Pharmacological effects of Lu AA21004: a novel multimodal compound for the treatment of major depressive disorder


H. Lundbeck A/S, Valby-Copenhagen, Denmark (AM, LTB, SMN, ABL, CB, SH, BBA, TBS), Lundbeck Research USA Inc., New Jersey, USA (AP, HZ, SM, LW, NJB, CS) and Centre for Psychiatric Research, University of Aarhus, Risskov, Denmark (CWF, NL, GW)
Running title page

Effects of Lu AA21004 on transmitter function and behaviour

Corresponding author:
Arne Mørk
Synaptic Transmission 1
H. Lundbeck A/S
Ottiliavej 9
DK-2500 Copenhagen-Valby
Denmark
Phone: +45 36 43 22 89
Fax: +45 36 43 82 32
E-mail: arm@lundbeck.com

Number of text pages: 42
Number of tables: 2
Number of figures: 9
Number of references: 40
Number of words:
  Abstract: 245
  Introduction: 726
  Discussion: 1500

Nonstandard abbreviations: (1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine: Lu AA21004, SERT: 5-HT transporter, IA: intrinsic activity; FSL: Flinders sensitive line.

Recommended section assignment: Neuropharmacology
ABSTRACT

Lu AA21004 (1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine) is a human serotonin (h5-HT)\textsubscript{3A} receptor antagonist (K\textsubscript{i}=3.7nM), h5-HT\textsubscript{7} receptor antagonist (K\textsubscript{i}=19nM), h5-HT\textsubscript{1B} receptor partial agonist (K\textsubscript{i}=33nM), h5-HT\textsubscript{1A} receptor agonist (K\textsubscript{i}=15nM) and a h5-HT transporter (hSERT) inhibitor (K\textsubscript{i}=1.6nM) (Bang-Andersen et al., 2011). Here we confirm that Lu AA21004 is a partial h5-HT\textsubscript{1B} receptor agonist (EC\textsubscript{50}=460nM, IA=22\%) using a whole cell cAMP-based assay and demonstrate that Lu AA21004 is a rat (r)5-HT\textsubscript{7} receptor antagonist (K\textsubscript{i}=200nM and IC\textsubscript{50}=2080nM). In vivo, Lu AA21004 occupies the r5-HT\textsubscript{1B} receptor and rSERT (ED\textsubscript{50}=3.2mg/kg and 0.4mg/kg, respectively) after subcutaneous (sc) administration and is a 5-HT\textsubscript{3} receptor antagonist in the Bezold-Jarisch reflex assay (ED\textsubscript{50}=0.11mg/kg sc). In rat microdialysis experiments, Lu AA21004 (2.5-10.0mg/kg sc) increased extracellular 5-HT, dopamine and noradrenaline in the medial prefrontal cortex and ventral hippocampus. Lu AA21004, 5mg/kg/day for 3 days (minipumps sc), corresponding to 41\% rSERT occupancy, significantly increased extracellular 5-HT in the ventral hippocampus. Furthermore, the 5-HT\textsubscript{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 3.9mg/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 where
targets in addition to the SERT are occupied. The multimodal activity profile of Lu AA21004 is distinct from current antidepressants.
INTRODUCTION

Major depressive disorder is a highly prevalent, disabling disease with negative impact on medical health, life quality and productivity (Baune et al., 2007; Kessler et al., 2006). 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 transmitter systems. However, multi-transmitter 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 to selective compounds; see review by 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 remitted patients (Booij et al., 2005).

Encouraging clinical data demonstrated an accelerated time to effect of SSRIs by adjunctive use of the 5-HT1A receptor partial agonist, pindolol (Artigas et al., 2006), which inhibits somatodendritic 5-HT1A autoreceptors and augments 5-HT levels in the forebrain following acute SSRI administration (Gardier et al., 1996). Moreover, 5-HT1B autoreceptors located on serotonergic nerve terminals modulate 5-HT neurotransmission. Thus, a 5-HT1B receptor agonist decreases extracellular 5-HT levels in the mouse prefrontal cortex and a 5-HT1B receptor antagonist augments SSRI-induced increases in cortical 5-HT levels in microdialysis studies (De Groote et al., 2003). 5-HT3 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-HT7 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, since the FSL rat has lower density of 5-HT₁₆ receptors but a higher density of 5-HT₁₅ receptors in several brain regions compared to 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) vocalisation in rats seems to reflect their emotional state and drug-induced reduction of ultrasonic vocalization induced by an aversive stimulus is used as an index of anxiolytic activity (Kikusui et al., 2001).

Lu AA21004 ((1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine) is a h5-HT₃₆ receptor antagonist (Kᵢ=3.7nM), h5-HT₇ receptor antagonist (Kᵢ=19nM), h5-HT₁₅ receptor partial agonist (Kᵢ=33nM), h5-HT₁₆ receptor agonist (Kᵢ=15nM) and a h5-HT transporter (hSERT) inhibitor (Kᵢ=1.6nM) (Bang-Andersen et al., 2011).

Here, we elaborate 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-HT₁₅ receptor.
MATERIALS AND METHODS

Materials

1-[2-(2,4-Dimethyl-phenylsulfanyl)-phenyl]piperazine, HBr (Lu AA21004) was synthesized at the Department of Medicinal Chemistry Research, H. Lundbeck A/S (Figure 1) (Bang-Andersen et al., 2011). Lu AA21004 (mw=379.4) was dissolved in 10% beta-hydroxypropyl cyclodextrin and administered as single injections sc, po or administered via minipumps for 3 days prior to the experiments. Imipramine (Sigma Aldrich) was dissolved in 10% beta-hydroxypropyl cyclodextrin and administered intraperitoneally (ip). Ondansetron (Sequoia Research) was dissolved in saline and administered sc. In the Bezold-Jarisch reflex studies, ondansetron was dissolved in 10% beta-hydroxypropyl cyclodextrin and administered sc. All doses of Lu AA21004 are expressed in mg base per kg body weight. For the three-day treatment, osmotic minipumps (Alzet, 2ML1) were filled under aseptic conditions and implanted sc under hypnorm/dormicum anaesthesia. The experiments were carried out with the minipumps onboard.

Animals

The rats were pair housed in temperature and humidity controlled environment under a 12-h light/dark cycle (lights on at 06.00 a.m.). Food and water were available ad libitum. The rats had a minimum of 5 days adaptation in the animal facility prior to the initiation of experiments. All animal procedures were carried out in compliance with EC 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 for Health Guide for the Care and Use of Laboratory animals and approved by the local IACUC.

**Functional assay for the human 5HT₁B receptor**

The functional intrinsic activity (IA) was evaluated using a functional whole cell assay performed as a cAMP assay. HELA cells stably expressing human serotonin 5-HT₁B receptors were harvested using Sigma cell dissociation buffer and counted on a NucleoCounter (Chemometec). To each well of a 384-well Optiplates was added 3µl of test compound followed by 10µl of cell suspension containing Lance cAMP antibody and 2000 cells. The plates were incubated at 16°C for 20min before 3µl of a rolipram/forskolin solution was added (final concentration of rolipram and forskolin were 10µM and 1µM, respectively). The plates were incubated for additional 20min at 16°C followed by 10min at RT prior to addition of 15µl of Lance detection buffer. The plates were incubated at RT for 1-3h before counting on an Envision instrument. All data points were normalized using the minimum and maximum response level defined by each 5-HT curve and analyzed by sigmoidal dose response curve-fittings using Graph Pad Prism 4 to determine the EC₅₀ and IA.

**Binding assay for the rat 5HT₇ receptor**

[^3H]LSD (lysergic acid diethylamide) binding to r5-HT₇ receptor was determined in membranes from a polyclonal HEK-293 cell line expressing the r5-HT₇ receptor. Nonspecific binding was obtained in the presence of 10µM Lu AA21004. Competition binding studies were conducted by displacement of[^3H]LSD using assay conditions recommended by Perkin Elmer in technical data sheet for membranes containing r5-HT₇ receptor (Product No. RBRS7M400UA). Briefly, to each well of a Costar 3365 96-well
microtiter plate, the following were added: 25µl diluted drug (starting concentration of 10µM), 25µl of the [3H]-LSD and 200µl of membrane (5µg/well). The plates were shaken slightly and placed in a 37°C incubator for 1h. The plates were then filtered using a Brandel Harvester and the “filter mat” dried in a convection oven at 55°C for 20min. The filter mat was attached to a Meltilex (solid scintillant) and sealed with a heat sealer before obtaining the counts in a Trilux LSC counter. Lu AA21004 was tested at least three times over a 6 log concentration range. IC\textsubscript{50} values were determined by nonlinear regression analysis using a sigmoidal variable slope curve fitting. The dissociation constant (K\textsubscript{i}) was calculated from the Cheng-Prusoff equation (K\textsubscript{i}=IC\textsubscript{50}/(1 + ([L]/K\textsubscript{D})), where L=concentration of radioligand in the assay, and K\textsubscript{D}=affinity of the radioligand for the receptor; using GraphPad Prism 4.

**Functional assay for the rat 5HT\textsubscript{7} receptor**

The functional activity of r5HT\textsubscript{7} receptor was determined using whole cell cAMP HiRange HTRF assay (Cisbio Bioassays, two step protocol). Briefly, polyclonal HEK293 cell line expressing r5-HT\textsubscript{7} receptor were resuspended in stimulation buffer (1xHBSS with CaCl\textsubscript{2} and MgCl\textsubscript{2}, 5mM HEPES pH=7.4, 0.075% BSA) and counted on a Guava EasyCyte (Millipore). In each well of a 384-well Optiplate, the following were added: 5µl of cells (30000 cells), 2.5µl drug, 2.5µl 5-HT, 5µl of cAMP-D2 conjugate and 5µl Cryptate conjugate as recommended by the kit manufacturer. Plates were incubated with drugs for 30 min (final concentration of Lu AA21004 and metitepine were 100µM and 10µM, respectively). The plates were then incubated for 30min with 5-HT (at EC\textsubscript{80} concentration) at 37°C, and additional 10min at room temperature. Following addition of the conjugates, the plates were incubated for 1h at room temperature before the cell
cAMP content was measured using EnVision 2104 Multilabel Reader (Perkin Elmer, Wallac). Results were calculated from the ratio of absorbance at 665nm/620nm. All data points were normalized using the minimum and maximum response 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 (iv) (1.5mg/kg), sc (2mg/kg) and po (6mg/kg) administration to male Sprague Dawley rats (n=9 per 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 MS/MS detection. One-compartment models were fitted to pooled Lu AA21004 plasma concentration data from sc administration whereas non-compartmental analysis was applied for pooled plasma concentration data obtained after iv and po administration. WinNonlin® (Vers. 5.2, Pharsight Corp.) was used during all pharmacokinetic analyses.

**Ex vivo 5HT$_{1B}$ receptor and SERT occupancy assays**

Adult male Sprague Dawley rats (Charles River labs) were used. Animals (n=3 per dose) were sacrificed by decapitation 1h after the injections and the brains were dissected and flash frozen on powdered dry ice and afterwards stored at -20°C until used.

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

5-HT\textsubscript{1B} receptor occupancy: Lu AA21004 had been injected at doses of 2.0, 4.0, 8.0 or 16.0mg/kg sc, and SB216641 administered at a dose of 7.5mg/kg sc. Slides were preincubated for 3min in a buffer containing 170mM TrisHCl, 4mM CaCl\textsubscript{2}, 0.1% L-ascorbic acid, pH7.4 and were then air-dried at RT for 30-45min. Slides were incubated for 60min in the buffer noted above with 10µM pargyline and 1nM \[^{3}H\]GR125743 (N-(4-methoxy-3-(4-methylpiperazin-1-yl)phenyl)-3-methyl-4-(pyridin-4-yl)benzamide); specific activity 76Ci/mmol; 0.1mCi/ml. Nonspecific binding was determined using 10µM SB 216641 (N-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1'-biphenyl]-4-carboxamide hydrochloride). Slides were washed twice in ice-cold buffer for 5min, then briefly dipped in distilled water and air dried. The slides were transferred into a desiccator and dried for at least 60min. Finally, the slides were exposed using a Beta imager (Biospace, France) for 20h prior to analysis using \(\beta\)-Vision software (Biospace, France) for quantification. Surface radioactivity (expressed as cpm/mm\(^2\)) was measured from a region of interest (ROI) that was defined \textit{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\textsubscript{50} analysis was conducted using GraphPad Prism 4. Briefly, a log transformation was performed fro each LuAA21004 dose. A non-linear 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, while 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 8.0mg/kg sc. Slides were incubated for 90min at RT in buffer (50mM Tris-HCl, 150mM NaCl, and 5mM KCl, pH7.4) containing 0.5nM \[^3\text{H}\]DASB (3-amino-4-[2-[(di(methyl)amino)methyl]phenyl]sulfanylbenzonitrile); 80Ci/mmol; 1mCi/mL. 1µM escitalopram was used to measure nonspecific binding. Slides were washed 3 times in buffer at 4°C for 5min, then briefly dipped in distilled water and air dried. The slides were transferred into a desiccator and dried for at least 60min. Finally, the slides were exposed using a Beta imager for at least 16h prior to analysis as noted above using a separate ROI.

**Bezold-Jarisch reflex**

The 5-HT\(_3\) receptor effect of Lu AA21004 was evaluated in vivo on the Bezold-Jarisch reflex in anaesthetised male Wistar rats (Charles River Laboratories, Germany) and compared to the specific 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 (Villalon and Centurion, 2007). Compounds with 5-HT\(_3\) antagonistic effects inhibit the Bezold-Jarisch reflex. Rats were anaesthetised by inhalation of 5% isoflurane in oxygen in an induction chamber and anaesthesia was maintained with 2% isoflurane in oxygen. The rats were placed on a heating pad and catheters were introduced into 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 10min before a 3s bolus
of 5-HT (0.06mg/kg iv) was administered. The decrease in heart rate induced by 5-HT was measured and set as the maximum (100%) response. Five min after the 5-HT bolus, Lu AA21004 (0.01, 0.03, 0.08, 0.1, 0.3, 0.8 or 3.0mg/kg sc, n=6 per dose) or ondansetron (0.003, 0.01, 0.02, 0.03, 0.1 or 0.3mg/kg sc, n=6-8 per dose) was administered and was followed by a 5-HT bolus injection (0.06mg/kg iv) 30min later. The decrease in heart rate induced by 5-HT was measured and the difference from the previous value was quantified as % inhibition of the Bezold-Jarisch reflex. ED50 values were calculated by non-linear 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 anaesthetised with hypnorm/dormicum (2ml/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: 5.6mm posterior to bregma, lateral -5.0mm, 7.0mm ventral to dura) or the medial prefrontal cortex (coordinates: -3.4mm posterior to bregma, lateral -0.8mm, 5.0mm 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, 0.5mm diameter, 3mm 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 solution (145mM NaCl, 3mM KCl, 1mM MgCl2, 1.2mM CaCl2) 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 1µl/min. After 180min of stabilization, the experiments were initiated and dialysates were collected every 20min. After the experiments, the animals were sacrificed, their brains removed, frozen and sliced for probe placement verification.

In the microdialysis studies, the mean value from 3 or 4 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 while the AUC for samples immediately after compound administration served as the response outcome. Prior to analysis baseline and post-baseline AUCs were log-transformed. All AUCs, regardless of whether being a baseline or post-baseline AUC, 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 AA21004 as 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 150x3mm, 3µm, ESA). The mobile phase consisted of 75mM NaH₂PO₄, 150ml/l sodium octanesulfonic acid, 100µl/l triethylamine and 10% acetonitrile (pH=3.0) at a flow rate of 0.4ml/min. Electrochemical detection was accomplished using a coulometric detector; potential set at 250mV (guard cell at 350mV) (Coulochem II, ESA).
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, 150x2.1mm, 3µm, Keystone Scientific, USA). The mobile phase consisted of sodium acetate buffer (4.1g/l) with methanol (2.5% v/v), Titriplex (150mg/l), sodium octanesulfonic acid (150mg/l) and trimethylamine (150mg/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 pre-treatment with 5mg/kg/day Lu AA21004 for 3 days. The day after the dialysis experiment rats were injected with 60µCi [3H]MADAM (2-(2-dimethylaminomethyl-phenylsulphanyl)-5-methyl-phenylamine), a selective SERT ligand, and were sacrificed 15min after ligand injection. The brain was quickly removed and the cortex was dissected out and homogenized in ice cold buffer (50mM Tris, 120mM NaCl, 5mM KCl, pH=7.4) and the homogenate filtered through Whatman GF/C filters to remove unbound ligand. The level of non-specific binding was determined as the percentage of binding in a group of animals that had been acutely pre-treated with 10mg/kg escitalopram sc; previously shown to induce full occupancy (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 280-320g were cage-housed in pairs at
20±2°C in a 12h light/dark cycle (lights on at 7.00 a.m.). 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 7.8mg/kg sc) and imipramine (15mg/kg ip) were administered 24h, 6h and 1h prior to the forced swim test procedure. A modified 2-day Porsolt swim test (Porsolt et al., 1978) was used as behavioral endpoint. Briefly, the diameter of the cylinder was 24cm, water depth 40cm and temperature 25°C. Mild lighting was placed above the cylinder (120Lux). 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 5min (open field, 1mx1m) with dark floor and dim lighting (40-50Lux). The swim and locomotor behavior on the (second) test day was manually scored by an observer blind to the treatment of the animals (forced swim test) and by using Noldus Ethovision XT 6 for the open field. Output is given as immobility, climbing and swimming in % of total test time (forced swim test) and distance moved (open field). Data were analyzed using two-way analyses of variance (ANOVA) with post-hoc Bonferroni tests to compare the different treatment conditions and Student t-tests to compare the vehicle-treated FSL and FRL rats.

**Social interaction test**

Male Sprague Dawley rats (Charles River, Wilmington, MA) weighing 200–225g at the start of the study were handled daily for 4 days prior to the social interaction test. On the fourth day animals were weighed for the study on day 5. The following day, pairs of weight-matched animals received Lu AA21004 (0.25, 0.5, 1.0, 2.0, 4.0 or 8.0mg/kg
po), vehicle po or chlordiazepoxide (5.0mg/kg po) as the positive control. Sixty min later pairs of rats were introduced into a brightly lit (425Lux) test chamber (54x36x30cm) and their behavior recorded for 10min. 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 behaviour 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, USA). Means and standard errors (SEM) were calculated for each parameter and treatment group. Data were analyzed using one-way analysis of variance (ANOVA) with post-hoc Dunnett’s tests where appropriate (GraphPad Prism 4).

**Conditioned fear-induced vocalisation**

Male Sprague Dawley rats (Charles River, Wilmington, MA) with initial weights of 175–200g were used. The animals were tested in this assay with various compounds over a period of several weeks; with wash-out times determined by the properties of the drugs tested.

The test equipment consisted of 4 chambers, each with grid floors, waste pan, house light, small fan for white noise, video camera, and ultrasonic sound detector. On the first day, animals were put into a chamber, and shock conditioning was administered as 6 presentations of a 8s, 0.8mA shock, using the “varcond6_8sec” protocol in the Freeze Frame software program. The shocks were delivered at varying time points throughout the conditioning session. The total length of the conditioning session was
eight min with an initial acclimation period of approximately 1.5 min and approximately 40 s between shocks. On the second day, the rats were pretreated with Lu AA21004 (1.9, 3.9 or 7.9 mg/kg sc), vehicle sc, or the positive control, buspirone (1.0 mg/kg sc). Thirty min 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 emptied between each session. After completion of the experimental trials, test animals were returned to their home cages. Means and standard deviations were calculated for duration of vocalizations in seconds for each treatment group. Data was analyzed using analysis of variance (ANOVA) followed by Dunnett’s test where appropriate (GraphPad Prism 4).
RESULTS

Effects on the human 5-HT\textsubscript{1B} and rat 5-HT\textsubscript{7} receptor in cloned receptor systems

In a whole cell cAMP-based assay, Lu AA21004 displayed a partial agonistic response with an $EC_{50}=460\pm110\text{nM}$ (SEM, n=12) and an IA of 22 % (Table 1). Furthermore, Lu AA21004 bound to the r5-HT\textsubscript{7} receptor with a $K_i$-value of $200\pm27\text{nM}$ (SEM, n=4) and was a functional antagonist at the r5-HT\textsubscript{7} receptor with an $IC_{50}=2080\pm18\text{nM}$ (SEM, n=4) in an \textit{in vitro} whole cell cAMP assay (Table 1).

Pharmacokinetics

In the \textit{in vivo} studies of Lu AA21004 doses and pretreatment times were selected based on pharmacokinetic parameters and target occupancy. Relevant pharmacokinetic parameters are summarised in Table 2. Maximal plasma levels of Lu AA21004 in the rat were reached following 0.8 and 1h after sc and po administration, respectively. Furthermore, the plasma elimination half-life was approximately 3.2h. The bioavailability of Lu AA21004 was 10-fold lower after po administration compared to sc dosing, which may be ascribed to first-pass metabolism as a relatively high clearance, 3.7 l/h/kg, was observed after iv injection (Table 2).

Ex vivo occupancies at the rat 5-HT\textsubscript{1B} receptor and the rat SERT

One hour following sc administration of Lu AA21004, dose-dependent occupancies were measured at the targets with ED\textsubscript{50} values for the 5-HT\textsubscript{1B} receptor and the SERT of 3.2 and 0.4mg/kg, respectively (Fig 2). SERT occupancy 1h after po administration displayed dose-dependent response with an ED\textsubscript{50} value of 11.7 mg/kg (results not shown).

Bezold-Jarisch-like reflex
Bolus administration of 5-HT (0.06mg/kg iv) in anaesthetised rats induced a transient decrease in heart rate (bradycardia). The mean percentage reductions in heart rate (±SD) after the first 5-HT bolus were 31±8 and 32±11 % in the Lu AA21004 (n=42) and ondansetron-treated (n=38) groups, respectively. Lu AA21004 (0.01–3.0mg/kg sc) and the selective 5-HT3 receptor antagonist, ondansetron (0.003–0.3mg/kg sc) affected the Bezold-Jarisch reflex in the rat dose-dependently inhibiting transient bradycardia with ED50 (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±0.3 (n=74), 2.4±0.2 (n=70) and 18.3±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±1.5 (n=74), 7.6±0.8 (n=76) and 18.5±1.1 (n=72) fmol/sample.

Relative to vehicle, Lu AA21004 (2.5–10.0mg/kg sc) increased extracellular levels of 5-HT, DA, and NA in the medial prefrontal cortex and in the ventral hippocampus (Fig 4a,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 10.0mg/kg increased NA levels, whilst 5.0mg/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 10mg/kg.

Ondansetron potentiates the effect of citalopram on 5-HT levels
A single injection of ondansetron (1.6 mg/kg sc) did not affect the extracellular 5-HT levels. Injection of citalopram alone (4.0 mg/kg sc) 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 5a,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 three days with Lu AA21004 (5.0 mg/kg/day, minipumps sc). A significant increase in the extracellular levels of 5-HT (200±38%) in the ventral hippocampus was observed after 3 days of treatment (P<0.05 vs 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±9% (Fig 6).

**Rat forced swim model**

FSL rats displayed significantly increased immobility and decreased swimming and climbing behaviour compared to FRL rats (Fig 7a,b,c). Lu AA21004 at 7.8 mg/kg sc (P<0.05) and imipramine at 15.0 mg/kg ip (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 behaviour in neither FSL nor FRL rats (Fig 7b). Imipramine significantly increased the climbing behaviour in both FSL and FRL rats (P<0.001), whilst 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).

**Social interaction**

Lu AA21004 (2.0, 4.0 or 8.0mg/kg po) produced an increase in social interaction. The magnitude of the response was similar to that observed with the anxiolytic chlordiazepoxide (5.0mg/kg ip) (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 vocalisation**

Lu AA21004 showed a dose dependent anxiolytic-like effect in the conditioned fear assay, in which animals receiving doses of 3.9 and 7.9 mg/kg sc 30 min prior to the test session emitted significant less fear-induced ultrasonic vocalisation compared to vehicle controls (P<0.05) (Fig 9). The positive control buspirone at 1.0mg/kg sc also reduced fear-induced vocalisation (P<0.01) (Fig 9).
DISCUSSION

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

The pharmacokinetics of Lu AA21004 obtained after sc administration showed that high systemic plasma levels could be obtained within the first hour 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 1h 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$_{1B}$ receptors in vivo and possibly play a role in the clinical effect of Lu AA21004, as genetic and post-mortem 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 antidepressive-like effect, augment the actions of SSRIs, and enhance the tonic activation of postsynaptic 5-HT_{1A} receptors in the hippocampus (Sarkisyan et al., 2010; Bonaventure et al., 2007). The 5-HT_{7} receptor is also involved in sleep regulation, since the 5-HT_{7} receptor antagonist, SB-269970 augments rapid eye movement (REM) latency and decreases REM sleep duration (Bonaventure et al., 2007). Currently we have no direct evidence that the 5-HT_{7} receptor is involved in the modulation of neurotransmitter levels or the behavioural effects in our preclinical models. The 4 to 10-fold lower \textit{in vitro} potency of Lu AA21004 at the r5-HT_{7} receptor compared to the h5-HT_{7} receptor may result in an underestimation of its contribution in these models and not fully address possible contribution to its overall effect in humans.

Lu AA21004 is an agonist at the h5-HT_{1A} receptor (K_{i}=15 \text{nM}; EC_{50}=200 \text{nM}; IA=96%), but is considerably weaker at the r5-HT_{1A} receptor (K_{i}=230 \text{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 have been proposed to be responsible for the delay in onset of the therapeutic effect of antidepressants (Blier and Ward, 2003). Therefore, blocking 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). Alternatively, a 5-HT_{1A}
agonist may rapidly lead to desensitization of the somatodendritic 5-HT\textsubscript{1A} receptors (Assie et al., 2006) and, at the same time, activate postsynaptic 5-HT\textsubscript{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\textsubscript{1A} receptors.

Lu AA21004 displays high affinity binding for the h5-HT\textsubscript{3A} receptor and potent functional antagonism at cloned rat and human 5-HT\textsubscript{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\textsubscript{3} receptor antagonist \textit{in vivo}. In the forebrain, 5-HT\textsubscript{3} receptors are primarily located on inhibitory GABAergic interneurons (Yan, 2002). Local infusion of 5-HT or the selective 5-HT\textsubscript{3} receptor agonist, 2-methyl-5-HT into the rat hippocampus inhibits the K\textsuperscript{+}-evoked release of NA and this is counteracted by the 5-HT\textsubscript{3} receptor antagonist, ondansetron (Matsumoto et al., 1995). This suggests that NA release is under inhibitory serotonergic control through the 5-HT\textsubscript{3} receptor. Furthermore, local infusion of the 5-HT\textsubscript{3} receptor agonist, SR57227 reduced the firing of locus coeruleus neurons and decreased extracellular levels of NA in the prefrontal cortex; an effect blocked by the 5-HT\textsubscript{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\textsubscript{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 previously described, 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, since Lu AA21004 shows low potency in functional assays of NA and DA transporter inhibition (IC$_{50}$=140 and 890nM, 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 (5mg/kg/day for 3 days) revealed a significant increase in extracellular levels of 5-HT in the rat ventral hippocampus, while 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 PET study of Lu AA21004 in healthy men 2.5, 10 and 60mg/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 PET study, it was predicted that doses of 5 mg/day (corresponding to approximately 40% SERT occupancy) or higher would be clinically effective. This 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 behavioural immobility in the forced swim test, those that predominantly increase serotonergic neurotransmission mainly increase swimming behaviour, whereas compounds that block NA reuptake increase climbing behaviour (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 behaviour in this FSL/FRL rat model. Furthermore, Lu AA21004 had no significant effect on climbing behaviour in spite of producing increased extracellular NA levels in the brain. Whether this 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 acute 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 (Starr et al., 2007; Artaiz et al., 2005). The selective 5-HT$_3$ antagonist, BRL 46470A exerts an acute anxiolytic-like activity in the rat social interaction test (Blackburn et al., 1993). Due to the latter observation, the low bioavailability and SERT occupancy below 50% following the used po 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 vocalisation when the rats are placed in the test chamber the following day. Lu AA21004 and the 5-HT$_{1A}$ receptor agonist, buspirone reduced the
ultrasonic vocalisation. Given the low affinity of Lu AA21004 for the r5-HT1A 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-HT1A, 5-HT1B, 5-HT3, and 5-HT7 receptors. Lu AA21004 increased brain levels of multiple neurotransmitters and had antidepressant- and anxiolytic-like profiles in preclinical models at doses where various targets are occupied. The multimodal activity profile of Lu AA21004 is different from that of current antidepressants. This may translate into a unique therapeutic profile. Clinical testing is currently in progress to assess this possibility.
Acknowledgements

The skilful technical assistance of Anette Frederiksen, Mary Dovlatyan, Nina Guldhammer, Lærke B. Jørgensen, Mathias Madsen, Louise Tarpø, Olle Åkerlund is gratefully acknowledged. Furthermore, we thank our colleagues in the Department of Bioanalysis, H. Lundbeck A/S for the generation of the pharmacokinetic data.
Author Contributions

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

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

Contributed new reagents or analytical tools: Wegener.

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

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


data from a naturalistic study on a large sample of inpatients with major depression. *J Affect Disord* **115**:439-449.


Nishi K, Kanemaru K and Diksic M (2009) A genetic rat model of depression, Flinders sensitive line, has a lower density of 5-HT(1A) receptors, but a higher density of 5-HT(1B) receptors, compared to control rats. *Neurochem Int* **54**:299-307.


Legends for figures

Fig 1: Structure of Lu AA21004.

Fig. 2: SERT and r5-HT1B receptor occupancy *ex vivo* after administration of Lu AA21004 sc. Animals were pretreated with the compounds for 1h. Data are expressed as mean±SEM (n=3 per dose).

Fig 3: Effect of Lu AA21004 and ondansetron on the Bezold-Jarisch reflex in anaesthetised rats. Lu AA21004 (n=6 per dose) and ondansetron (n=6-8 per dose) dose-dependently inhibited the transient bradycardia with ED50 (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 mean±SEM.

Fig 4: Effects of subcutaneous Lu AA21004 (2.5mg/kg, n=5, 5mg/kg, n=5, 10mg/kg, n=5) or vehicle (Veh, n=5-7) administration on extracellular levels of 5-HT, DA and NA in a) the rat medial prefrontal cortex or b) the ventral hippocampus. All AUCs, regardless of whether being a baseline or post-baseline AUC, 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 AA21004 as categorical variable. Post-hoc pairwise analyses were performed using Dunnett's correction. Data are expressed as mean±SEM. *P<0.05 vs Veh; **P<0.01 vs Veh; ***P< 0.001 vs Veh.
Fig 5: Effect of ondansetron (Ond) (0.16 or 1.6mg/kg sc) and citalopram (Cit) (4.0mg/kg sc) on extracellular levels of 5-HT in (a) the rat ventral hippocampus and (b) the medial prefrontal cortex. The AUC for samples immediately preceding compound administration served as the baseline AUC while the AUC for samples immediately after compound administration served as the response outcome. Prior to analysis baseline and post-baseline AUCs were log-transformed. All AUCs, regardless of whether being a baseline or post-baseline AUC, 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 categorical variable. Post-hoc pairwise analyses were performed using Dunnett's correction. Data are expressed as mean±SEM. a) P(Cit+Ond 0.16 vs Cit+Veh)=0.064; P(Cit+Ond 1.6 vs Cit+Veh)=0.0463, b) P(Cit+Ond 1.6 vs Cit+Veh)=0.0144.

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 (5mg/kg/day sc, n=8) or vehicle (Veh, n=12) using minipumps. Data are expressed as mean±SEM. *P<0.05, 5 mg/kg vs vehicle group.

Fig 7a: Effects of Lu AA21004 (2.3 or 7.8mg/kg sc), imipramine (IMI; 15mg/kg ip) or vehicle (Veh) on the immobility time in the rat forced swim test in FSL and FRL rats. Compounds were administered 24h, 6h and 1h prior to the forced swim test procedure.
Data are expressed as mean±SEM (n=7-10). *P<0.05, ***P<0.001 vs FSL Veh. ***P<0.001, FSL Veh vs 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 vs FRL Veh. c) Effects of Lu AA21004, IMI or Veh on climbing activity in the rat forced swim test (n=7-10). ***P<0.001 vs FSL/FRL Veh. *P<0.05, FSL Veh vs 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 8.0mg/kg po), vehicle (Veh) po or chlordiazepoxide (CDP, 5mg/kg po) was administered 1h prior to the test. Data are expressed as mean±SEM (n=5) *P<0.05 vs vehicle. ***P<0.001 vs Veh.

Fig 9: Effects of Lu AA21004 on conditioned fear-induced ultrasonic vocalisation. The rats were pretreated with Lu AA21004 (1.9, 3.9, or 7.9mg/kg sc), vehicle (Veh) sc, or the positive control, buspirone (1mg/kg sc) 30min prior to the test. Data are expressed as mean±SEM (n=8). *P<0.05 vs Veh. **P<0.01 vs Veh.
Table 1. In vitro effects of Lu AA21004 on 5-HT\textsubscript{1B} and 5-HT\textsubscript{7} receptors.

<table>
<thead>
<tr>
<th></th>
<th>Ki (nM)</th>
<th>IC\textsubscript{50} (nM)</th>
<th>IA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h5-HT\textsubscript{1B}</td>
<td>33\textsuperscript{a}</td>
<td>120\textsuperscript{a,b}</td>
<td>55\textsuperscript{a,b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>460\textsuperscript{c}</td>
<td>22\textsuperscript{c}</td>
</tr>
<tr>
<td>r5-HT\textsubscript{1B}</td>
<td>16\textsuperscript{d}</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>h5-HT\textsubscript{7}</td>
<td>19\textsuperscript{a}</td>
<td>450\textsuperscript{a}</td>
<td>0</td>
</tr>
<tr>
<td>r5-HT\textsubscript{7}</td>
<td>200</td>
<td>2080</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Bang-Andersen et al (2011); \textsuperscript{b}GTP\textgamma S assay; \textsuperscript{c}cAMP assay; \textsuperscript{d}CEREP study no. 841078; IA: intrinsic activity; n.d.: not determined
Table 2. Overview of pharmacokinetic parameters of Lu AA211004 obtained in rats following intravenous (iv) (1.5mg/kg), subcutaneous (sc) (2mg/kg) or oral (po) (6mg/kg) administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>iv dosing</strong></td>
<td></td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>3.7</td>
</tr>
<tr>
<td>Vz (l/kg)</td>
<td>12.7</td>
</tr>
<tr>
<td><strong>sc dosing</strong></td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.8</td>
</tr>
<tr>
<td>Cmax (nM)</td>
<td>281</td>
</tr>
<tr>
<td>F (%)</td>
<td>70</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>po dosing</strong></td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cmax (nM)</td>
<td>70</td>
</tr>
<tr>
<td>F (%)</td>
<td>7</td>
</tr>
<tr>
<td>T½ (h)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

CL: clearance; Vz: volume of distribution during terminal phase; F: bioavailability; T½: elimination half-life.
Fig 1
Fig 2

- SERT ED$_{50}$ = 0.4 mg/kg
- 5HT$_1B$ ED$_{50}$ = 3.2 mg/kg

% Target occupancy vs. Lu AA21004 dose (mg/kg sc)
Fig 4

Area under the curve

- 5-HT
- DA
- NA

Veh 2.5 5.0 10.0

** ***
Fig 6

5-HT in dialysate (% of vehicle group)

Veh

LU AA21045 mg/kg/day

SERT occupancy

% 6 ± 9%

*