Pharmacological Effects of Lu AA21004: A Novel Multimodal Compound for the Treatment of Major Depressive Disorder

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ABSTRACT

1-[2-(2,4-Dimethylphenyl-sulfanyl)-phenyl]-piperazine (Lu AA21004) is a human (h) serotonin (5-HT)$_{3A}$ receptor antagonist (K_i = 3.7 nM), h5-HT$_7$ receptor antagonist (K_i = 19 nM), h5-HT$_{1B}$ receptor partial agonist (K_i = 33 nM), h5-HT$_{1A}$ receptor agonist (K_i = 15 nM), and a human 5-HT transporter (SERT) inhibitor (K_i = 1.6 nM) (*J Med Chem* **54**:3206–3221, 2011). Here, we confirm that Lu AA21004 is a partial h5-HT$_{1B}$ receptor agonist [EC$_{50}$ = 460 nM, intrinsic activity = 22%] using a whole-cell cAMP-based assay and demonstrate that Lu AA21004 is a rat (r) 5-HT$_7$ receptor antagonist (K_i = 200 nM and IC$_{50}$ = 2080 nM). In vivo, Lu AA21004 occupies the r5-HT$_{1B}$ receptor and rSERT (ED$_{50}$ = 3.2 and 0.4 mg/kg, respectively) after subcutaneous administration and is a 5-HT$_3$ receptor antagonist in the Bezold-Jarisch reflex assay (ED$_{50}$ = 0.11 mg/kg s.c.). In rat microdialysis experiments, Lu AA21004 (2.5–10.0 mg/kg s.c.) increased extracellular 5-HT, dopamine, and noradrenaline in the medial prefrontal cortex and ventral hippocampus. Lu AA21004 (5 mg/kg per day for 3 days; minipump subcutaneously), corresponding to 41% rSERT occupancy, significantly increased extracellular 5-HT in the ventral hippocampus. Furthermore, the 5-HT$_3$ receptor antagonist, ondansetron, potentiated the increase in extracellular levels of 5-HT induced by citalopram. Lu AA21004 has antidepressant- and anxiolytic-like effects in the rat forced swim (Flinders Sensitive Line) and social interaction and conditioned fear tests (minimal effective doses: 7.8, 2.0, and 3.9 mg/kg). In conclusion, Lu AA21004 mediates its pharmacological effects via two pharmacological modalities: SERT inhibition and 5-HT receptor modulation. In vivo, this results in enhanced release of several neurotransmitters and antidepressant- and anxiolytic-like profiles at doses for which targets in addition to the SERT are occupied. The multimodal activity profile of Lu AA21004 is distinct from that of current antidepressants.

Introduction

Major depressive disorder is a highly prevalent, disabling disease with negative impact on medical health, life quality, and productivity (Kessler et al., 2006; Baune et al., 2007). The introduction of selective serotonin (5-HT) reuptake inhibitors (SSRIs) and 5-HT and noradrenaline (NA) reuptake inhibitors (SNRIs) produced therapeutic benefits without the serious side effects associated with the tricyclic antidepressants (Cassano and Fava, 2004). However, a therapeutic improvement is only apparent after several weeks of treatment, and many patients respond only partially to treatment and some fail to respond at all (Henkel et al., 2009). Moreover, side effects such as sexual dysfunction, sleep disturbances, and gastrointestinal disturbances have been reported (Cassano and Fava, 2004).

With the advent of SNRIs and more recently triple reuptake [dopamine (DA), NA, and 5-HT] inhibitors, attention has shifted to antidepressants that influence multiple trans-

ABBREVIATIONS: 5-HT, serotonin; SSRI, selective serotonin reuptake inhibitor; SNRI, 5-HT and noradrenaline reuptake inhibitors; NA, noradrenaline; DA, dopamine; SERT, 5-HT transporter; FSL, Flinders Sensitive Line; FRL, Flinders Resistant Line; Lu AA21004, 1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine; h, human; IA, intrinsic activity; LSD, lysergic acid diethylamide; r, rat; HPLC, high-performance liquid chromatography; SB216641, *N*-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride; GR125743, *N*-(4-methoxy-3-(4-methylpiperazin-1-yl)phenyl)-3-methyl-4-(pyridin-4-yl)benzamide; AUC, area under the curve; ANCOVA, analysis of covariance; ANOVA, analysis of variance; SB-269970, (2*R*)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]-pyrrolidine) hydrochloride; SR57227, 1-(6-chloro-2-pyridinyl)-4-piperidinamine hydrochloride; Y-25130, *N*-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide hydrochloride; BRL 46470A, endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)2,3-dihydro-3,3 dimethyl-indole-1-carboxamide hydrochloride.

mitter systems. However, multitransmitter reuptake inhibitors produce widespread increases in transmitter levels both centrally and peripherally, which may lead to tolerability issues and peripheral sympathomimetic side effects that may limit the possibility of reaching therapeutically effective doses. An alternative approach is to develop compounds that, for example, inhibit the 5-HT transporter (SERT) and modulate receptors that control neurotransmitter function (Millan, 2006; Butler and Meegan, 2008). Several observations support the idea that compounds working through multitarget mechanisms will have a superior effect on both cardinal and comorbid symptoms of depression compared with selective compounds (for review, see Millan, 2006).

The delayed therapeutic effect of SSRIs or SNRIs may be ascribed to adaptations in downstream signaling pathways. However, the importance of extracellular 5-HT levels is supported by the fast onset of effect of the 5-HT-releasing compound, d-fenfluramine, in patients with seasonal affective disorder (O'Rourke et al., 1989) and the rapid relapse induced by acute tryptophan depletion in SSRI-treated patients in remission (Booij et al., 2005).

Encouraging clinical data demonstrated an accelerated time to effect of SSRIs by adjunctive use of the 5-HT$_{1A}$ receptor partial agonist, pindolol (Artigas et al., 2006), which inhibits somatodendritic 5-HT$_{1A}$ autoreceptors and augments 5-HT levels in the forebrain after acute SSRI administration (Gardier et al., 1996). Moreover, 5-HT$_{1B}$ autoreceptors located on serotonergic nerve terminals modulate 5-HT neurotransmission. Thus, a 5-HT$_{1B}$ receptor agonist decreases extracellular 5-HT levels in the mouse prefrontal cortex, and a 5-HT$_{1B}$ receptor antagonist augments SSRI-induced increases in cortical 5-HT levels in microdialysis studies (de Groote et al., 2003). 5-HT$_3$ receptor antagonists exert antidepressant- and anxiolytic-like effects in preclinical settings (Costall and Naylor, 2004) and enhance the effects of SSRIs and SNRIs in the forced swim test (Ramamoorthy et al., 2008). 5-HT$_7$ receptor antagonists show antidepressant-like activity in the mouse forced swim and tail-suspension tests (Sarkisyan et al., 2010) and a synergistic augmentation of extracellular 5-HT levels in the rat prefrontal cortex when combined with an SSRI (Bonaventure et al., 2007). Thus, by blocking feedback systems and modulating relevant receptors, it appears that the efficacy of current antidepressant treatments may be enhanced.

Regarding animal models, the Flinders Sensitive Line (FSL) rat and its control counterpart, the Flinders Resistant Line (FRL) rat, have been selectively bred for high and low sensitivity to cholinergic agonism, respectively (Wegener et al., 2011). The cholinergic hypersensitivity gives rise to changes in other neurotransmitter systems, because the FSL rat has lower density of 5-HT$_{1A}$ receptors but a higher density of 5-HT$_{1B}$ receptors in several brain regions compared with those of the FRL rat (Nishi et al., 2009). In the rat forced swim test, FSL rats display depressive-like behavior that is reversed by antidepressants (Wegener et al., 2011). The rat social interaction test is a widely used assay that assesses the time two unfamiliar rats spend actively interacting in a novel aversive environment; compounds that have anxiolytic properties increase that time (File and Seth, 2003). Moreover, ultrasonic (22 kHz) vocalization in rats seems to reflect their emotional state and drug-induced reduction of ultrasonic vo-

calization induced by an aversive stimulus is used as an index of anxiolytic activity (Kikusui et al., 2001).

1-[2-(2,4-Dimethylphenyl-sulfanyl)-phenyl]-piperazine (Lu AA21004) is a h5-HT$_{3A}$ receptor antagonist (K_i = 3.7 nM), h5-HT$_7$ receptor antagonist (K_i = 19 nM), h5-HT$_{1B}$ receptor partial agonist (K_i = 33 nM), h5-HT$_{1A}$ receptor agonist (K_i = 15 nM), and hSERT inhibitor (K_i = 1.6 nM) (Bang-Andersen et al., 2011).

Here, we elaborate on the in vitro profile of Lu AA21004 and assess its effects on brain neurotransmitter levels in freely moving rats and in animal models predictive of antidepressant and anxiolytic-like activity and relate these effects to occupancy levels of the SERT and the 5-HT$_{1B}$ receptor.

Materials and Methods

Materials

Lu AA21004 was synthesized at the Department of Medicinal Chemistry Research, H. Lundbeck A/S (Fig. 1) (Bang-Andersen et al., 2011). Lu AA21004 (molecular weight = 379.4) was dissolved in 10% β-hydroxypropyl cyclodextrin and administered as a single injection subcutaneously or orally or administered via minipumps for 3 days before the experiments. Imipramine (Sigma-Aldrich, St. Louis, MO) was dissolved in 10% β-hydroxypropyl cyclodextrin and administered intraperitoneally. Ondansetron (Sequoia Research, St. James Close, Pangbourne, UK) was dissolved in saline and administered subcutaneously. In the Bezold-Jarisch reflex studies, ondansetron was dissolved in 10% β-hydroxypropyl cyclodextrin and administered subcutaneously. All doses of Lu AA21004 are expressed in milligrams of base per kilogram body weight. For the 3-day treatment, osmotic minipumps (2ML1; Alzet, Cupertino, CA) were filled under aseptic conditions and implanted subcutaneously under Hypnorm/Dormicum anesthesia. The experiments were carried out with the minipumps onboard.

Animals

The rats were pair-housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 6:00 AM). Food and water were available ad libitum. The rats had a minimum of 5 days' adaptation in the animal facility before the initiation of experiments. All animal procedures were carried out in compliance with European Commission Directive 86/609/EEC and with the laws regulating experiments on animals and for studies conducted at Lundbeck Research USA in compliance with the policies and standards of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and approved by the local institutional animal care and use committee.

Functional Assay for the Human 5-HT$_{1B}$ Receptor

Functional intrinsic activity (IA) was evaluated using a functional whole-cell assay performed as a cAMP assay. HeLa cells stably expressing human serotonin 5-HT$_{1B}$ receptors were harvested using Sigma-Aldrich cell dissociation buffer and counted on a Nucleo-Counter (ChemoMetec A/S, Allerød, Denmark). To each well of a 384-well OptiPlate was added 3 μl of test compound followed by 10 μl of cell suspension containing Lance cAMP antibody and 2000 cells.
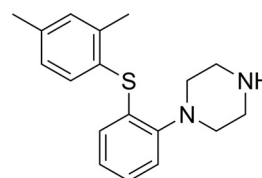

Fig. 1. Structure of Lu AA21004.

The plates were incubated at 16°C for 20 min before 3 μl of a rolipram-forskolin solution was added (final concentrations of rolipram and forskolin were 10 and 1 μM, respectively). The plates were incubated for additional 20 min at 16°C followed by 10 min at room temperature before addition of 15 μl of Lance detection buffer. The plates were incubated at room temperature for 1 to 3 h before counting on an EnVision instrument. All data points were normalized using the minimum and maximum response levels defined by each 5-HT curve and analyzed by sigmoidal dose-response curve-fittings using Graph-Pad Prism 4 to determine the EC_{50} and IA.

Binding Assay for the Rat 5-HT$_7$ Receptor

[^{3}H]lysergic acid diethylamide (LSD) binding to r5-HT$_7$ receptor was determined in membranes from a polyclonal human embryonic kidney 293 cell line expressing the r5-HT$_7$ receptor. Nonspecific binding was obtained in the presence of 10 μM Lu AA21004. Competition binding studies were conducted by displacement of [^{3}H]LSD using assay conditions recommended in the technical data sheet for membranes containing r5-HT$_7$ receptor (PerkinElmer Life and Analytical Sciences (Waltham, MA). In brief, to each well of a Costar 3365 96-well microtiter plate, the following were added: 25 μl of diluted drug (starting concentration of 10 μM), 25 μl of the [^{3}H]LSD, and 200 μl of membrane (5 μg/well). The plates were shaken slightly and placed in a 37°C incubator for 1 h. The plates were then filtered using a Brandel Harvester and the "filter mat" was dried in a convection oven at 55°C for 20 min. The filter mat was attached to MeltiLex (solid scintillant) and sealed with a heat sealer before counts were obtained in a TriLux liquid scintillation counter. Lu AA21004 was tested at least three times over a 6-log concentration range. IC_{50} values were determined by nonlinear regression analysis using a sigmoidal variable slope curve-fitting. The dissociation constant (K_i) was calculated from the Cheng-Prusoff equation [K_i = $IC_{50}/(1 + ([L]/K_D)$], where L is concentration of radioligand in the assay and K_D is affinity of the radioligand for the receptor using GraphPad Prism 4.

Functional Assay for the Rat 5-HT$_7$ Receptor

Functional activity of r5-HT$_7$ receptor was determined using a whole-cell cAMP HiRange HTRF assay (two-step protocol; Cisbio US, Bedford, MA). In brief, polyclonal human embryonic kidney 293 cells expressing r5-HT$_7$ receptors were resuspended in stimulation buffer (1× Hanks' balanced salt solution with CaCl$_2$ and MgCl$_2$, 5 mM HEPES, pH 7.4, and 0.075% bovine serum albumin) and counted on a Guava easyCyte system (Millipore Corporation, Billerica, MA). In each well of a 384-well OptiPlate, the following were added: 5 μl of cells (30,000 cells), 2.5 μl of drug, 2.5 μl of 5-HT, 5 μl of cAMP-D2 conjugate, and 5 μl of Cryptate conjugate as recommended by the kit manufacturer. Plates were incubated with drugs for 30 min (final concentration of Lu AA21004 and metitepine were 100 and 10 μM, respectively). The plates were then incubated for 30 min with 5-HT (at EC_{80} concentration) at 37°C and an additional 10 min at room temperature. After addition of the conjugates, the plates were incubated for 1 h at room temperature before the cell cAMP content was measured using an EnVision 2104 Multilabel Reader (PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland). Results were calculated from the ratio of absorbance at 665 nm/620 nm. All data points were normalized using the minimum and maximum responses defined by each 5-HT curve and analyzed by sigmoidal dose-response curve-fitting using GraphPad Prism 4 to determine IC_{50}.

Pharmacokinetics

The pharmacokinetics of Lu AA21004 was investigated after intravenous (1.5 mg/kg), subcutaneous (2 mg/kg), and oral (6 mg/kg) administration to male Sprague-Dawley rats (n = 9/dose). At appropriate time points after dosing, blood was sampled from three different rats. Each rat was sampled three times in total. Plasma samples were analyzed for concentrations of Lu AA21004 after solid-phase extraction followed by HPLC and tandem mass spectrometric detection. One-compartment models were fitted to pooled Lu AA21004 plasma concentration data from subcutaneous administration, whereas noncompartmental analysis was applied for pooled plasma concentration data obtained after intravenous and oral administration. WinNonlin (version 5.2; Pharsight, Mountain View, CA) was used during all pharmacokinetic analyses.

Ex Vivo 5-HT$_{1B}$ Receptor and SERT Occupancy Assays

Adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were used. Animals (n = 3/dose) were sacrificed by decapitation 1 h after the injections, and the brains were dissected and flash-frozen on powdered dry ice and afterward stored at −20°C until used.

Brains were sectioned coronally for autoradiography using a cryostat and mounted on microscope slides. Slices (n = 3/rat) were cut at 20-μm thickness and beginning at approximately 1.56 mm anterior to bregma. Slides were stored for at least 24 h at −20°C before being used in autoradiography experiments. On the day of the experiment, boxes containing slides were thawed at room temperature under a constant stream of dry air for 30 to 45 min before use.

5-HT$_{1B}$ Receptor Occupancy. Lu AA21004 had been injected at doses of 2.0, 4.0, 8.0, or 16.0 mg/kg s.c., and N-[3-[3-(dimethylamino) ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride (SB216641) was administered at a dose of 7.5 mg/kg s.c. Slides were preincubated for 3 min in a buffer containing 170 mM Tris-HCl, 4 mM CaCl$_2$, and 0.1% l-ascorbic acid, pH 7.4 and were then air-dried at room temperature for 30 to 45 min. Slides were incubated for 60 min in the buffer noted above with 10 μM pargyline and 1 nM [^{3}H] N-(4-methoxy-3-(4-methylpiperazin-1-yl)phenyl)-3-methyl-4-(pyridin-4-yl)benzamide (GR125743) (specific activity 76 Ci/mmol, 0.1 mCi/ml). Nonspecific binding was determined using 10 μM SB216641. Slides were washed twice in ice-cold buffer for 5min and then were briefly dipped in distilled water and air-dried. The slides were transferred to a desiccator and dried for at least 60 min. Finally, the slides were exposed using a Beta Imager (Biospace Lab, Paris, France) for 20 h before analysis using β-Vision software (Biospace Lab) for quantification. Surface radioactivity (expressed as counts per millimeter/square millimeter) was measured from a region of interest that was defined a priori and was consistent across each slice of brain tissue. Specific binding was determined by subtracting nonspecific binding from total binding. Subsequently, specific binding levels for each brain were expressed as a percentage of the average specific binding from vehicle-treated rats. These percentages were then subtracted from 100% to obtain percent receptor occupancy. ED_{50} analysis was conducted using GraphPad Prism 4. In brief, a log transformation was performed for each LuAA21004 dose. A nonlinear regression was then performed on occupancy values using a sigmoidal dose-response curve. The maximum and minimum values were constrained to 100 and 0, respectively, whereas the Hill coefficient was not constrained.

SERT Receptor Occupancy. Lu AA21004 had been injected at doses of 0.1, 0.2, 0.8, 2.0, 4.0, or 8.0 mg/kg s.c. Slides were incubated for 90 min at room temperature in buffer (50 mM Tris-HCl, 150 mM NaCl, and 5 mM KCl, pH 7.4) containing 0.5 nM [^{3}H]3-amino-4-[2-[(di(methyl)amino)methyl]phenyl]sulfanylbenzonitrile (80 Ci/mmol; 1 mCi/ml), and 1 μM escitalopram was used to measure nonspecific binding. Slides were washed 3 times in buffer at 4°C for 5 min and then were briefly dipped in distilled water and air-dried. The slides were transferred into a desiccator and dried for at least 60 min. Finally, the slides were exposed using a Beta Imager for at least 16 h before analysis as noted above using a separate region of interest.

Bezold-Jarisch Reflex

The 5-HT$_3$ receptor effect of Lu AA21004 was evaluated in vivo on the Bezold-Jarisch reflex in anesthetized male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) and compared with the spe-

cific 5-HT$_3$ antagonist, ondansetron. Intravenous administration of 5-HT induces bradycardia by eliciting the Bezold-Jarisch reflex mainly via 5-HT$_3$ receptors located on sensory vagal nerve endings in the heart (Villalón and Centurion, 2007). Compounds with 5-HT$_3$ antagonistic effects inhibit the Bezold-Jarisch reflex. Rats were anesthetized by inhalation of 5% isoflurane in oxygen in an induction chamber, and anesthesia was maintained with 2% isoflurane in oxygen. The rats were placed on a heating pad, and catheters were introduced into the vena jugularis for drug administration and aorta carotis for blood pressure measurement. Electrodes for ECG (lead II) recording were placed. Baseline ECG and blood pressure were recorded for 10 min before a 3-s bolus of 5-HT (0.06 mg/kg i.v.) was administered. The decrease in heart rate induced by 5-HT was measured and set as the maximum (100%) response. Five minutes after the 5-HT bolus, Lu AA21004 (0.01, 0.03, 0.08, 0.1, 0.3, 0.8, or 3.0 mg/kg s.c., $n = 6$/dose) or ondansetron (0.003, 0.01, 0.02, 0.03, 0.1, or 0.3 mg/kg s.c., $n = 6-8$/dose) was administered and was followed by a 5-HT bolus injection (0.06 mg/kg i.v.) 30 min later. The decrease in heart rate induced by 5-HT was measured, and the difference from the previous value was quantified as percent inhibition of the Bezold-Jarisch reflex. ED$_{50}$ values were calculated by nonlinear regression using GraphPad Prism 4, using a sigmoidal dose-response with free-floating Hill slope and minimum value constrained to zero.

Microdialysis

Male Sprague-Dawley rats were anesthetized with Hypnorm/Dormicum (2 ml/kg), and intracerebral guide cannulas (CMA/12) were stereotaxically implanted into the hippocampus, aiming to position the dialysis probe tip in the ventral hippocampus (coordinates: 5.6 mm posterior to bregma, lateral -5.0 mm, and 7.0 mm ventral to dura) or the medial prefrontal cortex (coordinates: -3.4 mm posterior to bregma, lateral -0.8 mm, and 5.0 mm ventral to dura) (Paxinos and Watson, 1998). Anchor screws and acrylic cement were used for fixation of the guide cannulas. The body temperature of the animals was maintained at 37°C and monitored by rectal probe. The rats were allowed to recover from surgery for 2 days, housed singly in cages. On the day of the experiment, a microdialysis probe (CMA/12, 0.5-mm diameter, 3-mm length) was inserted through the guide cannula. The probes were connected via a dual-channel swivel to a microinjection pump. Perfusion of the microdialysis probe with filtered Ringer's solution (145 mM NaCl, 3 mM KCl, 1 mM MgCl$_2$, and 1.2 mM CaCl$_2$) was begun shortly before insertion of the probe into the brain and continued for the duration of the experiment at a constant flow rate of 1 μl/min. After 180 min of stabilization, the experiments were initiated, and dialysates were collected every 20 min. After the experiments, the animals were sacrificed, and their brains were removed, frozen, and sliced for probe placement verification.

In the microdialysis studies, the mean value from three or four consecutive samples immediately preceding compound administration served as the basal level for each experiment. The area under the curve (AUC) for samples immediately preceding compound administration served as the baseline AUC, whereas the AUC for samples immediately after compound administration served as the response outcome. Before analysis, baseline and postbaseline AUCs were log-transformed. All AUCs, regardless of whether they were baseline or postbaseline AUCs, were calculated using the trapezoid rule based on the average value of each pair of points multiplied by the sample duration. Statistical analysis was performed using one-way ANCOVA with baseline AUC as a continuous covariate and doses of Lu AA21004 as a categorical variable. Post hoc pairwise analyses were performed using Dunnett's correction.

Analysis of Dialysate 5-HT

The concentration of 5-HT in the dialysates was analyzed by means of HPLC with electrochemical detection. The monoamines were separated by reverse-phase liquid chromatography (ODS column, 150 × 3 mm, 3 μm; ESA Inc., Chelmsford, MA). The mobile

phase consisted of 75 mM NaH$_2$PO$_4$, 150 ml/l sodium octanesulfonic acid, 100 μl/l triethylamine, and 10% acetonitrile (pH = 3.0) at a flow rate of 0.4 ml/min. Electrochemical detection was accomplished using a coulometric detector (potential set at 250 mV and guard cell at 350 mV; Coulochem II, ESA Inc.).

Analysis of Dialysate DA and NA

The concentration of DA and NA in the dialysates was analyzed by means of HPLC with electrochemical detection. The monoamines were separated by reverse-phase liquid chromatography (Thermo BDS Hypersil column, 150 × 2.1 mm, 3 μm; Thermo Fisher Scientific, Waltham, MA). The mobile phase consisted of sodium acetate buffer (4.1g/l) with methanol (2.5% v/v), Titriplex (150 mg/l), sodium octanesulfonic acid (150 mg/l), and trimethylamine (150 mg/l) (pH = 4.1) at a flow rate of 0.35 ml/min.

In Vivo Occupancy of the SERT in Rats Subjected to Microdialysis

Rats from the microdialysis experiment were included in an in vivo binding study in which the level of SERT occupancy was determined after subchronic pretreatment with 5 mg/kg per day Lu AA21004 for 3 days. The day after the dialysis experiment rats were injected with 60 μCi of [^{3}H]2-(2-dimethylaminomethyl-phenylsulfanyl)-5-methyl-phenylamine), a selective SERT ligand and were sacrificed 15 min after ligand injection. The brain was quickly removed, the cortex was dissected out and homogenized in ice-cold buffer (50 mM Tris, 120 mM NaCl, and 5 mM KCl, pH = 7.4), and the homogenate was filtered through Whatman GF/C filters to remove unbound ligand. The level of nonspecific binding was determined as the percentage of binding in a group of animals that had been acutely pretreated with 10 mg/kg escitalopram subcutaneously, previously shown to induce full occupancy (L. T. Brennum, unpublished data). A group of vehicle-treated animals were used to determine total binding.

Forced Swim Test in FSL Rats

Eighty male Flinders Line rats (FSL and FRL; age 9–10 weeks) from the colony maintained at University of Aarhus, weighing 280 to 320 g, were cage-housed in pairs at 20 ± 2°C in a 12-h light/dark cycle (lights on at 7:00 AM). Tap water and chow pellets were available ad libitum. The animal colony was protected from outside noise, and all experimental procedures were performed in specially equipped rooms within the animal house.

Lu AA21004 (2.3 or 7.8 mg/kg s.c.) and imipramine (15 mg/kg i.p.) were administered 24, 6, and 1 h before the forced swim procedure. A modified 2-day Porsolt swim test (Porsolt et al., 1978) was used as behavioral endpoint. In brief, the diameter of the cylinder was 24 cm, water depth was 40 cm, and temperature was 25°C. Mild lighting was placed above the cylinder (120 lux). All behavioral procedures were recorded using digital video. Just before entering the forced swim test, basal rat locomotion was explored using a simple open field during 5 min (open field, 1 m × 1 m) with dark floor and dim lighting (40–50 lux). The swim and locomotor behavior on the (2nd) test day was manually scored by an observer blind to the treatment of the animals (forced swim test) and by using EthoVision XT 6 (Noldus Information Technology. Leesburg, VA) for the open field. Output is given as immobility, climbing, and swimming as a percentage of total test time (forced swim test) and distance moved (open field). Data were analyzed using two-way analyses of variance (ANOVAs) with post hoc Bonferroni tests to compare the different treatment conditions and Student's t tests to compare the vehicle-treated FSL and FRL rats.

Social Interaction Test

Male Sprague-Dawley rats (Charles River Laboratories, Inc.) weighing 200 to 225 g at the start of the study were handled daily for 4 days before the social interaction test. On the 4th day, animals

were weighed for the study on day 5. On the following day, pairs of weight-matched animals received Lu AA21004 (0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 mg/kg p.o.), vehicle orally, or chlordiazepoxide (5.0 mg/kg p.o.) as the positive control. Sixty minutes later, pairs of rats were introduced into a brightly lit (425 lux) test chamber (54 × 36 × 30 cm), and their behavior was recorded for 10 min. The chamber was cleaned at the end of each test. A trained observer, unaware of drug treatments, reviewed the video image and recorded the time pairs of animals spent in active social interaction. Active social interaction behavior monitored included sniffing, licking, grooming, following, and crawling over and under the partner rat. Simply being in close proximity or just in contact was not considered active interaction and was not scored. Locomotor activity and rearing were automatically monitored in "real time" using the Cleversys tracking system (Cleversys Inc., Reston, VA). Means and S.E.M. were calculated for each parameter and treatment group. Data were analyzed using ANOVA with post hoc Dunnett's tests where appropriate (GraphPad Prism 4).

Conditioned Fear-Induced Vocalization

Male Sprague-Dawley rats (Charles River Laboratories, Inc.) with initial weights of 175 to 200 g were used. The animals were tested in this assay with various compounds over a period of several weeks, with washout times determined by the properties of the drugs tested.

The test equipment consisted of four chambers, each with grid floors, waste pan, house light, small fan for white noise, video camera, and ultrasonic sound detector. On the 1st day, animals were put into a chamber, and shock conditioning was administered as six presentations of a 8-s, 0.8-mA shock, using the "varcond6_8sec" protocol in the FreezeFrame software program. The shocks were delivered at various time points throughout the conditioning session. The total length of the conditioning session was 8 min with an initial acclimation period of approximately 1.5 min and approximately 40 s between shocks. On the 2nd day, the rats were pretreated with Lu AA21004 (1.9, 3.9, or 7.9 mg/kg s.c.), vehicle subcutaneously or the positive control, buspirone (1.0 mg/kg s.c.). Thirty minutes later, the rats were placed for 5 min in the test chamber with no shocks administered. Ultrasonic vocalizations (22 kHz frequency range) were recorded. All chambers were wiped clean, and waste pans were emptied between each session. After completion of the experimental trials, test animals were returned to their home cages. Means and S.D. were calculated for duration of vocalizations in seconds for each treatment group. Data were analyzed using ANOVA followed by Dunnett's test where appropriate (GraphPad Prism 4).

Results

Effects on the Human 5-HT$_{1B}$ and Rat 5-HT$_7$ Receptor in Cloned Receptor Systems. In a whole-cell cAMP-based assay, Lu AA21004 displayed a partial agonistic response with an EC$_{50}$ = 460 ± 110 nM (S.E.M., n = 12) and an IA of 22% (Table 1). Furthermore, Lu AA21004 bound to the r5-HT$_7$ receptor with a K_i value of 200 ± 27 nM (n = 4) and was a functional antagonist at the r5-HT$_7$ receptor with an

TABLE 1
In vitro effects of Lu AA21004 on 5-HT$_{1B}$ and 5-HT$_7$ receptors

	K_i	IC$_{50}$	IA
	nM		*%*
h5-HT$_{1B}$	33[a]	120[a,b]	55[a,b]
		460[c]	22[c]
r5-HT$_{1B}$	16[d]	N.D.	N.D.
h5-HT$_7$	19[a]	450[a]	0
r5-HT$_7$	200	2080	0

N.D., not determined.
[a] Data from Bang-Andersen et al. (2011).
[b] Guanosine 5′-*O*-(3-thio)triphosphate assay.
[c] cAMP assay.
[d] Study 841078, Cerep (Celle l'Evescault, France).

IC$_{50}$ = 2080 ± 18 nM (n = 4) in an in vitro whole-cell cAMP assay (Table 1).

Pharmacokinetics. In the in vivo studies of Lu AA21004, doses and pretreatment times were selected on the basis of pharmacokinetic parameters and target occupancy. Relevant pharmacokinetic parameters are summarized in Table 2. Maximal plasma levels of Lu AA21004 in the rat were reached 0.8 and 1 h after subcutaneous and oral administration, respectively. Furthermore, the plasma elimination half-life was approximately 3.2 h. The bioavailability of Lu AA21004 was 10-fold lower after oral administration compared with subcutaneous dosing, which may be ascribed to first-pass metabolism, because a relatively high clearance, 3.7 l · h^{-1} · kg^{-1}, was observed after intravenous injection (Table 2).

Ex Vivo Occupancies at the Rat 5-HT$_{1B}$ Receptor and the Rat SERT. One hour after subcutaneous administration of Lu AA21004, dose-dependent occupancies were measured at the targets with ED$_{50}$ values for the 5-HT$_{1B}$ receptor and the SERT of 3.2 and 0.4 mg/kg, respectively (Fig. 2). SERT occupancy 1 h after oral administration displayed dose-dependent response with an ED$_{50}$ value of 11.7 mg/kg (results not shown).

Bezold-Jarisch-Like Reflex. Bolus administration of 5-HT (0.06 mg/kg i.v.) in anesthetized rats induced a tran-

TABLE 2
Overview of pharmacokinetic parameters of Lu AA21004 obtained in rats after intravenous, subcutaneous, or oral administration

Parameter	Value
Intravenous dosing (1.5 mg/kg)	
CL, l · h^{-1} · kg^{-1}	3.7
Vz, l/kg	12.7
Subcutaneous dosing (2 mg/kg)	
T_{max}, h	0.8
C_{max}, nM	281
F, %	70
$t_{1/2}$, h	2.5
Oral dosing (6 mg/kg)	
T_{max}, h	1.0
C_{max}, nM	70
F, %	7
$t_{1/2}$, h	3.8

CL, clearance; Vz, volume of distribution during terminal phase; F, bioavailability; $t_{1/2}$, elimination half-life.
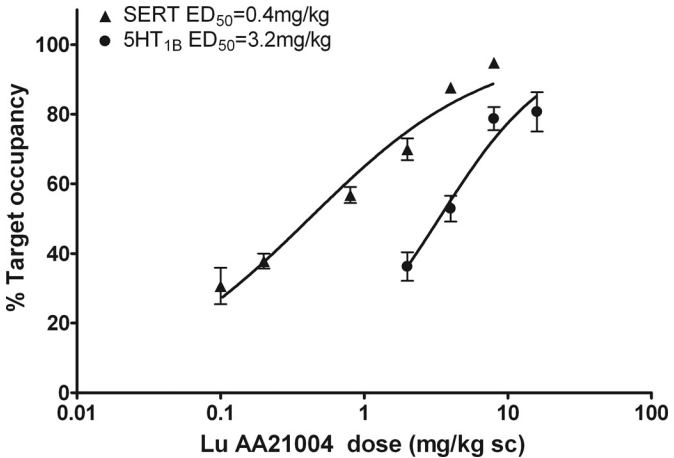

Fig. 2. SERT and r5-HT$_{1B}$ receptor occupancy ex vivo after administration of Lu AA21004 subcutaneously. Animals were pretreated with the compounds for 1 h. Data are expressed as means ± S.E.M. (n = 3/dose).

sient decrease in heart rate (bradycardia). The mean percentage reductions in heart rate ($\pm$S.D.) after the first 5-HT bolus were 31 $\pm$ 8 and 32 $\pm$ 11% in the Lu AA21004-treated ($n = 42$) and ondansetron-treated ($n = 38$) groups, respectively. Lu AA21004 (0.01–3.0 mg/kg s.c.) and the selective 5-HT$_3$ receptor antagonist, ondansetron (0.003–0.3 mg/kg s.c.), affected the Bezold-Jarisch reflex in the rat dose dependently, inhibiting transient bradycardia with ED$_{50}$ (95% confidence interval) values of 0.11 (0.07–0.16) and 0.021 (0.015–0.028) mg/kg, respectively (Fig. 3).

Effects of Lu AA21004 on Extracellular Levels of Monoamines. Basal levels of 5-HT, DA, and NA in the dialysis samples from the ventral hippocampus were 4.8 $\pm$ 0.3 ($n = 74$), 2.4 $\pm$ 0.2 ($n = 70$), and 18.3 $\pm$ 2.5 ($n = 87$) fmol/sample. Moreover, basal levels of 5-HT, DA, and NA in the dialysis samples from the medial prefrontal cortex were 8.6 $\pm$ 1.5 ($n = 74$), 7.6 $\pm$ 0.8 ($n = 76$), and 18.5 $\pm$ 1.1 ($n = 72$) fmol/sample.

Relative to vehicle, Lu AA21004 (2.5–10.0 mg/kg s.c.) increased extracellular levels of 5-HT, DA, and NA in the medial prefrontal cortex and in the ventral hippocampus (Fig. 4, a and b). Lu AA21004 exerted a dose-dependent increase in extracellular levels of the neurotransmitters. In the medial prefrontal cortex, all doses tested significantly increased the 5-HT levels, 5.0 and 10.0 mg/kg increased NA levels, and 5.0 mg/kg also increased the levels of DA. In the ventral hippocampus, all doses significantly increased the 5-HT levels. Significant increases in the levels of the DA and NA were observed at 10 mg/kg.

Ondansetron Potentiates the Effect of Citalopram on 5-HT Levels. A single injection of ondansetron (1.6 mg/kg s.c.) did not affect the extracellular 5-HT levels. Injection of citalopram alone (4.0 mg/kg s.c.) increased the extracellular 5-HT levels in both brain regions. When ondansetron was injected in combination with citalopram, ondansetron potentiated citalopram-induced increases in extracellular 5-HT levels in the ventral hippocampus ($P = 0.0463$) and the medial prefrontal cortex ($P = 0.0144$) (Fig. 5, a and b).

Extracellular 5-HT Levels and In Vivo SERT Occupancy after Subchronic Treatment with Lu AA21004. The subchronic effect of Lu AA21004 was studied by treating rats for 3 days with Lu AA21004 (5.0 mg/kg per day, minipump subcutaneously). A significant increase in the extra-
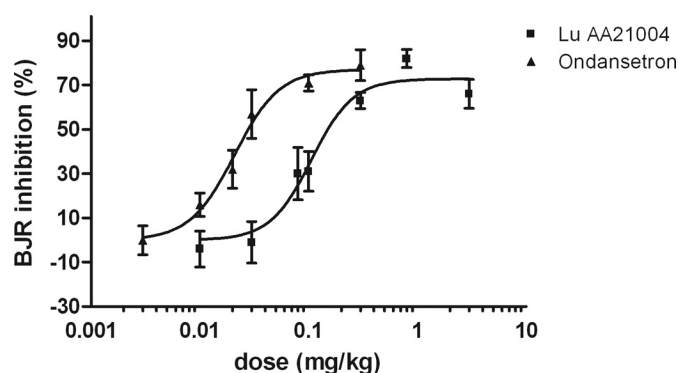

Fig. 3. Effect of Lu AA21004 and ondansetron on the Bezold-Jarisch reflex (BJR) in anesthetized rats. Lu AA21004 ($n = 6$/dose) and ondansetron ($n = 6$–8/dose) dose dependently inhibited the transient bradycardia with ED$_{50}$ (95% confidence interval) values of 0.11 (0.07–0.16) and 0.021 (0.015–0.028) mg/kg, respectively. Data are expressed as means $\pm$ S.E.M.
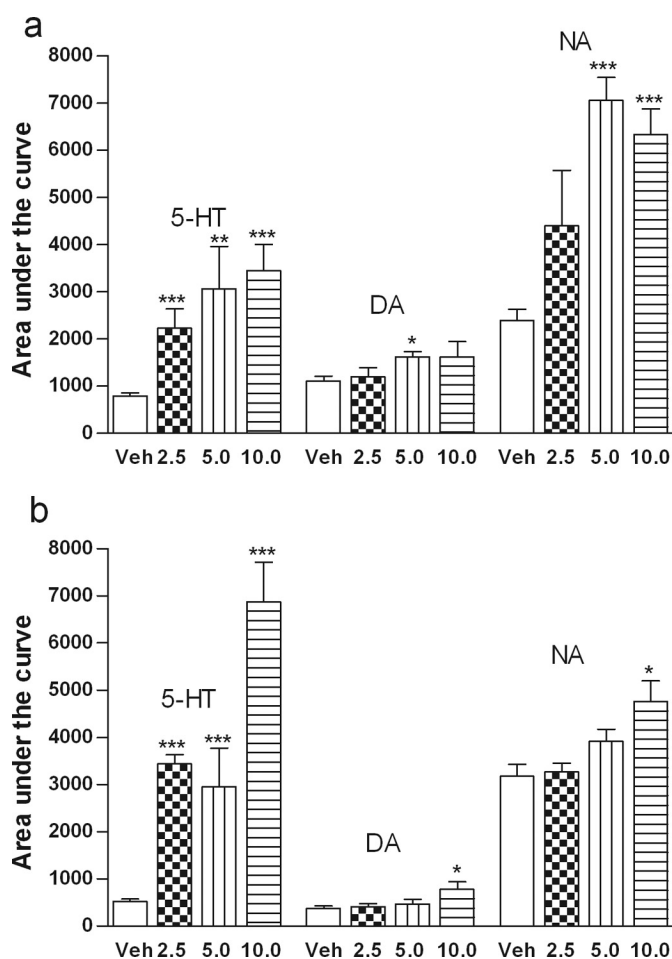

Fig. 4. Effects of subcutaneous Lu AA21004 (2.5 mg/kg, $n = 5$; 5 mg/kg, $n = 5$; 10 mg/kg, $n = 5$) or vehicle (Veh, $n = 5$–7) administration on extracellular levels of 5-HT, DA, and NA in the rat medial prefrontal cortex (a) or the ventral hippocampus (b). All AUCs, regardless of whether being baseline or postbaseline AUCs, were calculated using the trapezoid rule based on the average value of each pair of points multiplied by the sample duration. Statistical analysis was performed using one-way ANCOVA with baseline AUC as a continuous covariate and doses of Lu AA21004 as a categorical variable. Post hoc pairwise analyses were performed using Dunnett's correction. Data are expressed as means $\pm$ S.E.M. *, $P < 0.05$ versus vehicle; **, $P < 0.01$ versus vehicle; ***, $P < 0.001$ versus vehicle.

cellular levels of 5-HT (200 $\pm$ 38%) in the ventral hippocampus was observed after 3 days of treatment ($P < 0.05$ versus vehicle; one-way ANOVA followed by post hoc Bonferroni test). In these animals, the in vivo occupancy of Lu AA21004 at the SERT was 41 $\pm$ 9% (Fig. 6).

Rat Forced Swim Model. FSL rats displayed significantly increased immobility and decreased swimming and climbing behavior compared with those of FRL rats (Fig. 7, a–c). Lu AA21004 at 7.8 mg/kg s.c. ($P < 0.05$) and imipramine at 15.0 mg/kg i.p. ($P < 0.001$) significantly decreased the immobility time in the FSL rats but not in the FRL rats (Fig. 7a). No significant effect was seen for Lu AA21004 and imipramine with respect to swimming behavior in either FSL or FRL rats (Fig. 7b). Imipramine significantly increased the climbing behavior in both FSL and FRL rats ($P < 0.001$), whereas Lu AA21004 did not exert any change (Fig. 7c). Furthermore, imipramine, but not Lu AA21004, significantly reduced locomotor activity in both FSL and FRL rats in the open field test (data not shown).
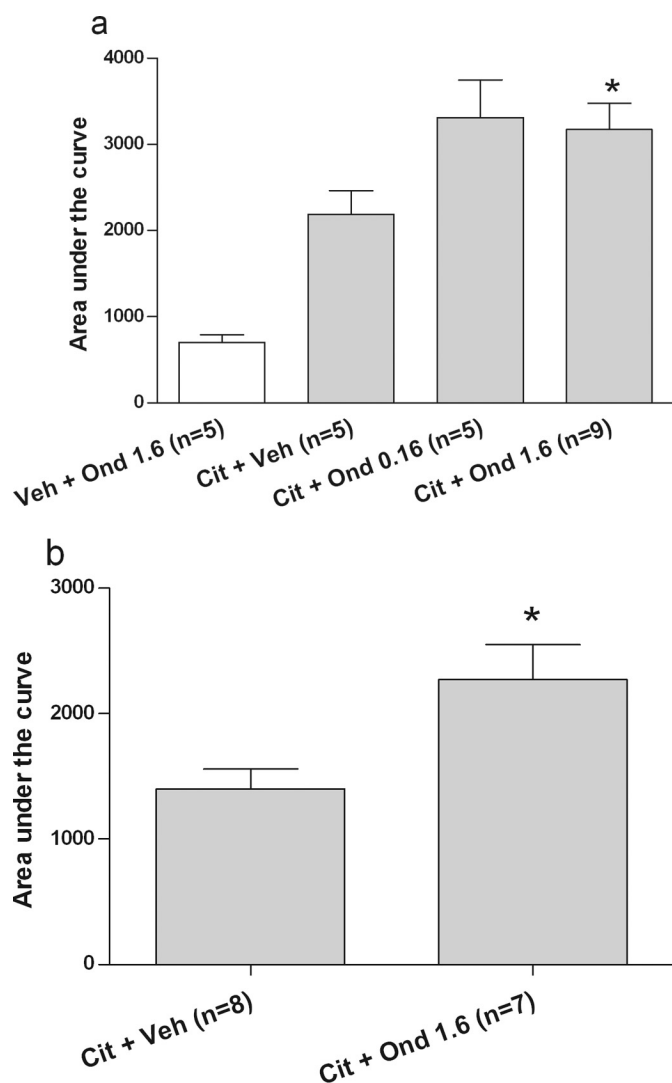
a

b

Fig. 5. Effect of ondansetron (Ond) (0.16 or 1.6 mg/kg s.c.) and citalopram (Cit) (4.0 mg/kg s.c.) on extracellular levels of 5-HT in the rat ventral hippocampus (a) and the medial prefrontal cortex (b). The AUC for samples immediately preceding compound administration served as the baseline AUC and the AUC for samples immediately after compound administration served as the response outcome. Before analysis baseline and postbaseline AUCs were log-transformed. All AUCs, regardless of whether being baseline or postbaseline AUCs, were calculated using the trapezoid rule based on the average value of each pair of points multiplied by the sample duration. Statistical analysis was performed using one-way ANCOVA with baseline AUC as a continuous covariate and doses of ondansetron as a categorical variable. Post hoc pairwise analyses were performed using Dunnett's correction. Data are expressed as means ± S.E.M. a, P (Cit + Ond 0.16 versus Cit + Veh) = 0.064; ∗, P (Cit + Ond 1.6 versus Cit + Veh) = 0.0463. b, ∗, P (Cit + Ond 1.6 versus Cit + Veh) = 0.0144. Veh, vehicle.

Social Interaction. Lu AA21004 (2.0, 4.0, or 8.0 mg/kg p.o.) produced an increase in social interaction. The magnitude of the response was similar to that observed with the anxiolytic chlordiazepoxide (5.0 mg/kg p.o.) (Fig. 8). Lu AA21004 also produced a small, but significant, increase in locomotor activity (result not shown). The positive control, chlordiazepoxide, produced a significant increase in social interaction without affecting motor activity or rearing.

Conditioned Fear-Induced Vocalization. Lu AA21004 showed a dose-dependent anxiolytic-like effect in the conditioned fear assay, in which animals receiving doses of 3.9 and
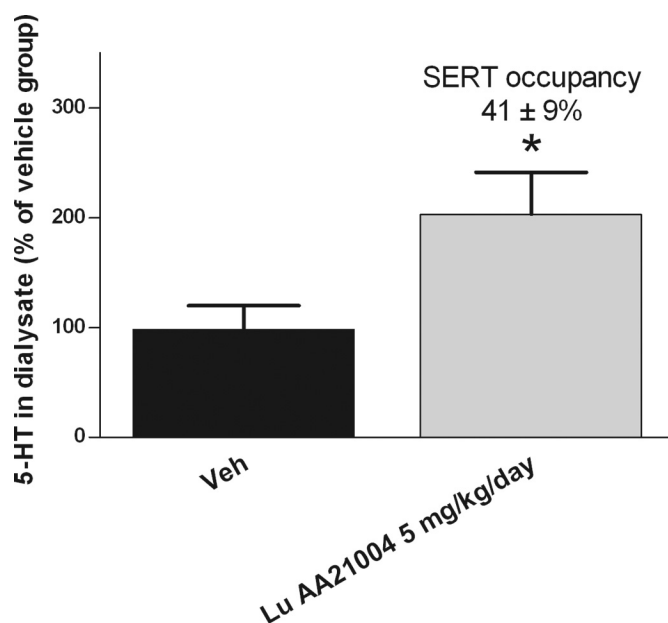

Fig. 6. Subchronic effect of Lu AA21004 on extracellular 5-HT in the ventral hippocampus was studied by treating rats for 3 days with Lu AA21004 (5 mg/kg per day s.c., n = 8) or vehicle (Veh, n = 12) using minipumps. Data are expressed as means ± S.E.M. ∗, P < 0.05, 5 mg/kg versus vehicle group.

7.9 mg/kg s.c. 30 min before the test session emitted significant less fear-induced ultrasonic vocalization compared with vehicle controls (P < 0.05) (Fig. 9). The positive control buspirone at 1.0 mg/kg s.c. also reduced fear-induced vocalization (P < 0.01) (Fig. 9).

Discussion

Lu AA21004 has previously been shown to be a h5-HT$_{3A}$ receptor antagonist (K_i = 3.7 nM), h5-HT$_7$ receptor antagonist (K_i = 19 nM), h5-HT$_{1B}$ receptor partial agonist (K_i = 33 nM), h5-HT$_{1A}$ receptor agonist (K_i = 15 nM), and hSERT inhibitor (K_i = 1.6 nM) (Bang-Andersen et al., 2011).

The pharmacokinetics of Lu AA21004 obtained after subcutaneous administration showed that high systemic plasma levels could be obtained within the 1st h after administration followed by a moderate plasma elimination half-life, corroborating with the time frame during which the in vivo experiments were conducted. Furthermore, the blood-brain barrier penetration was confirmed by measuring target occupancy of Lu AA21004 on the SERT and the 5-HT$_{1B}$ receptor 1 h after administration.

In the present study, we confirmed that Lu AA21004 displays a partial agonistic profile at the h5-HT$_{1B}$ receptor using a whole-cell-based cAMP assay. As measured by ex vivo binding, r5-HT$_{1B}$ receptors are engaged at the doses used in the in vivo studies and are therefore likely to contribute to the net effect of Lu AA21004. The combination of SERT blockade and 5-HT$_{1B}$ receptor antagonism increases the extracellular 5-HT levels in the prefrontal cortex (de Groote et al., 2003). Moreover, a 5-HT$_{1B}$ receptor agonist increases the firing rate of serotonergic neurons in the dorsal and medial raphe nucleus and increases extracellular levels of 5-HT in the median raphe nucleus (Adell et al., 2001). Thus, the partial agonistic effect of Lu AA21004 at the h5-HT$_{1B}$ receptor may translate into a modulatory control of 5-HT$_{1B}$ recep-
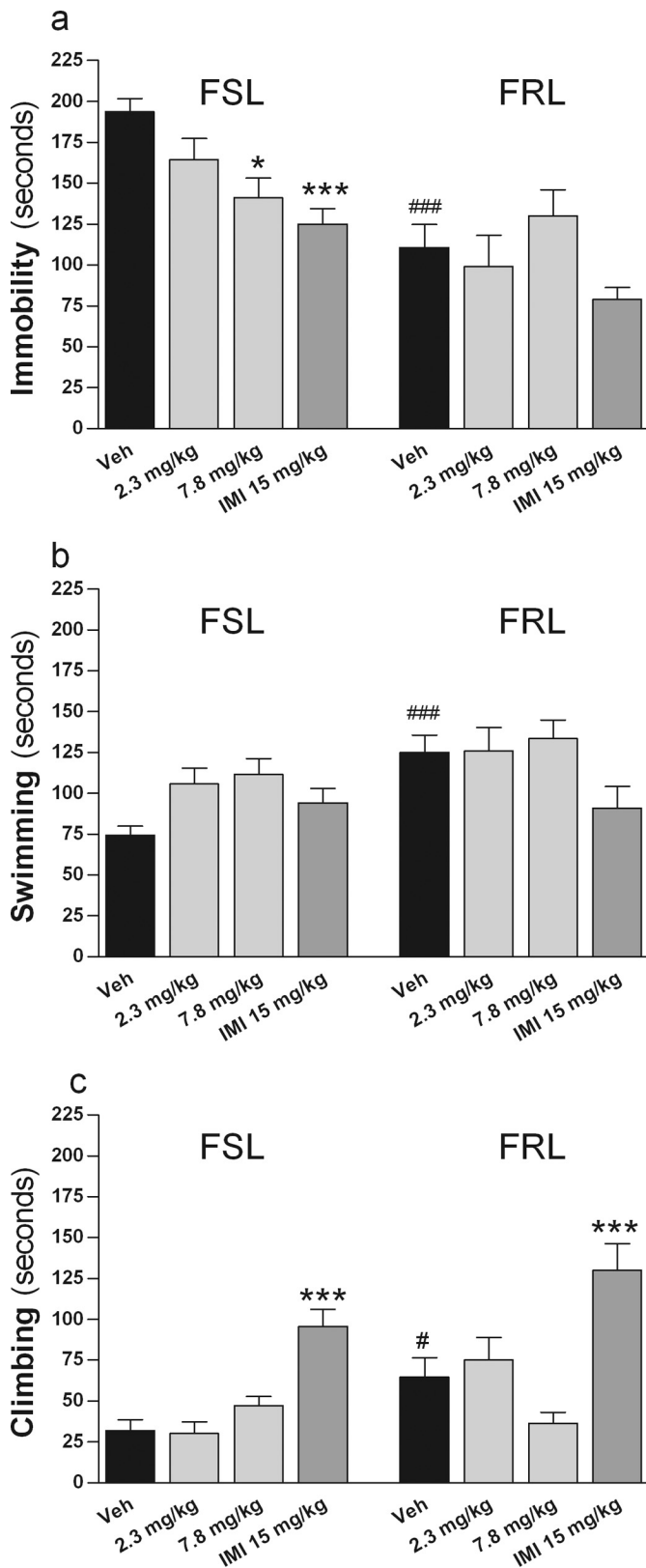
a

b

c

Fig. 7. a, effects of Lu AA21004 (2.3 or 7.8 mg/kg s.c.), imipramine (IMI) (15 mg/kg i.p.) or vehicle (Veh) on the immobility time in the rat forced swim test in FSL and FRL rats. Compounds were administered 24, 6, and 1 h before the forced swim test procedure. Data are expressed as means ± S.E.M. ($n = 7$–10). *, $P < 0.05$; ***, $P < 0.001$ versus FSL Veh; ###, $P < 0.001$, FSL Veh versus FRL Veh. b, effects of Lu AA21004, IMI, or Veh on swimming activity in the rat forced swim test ($n = 7$–10). ###, $P < 0.001$, FSL Veh versus FRL Veh.
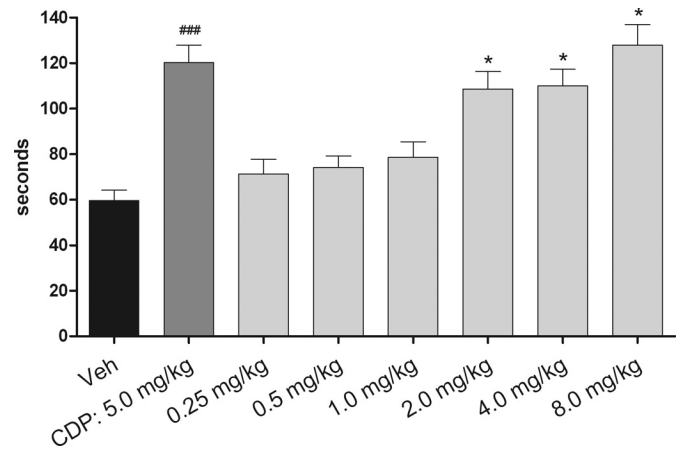

Fig. 8. Effect of Lu AA21004 on rat social interaction. Lu AA21004 (0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 mg/kg p.o.), vehicle (Veh) orally, or chlordiazepoxide (CDP) (5 mg/kg p.o.) was administered 1 h before the test. Data are expressed as means ± S.E.M. ($n = 5$). *, $P < 0.05$ versus vehicle; ###, $P < 0.001$ versus Veh.
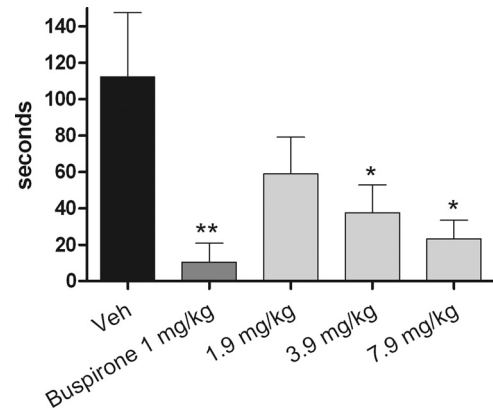

Fig. 9. Effects of Lu AA21004 on conditioned fear-induced ultrasonic vocalization. The rats were pretreated with Lu AA21004 (1.9, 3.9, or 7.9 mg/kg s.c.), vehicle (Veh) subcutaneously, or the positive control, buspirone (1 mg/kg s.c.) 30 min before the test. Data are expressed as means ± S.E.M. ($n = 8$). *, $P < 0.05$ versus Veh; **, $P < 0.01$ versus Veh.

tors in vivo and possibly play a role in the clinical effect of Lu AA21004, because genetic and postmortem studies indicate that mutations of this receptor subtype are implicated in psychiatric disorders, including major depression (Ruf and Bhagwagar, 2009).

Lu AA21004 is an antagonist at 5-HT$_7$ receptors. In preclinical models, 5-HT$_7$ receptor antagonists exert an antidepressive-like effect, augment the actions of SSRIs, and enhance the tonic activation of postsynaptic 5-HT$_{1A}$ receptors in the hippocampus (Bonaventure et al., 2007; Sarkisyan et al., 2010). The 5-HT$_7$ receptor is also involved in sleep regulation, because the 5-HT$_7$ receptor antagonist, (2R)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]-pyrrolidine) hydrochloride (SB-269970) augments rapid eye movement latency and decreases rapid eye movement sleep duration (Bonaventure et al., 2007). At present, we have no direct evidence that the 5-HT$_7$ receptor is involved in the modulation of neurotransmitter levels or the behavioral effects in our preclinical models. The 4- to 10-fold lower in vitro potency of Lu AA21004 at the r5-HT$_7$

c, effects of Lu AA21004, IMI, or Veh on climbing activity in the rat forced swim test ($n = 7$–10). ***, $P < 0.001$ versus FSL/FRL Veh; #, $P < 0.05$, FSL Veh versus FRL Veh.

receptor compared with the h5-HT$_7$ receptor may result in an underestimation of its contribution in these models and not fully address the possible contribution to its overall effect in humans.

Lu AA21004 is an agonist at the h5-HT$_{1A}$ receptor (K_i = 15 nM; EC$_{50}$ = 200 nM; IA = 96%) but is considerably weaker at the r5-HT$_{1A}$ receptor (K_i = 230 nM) (Bang-Andersen et al., 2011). The lower 5-HT$_{1A}$ receptor affinity in rodents may have an impact on the extrapolation of animal data to the clinical setting. By activating somatodendritic 5-HT$_{1A}$ receptors, endogenous 5-HT decreases the firing rate of 5-HT neurons in the raphe nuclei and, consequently, decreases terminal 5-HT release (Romero et al., 1996). This decrease has been proposed to be responsible for the delay in onset of the therapeutic effect of antidepressants (Blier and Ward, 2003). Therefore, blockade of somatodendritic 5-HT$_{1A}$ receptors has been suggested to reduce the delay in the onset of the effects of antidepressants in humans (Artigas et al., 2006). As an alternative, a 5-HT$_{1A}$ agonist may rapidly lead to desensitization of the somatodendritic 5-HT$_{1A}$ receptors (Assié et al., 2006) and, at the same time, activate postsynaptic 5-HT$_{1A}$ receptors that mediate at least part of the therapeutic actions of antidepressants (Blier and Ward, 2003). Thus, it is envisioned that Lu AA21004 may have an improved therapeutic effect by desensitizing the somatodendritic and activating postsynaptic 5-HT$_{1A}$ receptors.

Lu AA21004 displays high-affinity binding for the h5-HT$_{3A}$ receptor and potent functional antagonism at cloned rat and human 5-HT$_{3A}$ receptors (Bang-Andersen et al., 2011). The present data from the rat Bezold-Jarisch reflex assay demonstrate that Lu AA21004 is a potent 5-HT$_3$ receptor antagonist in vivo. In the forebrain, 5-HT$_3$ receptors are primarily located on inhibitory GABAergic interneurons (Yan, 2002). Local infusion of 5-HT or the selective 5-HT$_3$ receptor agonist, 2-methyl-5-HT, into the rat hippocampus inhibits the K$^+$-evoked release of NA, and this is counteracted by the 5-HT$_3$ receptor antagonist, ondansetron (Matsumoto et al., 1995). This result suggests that NA release is under inhibitory serotonergic control through the 5-HT$_3$ receptor. Furthermore, local infusion of the 5-HT$_3$ receptor agonist, SR57227 reduced the firing of locus ceruleus neurons and decreased extracellular levels of NA in the prefrontal cortex, an effect blocked by the 5-HT$_3$ receptor antagonist, Y25130 (Ortega et al., 2006).

Here, we show that ondansetron did not affect basal extracellular 5-HT levels. In contrast, ondansetron potentiated citalopram-induced increases in extracellular 5-HT levels, suggesting that the 5-HT$_3$ receptor exerts an inhibitory tone on the activated serotonergic system. It may be hypothesized that diminished GABAergic tone is responsible for the potentiation of the SSRI effect. As described previously, ondansetron augments the antidepressant-like effects of current antidepressants in preclinical models (Ramamoorthy et al., 2008).

Lu AA21004 shows high-affinity binding for the cloned hSERT transporter and potent inhibition of human and rat SERT (Bang-Andersen et al., 2011). In line with this, we found that systemic administration of Lu AA21004 produces a potent dose-dependent increase in occupancy at the SERT in the rat brain. In the microdialysis studies, Lu AA21004 increased extracellular levels of 5-HT, DA, and NA in both the medial prefrontal cortex and the ventral hippocampus, levels of 5-HT being most affected. The effect on the NA and DA levels is probably exerted indirectly through receptor modulation, because Lu AA21004 shows low potency in functional assays of NA and DA transporter inhibition (IC$_{50}$ = 140 and 890 nM, respectively) (Bang-Andersen et al., 2011). Studies on symptoms of major depressive disorder suggest a specific role also for NA and DA in this disorder (Moret and Briley, 2011; Wu et al., 2011).

Subchronic administration of Lu AA21004 (5 mg/kg per day for 3 days) revealed a significant increase in extracellular levels of 5-HT in the rat ventral hippocampus, whereas the in vivo occupancy of the SERT was 41%. Thus, in contrast to SSRIs and SNRIs, significant increases in 5-HT levels are obtained at low SERT occupancy with Lu AA21004. In a positron emission tomography study of Lu AA21004 in healthy men, 2.5, 10, and 60 mg/day resulted in SERT occupancy levels of 27, 54, and 68%, respectively (Areberg et al., 2009). From the relationship between the plasma concentrations of Lu AA21004 and SERT occupancy in the positron emission tomography study, it was predicted that doses of 5 mg/day (corresponding to approximately 40% SERT occupancy) or higher would be clinically effective. This prediction was confirmed in a phase II study in depressed patients, in which 5 and 10 mg/day were effective and well tolerated (Areberg et al., 2009; Alvarez et al., 2011). SSRI doses that separate from placebo in studies of major depressive episodes correspond to approximately 80% SERT occupancy (Meyer, 2007).

Although all known antidepressants reduce behavioral immobility in the forced swim test, those that predominantly increase serotonergic neurotransmission mainly increase swimming behavior, whereas compounds that block NA reuptake increase climbing behavior (Cryan et al., 2005). Lu AA21004 and imipramine showed dose-dependent antidepressant-like effects in FLS rats in the forced swim test by decreasing the immobility time, but the compounds did not exert any effect on the swimming behavior in this FSL/FRL rat model. Furthermore, Lu AA21004 had no significant effect on climbing behavior despite producing increased extracellular NA levels in the brain. Whether this result suggests qualitatively different pharmacological profiles for Lu AA21004 and NA reuptake blocking drugs remains to be studied in further detail. In the rat social interaction test, short-term administration of Lu AA21004 displayed an anxiolytic-like effect similar to that of chlordiazepoxide. In contrast, an anxiolytic-like effect by SSRIs or SNRIs usually requires subchronic or chronic treatment in this model (Artaiz et al., 2005; Starr et al., 2007). The selective 5-HT$_3$ antagonist BRL 46470A exerts an short-term anxiolytic-like activity in the rat social interaction test (Blackburn et al., 1993). As a result of the latter observation, the low bioavailability, and SERT occupancy below 50% after the oral doses, it may be speculated that the 5-HT$_3$ receptor is heavily involved in the positive effect of Lu AA21004 in this test. Shock conditioning of rats induces increased ultrasonic vocalization when the rats are placed in the test chamber on the following day. Lu AA21004 and the 5-HT$_{1A}$ receptor agonist, buspirone, reduced the ultrasonic vocalization. Given the low affinity of Lu AA21004 for the r5-HT$_{1A}$ receptor, the anxiolytic-like effect of Lu AA21004 in the conditioned fear test may be ascribed to its other targets.

In conclusion, Lu AA21004 displays a unique pharmacological profile via two pharmacological modalities, SERT inhibition and modulation of 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_3$, and 5-HT$_7$ receptors. Lu AA21004 increased brain levels of multiple neurotrans-

mitters and had antidepressant- and anxiolytic-like profiles in preclinical models at doses at which various targets are occupied. The multimodal activity profile of Lu AA21004 is different from that of current antidepressants, which may translate into a unique therapeutic profile. Clinical testing is currently in progress to assess this possibility.

Authorship Contributions

Participated in research design: Mørk, Pehrson, Brennum, Møller Nielsen, Zhong, Lassen, Miller, Westrich, Boyle, Sanchez, Wegener, Hogg, Bang-Andersen, and Bryan Stensbøl.

Conducted experiments: Mørk, Pehrson, Brennum, Møller Nielsen, Zhong, Lassen, Miller, Westrich, Boyle, Fischer, and Liebenberg.

Contributed new reagents or analytic tools: Wegener.

Performed data analysis: Mørk, Pehrson, Brennum, Møller Nielsen, Zhong, Lassen, Miller, Westrich, Boyle, Wegener, and Bundgaard.

Wrote or contributed to the writing of the manuscript: Mørk, Pehrson, Brennum, Møller Nielsen, Zhong, Lassen, Miller, Westrich, Boyle, Sanchez, Fischer, Liebenberg, Wegener, Bundgaard, Hogg, Bang-Andersen, and Bryan Stensbøl.

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