule solutions were transferred with a Tecan Freedom EVO liquid handling system equipped with a 100 nL pin tool (V&P Scientific). The controls were 3-dibutylamino-1-(4-hexyl-phenyl)-propan-1-one (25 mM in DMSO, positive control) and DMSO (negative control). The cells were incubated for 48 hours followed by the addition of CellTiter-Glo™, a luminescence-based cell viability assay (Promega, Madison, WI). All luminescence readings were performed on a Tecan Infinite M1000 plate reader. The assay was carried out in quadruplet with three independent runs. The data was normalized to the controls and analyzed by nonlinear regression (GraphPad Prism).

7. Rotorod assay

Swiss Webster mice were trained to maintain balance at a constant speed of 15 rpm on the rotarod apparatus (Omnitech Electronics Inc., Nova Scotia, Canada) until mice could perform for 3 minutes for three consecutive trials. Separate groups of nine mice received oral gavage of vehicle (2% hydroxypropyl methylcellulose and 2.5% polyethylene glycol) or compounds (40 mg/kg) in a volume of 200 µL. Control compound diazepam was given as an ip injection at 5 mg/kg in 10% DMSO, 40% propylene glycol, and 50% PBS. The mice were placed on the rotarod at three separate time points of 10, 30, and 60 minutes after each oral gavage drug administration. A fail was classified for each mouse falling twice prior to 3 minutes, as it is common for a mouse injected with vehicle to occasionally fall once. Hence after a second fall, it would be considered a fail, and that time point would be recorded.

8. Pharmacophore/receptor model[4,5]

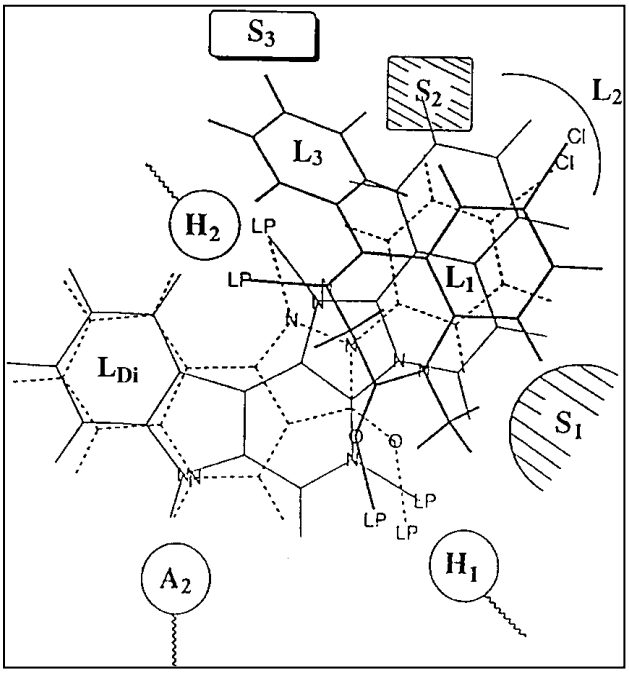

Figure S1. Relative locations of the descriptors and regions of the Unified pharmaco- phore/receptor model. The pyrazolo[*3,4-c*] quinoline-3-one CGS-9896 (dotted line), a diazadiindole (thin line), and diazepam (thick line) aligned within the Unified pharmacophore/receptor model for the Bz BS. H1 and H2 represent hydrogen bond donor sites within the Bz BS while A2 represents a hydrogen bond acceptor site necessary for potent inverse

agonist activity *in vivo*. L1, L2, L3 and LDi are four lipophilic regions and S1, S2, and S3 are regions of negative steric repulsion. LP = lone pair of electrons on the ligands.[4,5] This figure is modified from the one in reference 5.

9. References

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