Biosynthesis and identification of an N-oxide/N-glucuronide metabolite and first synthesis of an N-O-glucuronide metabolite of Lu AA21004

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Running title page.

a) Biosynthesis and ID of an N-oxide/N-glucuronide metabolite

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c) Text pages: 27
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   Abstract: 193 words
   Introduction: 419 words
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d) Non-standard abbreviations:

Abbreviations used are: 1, 1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine hydrobromide; 2, 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-ol; 3, 6-{4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-yloxy}-1-β-D-glucuronic acid; 4, 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-1-β-D-glucuronic acid-piperazine 1-oxide; 6, [(2-(2,4-dimethyl-phenylsulfanyl)-phenyl)-(2-hydroxy-ethyl)-amino]-ethanol; 7, 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-morpholin-2-one; 11, (2S,3S,4S,5R,6S)-3,4,5-triacetoxy-6-{4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-yloxy}-tetrahydro-pyran-2-carboxylic acid methyl ester; ELS, evaporative light scattering; HPLC, high-performance liquid chromatography; UDPGA, uridine 5′-diphosphoglucuronic acid; ES+, electrospray positive mode; MS, mass spectrometry; HRMS, high resolution mass spectrometry; IU, International
Units; THF, tetrahydrofuran; AcOEt, ethyl acetate; MeOH, methanol; TBME, \( t\)-butylmethyl ether; DIPEA, N,N-diisopropylethylamine; DMSO, dimethylsulfoxide; NMP, \( N\)-methyl-2-pyrrolidone; TFA, trifluoroacetic acid; CDCl\(_3\), chloroform-\( d\); CH\(_2\)Cl\(_2\), methylene chloride; pyr, pyridine; \( \text{Na}_2\text{SO}_4\), sodium sulfate; AcOH, acetic acid; \( \text{NaHCO}_3\), sodium hydrogen carbonate; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Coherence; ROESY, Rotating frame Overhauser Enhancement Spectroscopy; TOCSY, Total Correlation Spectroscopy
Abstract:

This article describes the biosynthesis and identification of a new class of metabolites, a piperazine N-oxide/N-glucuronide metabolite 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-1-β-D-glucuronic acid-piperazine 1-oxide (4). The metabolite was found in urine and plasma from humans and animals dosed with Lu AA21004, 1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine hydrobromide (1) as a novel multimodal antidepressant under development for treatment of depression. Human liver microsomes in combination with uridine 5’-diphosphoglucuronic acid were used as an in vitro system to generate enough material of (4) to perform 1 and 2D 1H and 13C NMR experiments for structure elucidation. Based on Rotating frame Overhauser Enhancement Spectroscopy NMR experiments, the distance correlation between a piperazine proton and the anomic proton of the glucuronic acid moiety is of a magnitude similar to that of the H-3’ and H-5’ protons, and can only be explained by proximity in space and the postulated structure (4). The structural analogue, the N-O-glucuronic acid conjugate 6-{4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-yloxy}-1-β-D-glucuronic acid (3) was also observed in biological samples from humans and animals and the first organic synthesis and structural identification of this metabolite is also reported. Treatment of the glucuronide metabolites (3) and (4) with β-glucuronidase gave mainly the expected hydrolysis product, the hydroxyl amine 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-ol (2).
Introduction.

Drugs that are piperazine derivatives typically generate piperazine ring carbon alcohols or metabolize at the piperazine nitrogen to generate hydroxylamines/N-oxides as phase I metabolites and N-glucuronides or N-O-glucuronides as phase II metabolites (Delbressine et al., 1992; Kassahun et al., 1996; Miller et al., 2004; Schaber et al., 2001). N-glucuronides are common metabolic products of drugs containing amino-groups or aromatic nitrogen heterocycles and in some cases tertiary, secondary and primary amines form N-glucuronides from a drug and its phase I metabolites (Dalgaard and Larsen, 1999). Porter et al. (1975) were the first to report the formation of quaternary ammonium-linked glucuronide from a tertiary amine in humans. The enzyme involved was later shown to be UGT1A4 (Green and Tephly, 1996). In other cases with tetrazoles and triazoles UGT1A6 (Huskey et al., 1994) or UGT1A9 (Omura et al., 2007) was involved. Also N-glucuronides of pyridine [synthesis, Dalgaard (1983)] and imidazoles [biosynthesis, Vashishtha et al. (2002)] are also known. It appears that liver microsomes from primates, guinea pigs and rabbits form imidazole glucuronides more readily than microsomes from rats and dogs. N-glucuronidation of tertiary piperazines resulting in quaternary structures seems to occur mainly in primates (Chiu and Huskey, 1998).

$N^+$-glucuronidation as a metabolic pathway, and the stability and spectroscopy of the glucuronides has been reviewed by Hawes (1998). A convenient route of synthesis of quaternary ammonium glucuronides has recently been described (Iddon et al., 2010). In some cases it can be a challenge to distinguish whether N- and O-glucuronidation has taken place, e.g., of a hydroxy isoxazole (Andersen et al., 1989) and requires more extensive investigation. The present study describes such a challenge. During investigation of the disposition of Lu AA21004 as a novel multimodal antidepressant agent 1 under development for treatment of
depression (Bang-Andersen et al., 2011) in humans, two major metabolites were observed in plasma and urine. The molecular weight and mass spectra of these metabolites indicate addition of oxygen and glucuronic acid to the piperazine ring of the parent drug. However, LC-MS data alone failed to provide sufficient evidence of the exact structures. The present study describes the identification of the two metabolites 3 and 4 (see Fig. 1) using a novel and unambiguous organic synthesis and biosynthetic approach, respectively. Biosynthesis was performed using UDPGA and human liver microsomes followed by semi-preparative HPLC purification and successful application of NMR spectroscopy. A sufficient amount of 4 was obtained to run NMR experiments in the carbon-observe mode, resulting in NMR data that led to the unambiguous identification of the structure of 4.
Materials and methods

Chemicals. 1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine hydrobromide 1, 4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-ol 2 and (2S, 3S, 4S, 5R, 6S)-6-{4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-yloxy}-3,4,5-trihydroxy-tetrahydro-pyran-2-carboxylic acid 3 were obtained from H. Lundbeck A/S (Copenhagen, Denmark). The purity of 1 and 2 were >99%. The purity of 3 was 81.6% (UV) and 100% (ELS). HPLC grade solvents were from Fisher Scientific Co. (Fair Lawn, NJ) and Sigma-Aldrich (St. Louis, MO). Uridine 5′-diphosphoglucuronic acid trisodium salt, alamethicin, D-saccharic acid 1,4 lactone monohydrate, magnesium chloride, Tris-HCl, β-glucuronidase (type VII-A from E. coli), ammonium acetate, deuterium oxide, and DMSO-d₆ were purchased from Sigma-Aldrich. Potassium dihydrogen phosphate, sodium hydroxide, acetic acid, hydrochloride acid 2N, ammonium hydroxide solution 25% aq, potassium hydroxide, ammonium acetate and formic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile-d₃ was purchased from Cambridge Isotope Laboratories (Andover, MA).

Biological preparations. Human liver microsomes, pool of mixed gender, 20 mg protein/ml was purchased from XenoTech (Lenexa, KS).

Preparative scale biosynthesis of N-oxide/N-glucuronide (4). Pooled human liver microsomes (mixed gender, final concentration, 0.5 mg protein/mL) were added to 100 mL of 100 mM Tris-HCl buffer, pH 7.4 containing 5 mM MgCl₂, 2.5 mM UDPGA, 12.5 μg/mL alamethicin, 5 mM D-saccharic acid 1,4 lactone and 200 μM of the hydroxyl amine 2. This suspension was incubated for 4 h at 37 °C and the reaction was stopped by the addition of ice-
cold (-20 °C) acetonitrile (100 mL). To obtain enough glucuronide metabolite 4 for further investigation, the biosynthesis was repeated 4 times. The ice-cold incubation suspension (200 mL) containing the glucuronide metabolite 4 was centrifuged (16,000 x g, 4 °C, 10 minutes) and evaporated at 35 °C under nitrogen in a TurboVap LV evaporator (Zymark Corp., Hopkinton, MA) until concentrated ca. 17 fold.

**Semi-preparative HPLC-MS purification.** The HPLC-MS system consisted of a G2258A Dual Loop Autosampler PS, a G1311A Quaternary Pump, a G1316A Thermostatted Column Compartment, a G1364B Fraction Collector Prep Scale and a G1956A LC/MSD Quad VL system (Agilent, Santa Clara, CA). The concentrate (approximately 12 mL from the biosynthesis) was diluted with 12 mL of mobile phase A and purified by five successive 5-mL injections on a 10 x 250 mm XBridge C18 column (Waters, Milford, MA) eluted at 4 mL/min with a linear gradient applied (0-20 min) from 70% to 40% mobile phase A (acetonitrile/water 10/90, 0.05% formic acid) and 30% to 60% mobile phase B (acetonitrile/water 90/10, 0.05% formic acid). Fractions containing the glucuronide metabolite 4 with m/z 491 (ES+) and retention time of approximately 11 min were collected and evaporated at 35 °C under a stream of nitrogen. The final yield from the four incubations and subsequent HPLC purifications was approximately 10 mg glucuronide metabolite 4 (yield 25%, needle shaped crystalline material) from 25 mg of starting material 2. See Fig. 10.
Analytical HPLC-MS. Ion trap. The HPLC-MS system consisted of a G1367 Autosampler, a G1312A Binary Pump, a G1316A Thermostatted Column Compartment, a G1322A Degasser (Agilent, Santa Clara, CA) and an LQC Deca XP MS (Thermo Scientific, Waltham, MA). Injections (10 μL) were made on a 2.1 x 100 mm Waters Symmetry C18 column (Waters, Milford, MA) eluted at 0.25 mL/min with a linear gradient applied (0-25 min) from 100% to 50% mobile phase A (acetonitrile/50 mM ammonium acetate pH 5.0 20/80) and 0% to 50% mobile phase B (acetonitrile/50 mM ammonium acetate pH 5.0 80/20) followed by a wash out (100% B, 5 min) and re-equilibration (100% A, 6 min).

MicrOTOF. Accurate masses were obtained on an HPLC-MS system that consisted of a G1313A Autosampler, a G1311A Quarternary Pump, a G1316A Column Oven, a G1322A Degasser (Agilent, Santa Clara, CA) and a Bruker Daltonics MicrOTOF (Bremen, Germany) using an ESI or APPI interface. Injections (5 μL) were made on a 4.5 x 50 mm Waters Sunfire C18 column, 3.5 μM (Waters, Milford, MA) eluted at 2.5 ml/min with a linear gradient applied (0-2 min) from 95% to 0% mobile phase A (water + 0.05% TFA) and 5% to 100% mobile phase B (water/acetonitile 5:95 + 0.03% TFA) followed by wash out (100% B, 0.5 min).

NMR spectroscopy. NMR spectra were recorded at 11.75 or 14.10 Tesla on Bruker instruments (Fällanden, Switzerland). The instruments were an Avance-I-500 equipped with a 5-mm four nucleus probe (1H, 19F, 13C, 15N) with a Z-axis gradient coil, an Avance DRX-500 equipped with a 1-mm triple resonance inverse probe (1H, 13C, 15N) with a Z-axis gradient coil or an Avance-III-600 equipped with a 5-mm triple resonance (1H, 13C, 15N) CryoProbe with a Z-axis gradient. The experiments were run at room temperature (25 °C). Tetramethylsilane or the residual signal from deuterated solvent was used for internal reference. The
compounds were dissolved in deuterated solvents. A mixture of deuterium oxide, acetonitrile-
$d_3$ and DMSO-$d_6$ was used to dissolve 4.

**Enzymatic hydrolysis of N-O-glucuronide (3) and N-oxide/N-glucuronide (4).**

The glucuronide metabolites 3 and 4 (10 μM) were each dissolved in 100 mM potassium
dihydrogen phosphate buffer solution, pH 6.8 and added to a β-glucuronidase solution (E.
coli, 1000 IU/ml). The suspensions were incubated at 37 °C for 30 min for complete
hydrolysis to mainly 2, and secondarily 1. Hydrolysis of 3 and 4 (10 μM) spiked to human
plasma required higher amounts of β-glucuronidase (up to 5000 IU/ml) and/or longer
incubation times (up to 4h).

**Synthesis of N-O-glucuronide (3).**

*Experimental.* {[2-(2,4-Dimethyl-phenylsulfanyl)-phenyl]-(2-hydroxy-ethyl)-amino}-ethanol
(6).

Aniline 5 (2.75g, 12.0 mmol) was dissolved in N-methylpyrrolidinone (20 mL), then
potassium iodide (4.13 g, 24.9 mmol) and ethyl bromoacetate (8.28 mL, 74.7 mmol) were
added. The mixture was warmed to 120 °C and stirred overnight (22h). Additional ethyl
bromoacetate (2 mL, 18 mmol) was added, and the mixture was stirred for an additional 4h.
The mixture was partitioned between water (100 mL) and TBME (100 mL) and the organic
phase was washed with water (2x50 mL) and brine (50 mL). The combined aq. phases were
extracted with TBME (50 mL) and the combined organic phases were dried (Na$_2$SO$_4$),
filtered, and evaporated to dryness *in vacuo*, affording a crude mixture of the corresponding
diester, which was taken directly to the next step.

Lithium borohydride in THF (2 M, 50 mL) was added to the diester at 0 °C and the mixture
was stirred overnight (18h). Subsequently, 2M NaOH (150 mL) was added and the mixture
was stirred for 1h, then partitioned between water (100 mL) and CH$_2$Cl$_2$ (150 mL). The aq. phase was extracted with CH$_2$Cl$_2$ (50 mL) and the combined organic phases were dried (Na$_2$SO$_4$), filtered, and evaporated to dryness in vacuo, affording a slightly yellow oil. The residue was purified using silica gel column chromatography (AcOEt:heptane, 1:19-1:1) affording diol 6 (1.8g, 46% over the two step sequence) as a colorless oil.

Data for 6:

$^1$H-NMR (CDCl$_3$, 500MHz) 7.44 (d, 1H, $J = 7.5$Hz), 7.26 (d, 1H, $J = 8.0$Hz), 7.19 (s, 1H), 7.13 (t, 1H, $J = 7.5$Hz), 7.08 (d, 1H, $J = 8.0$Hz), 7.00 (t, 1H, $J = 7.5$Hz), 6.47 (d, 1H, $J = 7.5$Hz), 3.62 (br. s, 4H), 3.24 (br. s, 4H), 3.09 (br. s, 2H), 2.39 (br. s, 4H), 2.32 (br. s, 4H).

$^{13}$C-NMR (CDCl$_3$, 125MHz) 146.3, 142.8, 140.2, 138.7, 137.0, 132.0, 128.0, 126.0, 125.1, 125.0, 60.1, 59.0, 21.2, 20.4.

HRMS: [M+H]$^+$ = 318.1522, found 318.1528

(2S,3S,4S,5R,6S)-3,4,5-Triacetoxy-6-{4-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazin-1-yloxy}-tetrahydro-pyran-2-carboxylic acid methyl ester (11)

Oxalyl chloride in CH$_2$Cl$_2$ (2 M, 3.54 mL) was added to CH$_2$Cl$_2$ (10 mL) and cooled to -78 °C followed by the addition of DMSO (1.00 mL, 14.2 mmol) and the mixture was stirred for 15 min, then diol 6 (900 mg, 2.835 mmol) in CH$_2$Cl$_2$ (1.0 mL + 0.5 mL rinse) was added dropwise. The mixture was stirred under nitrogen for 30min, then triethylamine (3.95 mL, 28.4 mmol) was added and the mixture was slowly warmed to -20 °C over 1h, then the cooling bath was removed and the mixture was stirred for 5 min at room temperature. In a separate vial MeOH (10 mL, 250 mmol) was added to [B] (2S,3S,4S,5R,6S)-3,4,5-triacetoxy-6-(1,3-dioxo-1,3-dihydro-isooindol-2-yloxy)-tetrahydro-pyran-2-carboxylic acid methyl ester (990 mg, 2.0 mmol) followed by hydrazine monohydrate (1:1, hydrazine:water, 115.1 uL) and
after 5 min, the mixture was transferred to the vial containing the above described Swern oxidation mixture followed by the addition of AcOH (5.8 mL) and sodium cyanoborohydride (0.75 g, 12 mmol), and the mixture was stirred for 2h. The mixture was concentrated to half volume, then AcOH (6 mL) and sodium cyanoborohydride (0.35g) were added and the mixture was stirred at 45 °C overnight (19h). The mixture was partitioned between AcOEt (20 mL) and sat. aq. NaHCO₃ (20 mL) and the organic phase was washed with sat. aq. NaHCO₃ (2x10 mL). The combined aq. phases were extracted with AcOEt (10 mL) and the combined organic phases were dried (Na₂SO₄), filtered, and evaporated to dryness in vacuo. The residue was subjected to silica gel column chromatography (AcOEt:heptane, 1:9-1:0) affording an off-white foam (245 mg). The product was precipitated from diethyl ether (1 mL) and the white solid was a 2:1 mixture of by-product (from the phthalimide) and desired product, while the filtrate was an impure mixture of desired product 11, hydroxyl amine 2, and unknown phthalimide by-products. The filtrate was evaporated to dryness and subjected to silica gel column chromatography (MeOH:CH₂Cl₂, 1:99-1:9), which removed the hydroxylamine 2 (200.3mg, 31%), but not the other by-products. The residue was then subjected to preparative TLC (MeOH:CH₂Cl₂, 3:97) which removed the phthalimide by-product. Finally, the mixture was precipitated in cold (-20 °C) diethyl ether (0.5 mL), affording glucuronic methyl ester 11 (105 mg, yield = 8.1%).

Data for 2:

¹H-NMR (CDCl₃, 500 MHz) 8.11 (s, 1H), 7.35 (s, 1H, J = 7.5Hz), 7.25 (s, 1H), 7.16 (d, 1H, J = 7.5Hz), 7.13-7.09 (m, 2H), 6.92 (t, 1H, J = 7.5Hz), 6.41 (d, 1H, J = 7.5Hz), 3.26 (d, 2H, J = 10.5Hz), 3.14 (d, 2H, J = 10.5Hz), 2.89 (t, 2H, J = 11.0Hz), 2.68 (t, 2H, J = 9.5 Hz), 2.34 (s, 3H), 2.26 (s, 3H).

¹³C-NMR (CDCl₃, 125 MHz) 150.3, 143.4, 140.3, 137.4, 135.0, 133.4, 129.8, 129.0, 127.6, 127.4, 126.1, 121.9, 59.8, 51.8, 22.5, 21.8.
HRMS: HRMS: [M+H]$^+$ = 315.15256, found 315.15177

Data for 11:

$^1$H-NMR (CDCl$_3$, 600 MHz) 7.35 (d, 1H, $J$ = 8.5 Hz), 7.14 (s, 1H), 7.07-7.01 (m, 3H), 6.86 (dt, 1H, $J$ = 1.5, 7.5 Hz), 6.40 (dd, 1H, $J$ = 1.5, 8.0 Hz), 5.28 (t, 1H, $J$ = 9.5 Hz), 5.19 (t, 1H, $J$ = 9.5 Hz), 5.06 (dd, 1H, $J$ = 8.5, 9.5 Hz), 4.96 (d, 1H, $J$ = 8.5 Hz), 4.09 (d, 1H, $J$ = 10.0 Hz), 3.76 (s, 3H) 3.47 (br. d, 1H, $J$ = 9.0 Hz), 3.34-3.27 (m, 3H), 3.14 (br. s, 1H), 3.03-2.95 (m, 3H), 2.36 (s, 3H), 2.30 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H).

$^{13}$C-NMR (CDCl$_3$, 150 MHz) 170.2, 169.5, 169.1, 167.3, 142.4, 139.3, 136.2, 134.7, 131.7, 127.8, 126.3, 125.5, 124.6, 119.9, 102.8, 72.5, 72.3, 69.7, 69.5, 52.9, 21.2, 20.7, 20.6, 20.5.

HRMS: HRMS: [M+H]$^+$ = 631.2320, found 631.2321

4-[2-(2,4-Dimethyl-phenylsulfanyl)-phenyl]-morpholin-2-one (7).

Diol 6 (70.1 mg, 0.221 mmol) was dissolved in CH$_2$Cl$_2$ (0.80 mL) and DMSO (0.150 mL, 2.11 mmol) and N,N-diisopropylethylamine (115 uL, 0.662 mmol) were added and the reaction mixture was stirred at -10 °C under nitrogen. Sulfur trioxide-pyridine(87.9 mg) was dissolved in DMSO (0.300 mL, 4.23 mmol) and added to the reaction mixture. The mixture was stirred for 30 min, while warmed to 0 °C. The mixture was purified by directly pouring it onto a silica gel column (AcOEt:heptane 1:4) affording the undesired lactone 7 (51 mg, 74%) as a colorless oil.

Data for 7:

$^1$H-NMR (CDCl$_3$, 500 MHz) 7.34 (d, 1H, $J$ = 8.0 Hz), 7.16 (s, 1H), 7.12 (t, 1H, $J$ = 7.5 Hz), 7.05-7.02 (m, 2H), 6.95 (t, 1H, $J$ = 8.0 Hz), 6.59 (d, 1H, $J$ = 8.0 Hz), 4.47 (t, 2H, $J$ = 4.5 Hz), 4.00 (s, 2H) 3.36 (t, 2H, $J$ = 4.5 Hz), 2.37 (impurity), 2.31 (impurity)

$^{13}$C-NMR (CDCl$_3$, 125 MHz) 167.5, 145.6, 142.3, 139.5, 136.0, 134.8, 131.9, 128.0, 126.9, 125.8, 125.7, 119.8, 68.9, 53.6, 48.5.
N-O-Glucuronide 3.
The tetra acetate 11 (84.1 mg, 0.133 mmol) was dissolved in MeOH (3.0 mL, 74 mmol), 5.4 M sodium methoxide in MeOH (24.69 uL) was added and the reaction mixture was stirred under nitrogen for 1h, then Amberlyst 15 (ca. 10 beads) was added and the mixture was concentrated and purified directly using silica gel column chromatography (MeOH:CH₂Cl₂, 2:98-10:90), affording the corresponding methyl ester (52 mg, 77%). The methyl ester (25 mg, 0.050 mmol) was dissolved in acetone (2.5 mL, 34 mmol) and 1.0 M sodium hydroxide in water (0.248 mL) was added resulting in a white precipitate (presumably the sodium salt) and the reaction mixture was stirred for 5 min. Subsequently, the solvent was decanted off. The residue was dissolved in MeOH:DMSO:H₂O (1:1:4) and filtered. The clear solution was subjected to preparative HPLC, affording N-O-glucuronide 3 (13.2 mg, 54%, solid). See Fig. 2.
Results

Humans dosed orally with 1 afforded a number of metabolites in plasma and urine including two compounds verified to be 3 and 4 both with (M+H)+ = m/z 491 using HPLC-MS (Fig 3). Fragmentation of the m/z 491 ions in the IonTrap MS resulted in the MS2 spectra shown in Fig 4. The ions at m/z 315, 299, 298 and 240 for 4 and 299, 298, 256 and 240 for 3 correspond to the loss of a glucuronic acid moiety (m/z 315), loss of a glucuronic acid moiety and oxygen (m/z 299), and loss of an O-glucuronic acid moiety and fragmentation of the piperazine ring (m/z 240 and 256). This pattern is consistent with the fragmentation of glucuronic acid conjugate metabolites of a monooxygenated piperazine derivative (Delbressine et al., 1992; Schaber et al., 2001; and Miller et al., 2004). Analysis of the (bio)synthesized standards containing 3 and 4 using MicrOTOF MS showed the compounds to have exact mass values of 491.18608 and 491.18325, respectively. The observed values are within 2.9 ppm and 2.8 ppm (for 3 and 4, respectively) of a theoretical exact mass of C24H31N2O7S, corresponding to a monooxygenated glucuronide metabolite of 1. Analysis of the starting material 2, the hydroxylamine, for the biosynthesis of 4, shows the compound to have exact mass value of 315.15177. The observed value is within 2.5 ppm of the theoretical exact mass of C18H23N2OS, corresponding to a monooxygenated metabolite of 1. 1H and 13C NMR spectra were obtained for 2 for comparative reasons (see Figs 5 and 6).

Metabolite 3 was unambiguously prepared using a novel organic synthesis and various NMR experiments were performed for structural ID purposes. See Tables 1 and 2 for chemical shift values and Figs 7 and 8 for 1H and 13C NMR spectra.

Treatment of 3 and 4 with β-glucuronidase resulted in hydrolysis mainly to a compound with (M+H)+ = m/z 315 and to a lesser extent to a compound with (M+H)+ = m/z 299. The retention times and MS spectra were consistent with those obtained for the hydroxyl amine 2 and the parent drug 1.
Organic synthesis of 4 was unsuccessful but in vitro biosynthesis using human liver microsomes, UDPGA, alamethicin (antibiotic, precautionary measure) and saccharic acid lactone (β-glucuronidase inhibitor, precautionary measure) (Bowalgaha et al., 2007; Hintikka et al., 2008; Miller et al., 2004; Picard et al., 2005 and Wen et al., 2007 a and b) and subsequent semi-preparative HPLC-MS resulted in the production of approximately 10 mg of the metabolite 4. During evaporation of the fractions collected during semi-preparative HPLC-MS, 4 started precipitating and was collected in sufficiently pure form for 1 and 2D NMR experiments. The isolated metabolite was confirmed to be identical to the metabolite (M+H)$^+$ = 491 observed in various in vitro and in vivo samples, including human urine, based on identical retention times and MS fragmentation pattern. See Figs 9 and 10.

The structure of 4, including assignment of all protons, was determined using a combination of 1D ($^1$H, decoupled $^{13}$C) and 2D techniques (TOCSY (Bax and Davis, 1985) for $^1$H-$^1$H correlations in coupling networks, gradient selected HSQC (Key et al., 1992, Wilker et al., 1993) for direct correlations between $^1$H and $^{13}$C, gradient selected HMBC (Bax and Davis, 1985; Wilker et al., 1993 and Cicero et al., 2001) for long-range correlations between $^1$H and $^{13}$C, and ROESY (Bax and Davis, 1985 and Hwang and Shaka, 1992) for correlating protons close in space). See $^1$H and $^{13}$C NMR spectra in Figs 11 and 12. The anomeric proton of the glucuronic acid moiety was observed at 4.93 ppm and with a coupling constant of $J_{H-1', H-2'}$ of 6.6 Hz. See Tables 1 and 2 for chemical shift values for all hydrogen and carbon atoms in compounds 2, 3 and 4.
Hydroxyl amines are the intermediate step towards N-O-glucuronides and 2 was the obvious starting point of the biosynthesis of 3 and 4 as they both were hydrolysed to the hydroxyl amine with β-glucuronidase. The further reaction of this substrate with human liver microsomes and UDPGA resulted in two glucuronides both with (M+H)^+ = m/z 491 observed in ES positive mode in the MS. One glucuronide was identified to be the N-O-glucuronide 3 based on identical retention times and MS spectrum in the HPLC MS analysis of the standard and the biosynthetic product.

The second metabolite with (M+H)^+ = m/z 491 was speculated to be the N-O, N-glucuronide 4. Other research groups have speculated about the possibility of an N-oxide, N-glucuronide metabolite, but none have described such a metabolite (Straub et al., 1988; Miller et al., 2004).

The exact mass data obtained for 3 and 4 (corresponding to C_{24}H_{31}N_{2}O_{7}S) and their fragmentation patterns are consistent with monooxygenated glucuronide metabolites of the piperazine 1 (Delbressine et al., 1992; Schaber et al., 2001; and Miller et al., 2004).

The anomeric protons of 3 and 4 were observed at 4.57 and 4.93 ppm and coupling constants J_{H-1',H-2'} of 8.5 and 6.6 Hz, respectively. Chemical shifts and coupling constants of tertiary N-glucuronides and β-anomers are found at 5.33-6.31 ppm and 8.3-9.5 Hz, respectively (Andersen et al., 1989; Keating et al., 2006; Nakazawa et al., 2006; Yan et al., 2006), which are at lower field (higher ppm) than the anomeric proton of N-O-glucuronides (range chemical shift 4.54-4.84 ppm and coupling constants of 8.4-9 Hz) (Straub et al., 1988; Delbressine et al., 1992; Turgeon et al., 1992; Schaber et al., 2001; and Miller et al., 2004).
The N-O-glucuronide 3 fits the published range. For quarternary N-glucuronides chemical shift values for the anomic protons of 4.39-4.6 ppm with coupling constants of approximately 9 Hz have been reported (Breyer-Pfaff, 1990; Kaku et al., 2004). The proposed N-oxide/N-glucuronide 4 does not completely fit any of the ranges. The final evidence for the proposed structure can be found in the distance correlations observed in the ROESY spectra (Hwang and Shaka, 1992) of 3 and 4. See Figs 13 and 14 and Table 1 for chemical shift values.

The lower graph in each spectrum shows the intensity of the distance correlations between the anomic proton and the protons close in space. For 3, the correlation peaks of H-1' (4.57 ppm), H-3' (3.22 ppm) and H-5' (3.65 ppm) in the glucuronide moiety are equivalent in size, while a weaker correlation is seen to H-2'. As expected, no correlations to the piperazine protons are observed for the anomic proton in 3. However for 4, apart from the expected correlations between H-1' (4.93 ppm), H-3' (3.44 ppm) and H-5' (4.02 ppm) of the glucuronide moiety, an additional distance correlation is observed between H-1' and the axial piperazine proton H-3''ax (3.68 ppm). This latter correlation shows that the glucuronide and piperazine moieties have a spatial distance that can only be explained by the structure shown in Fig 11. The N-oxide/N-glucuronide metabolite 4 represents a new class of quaternary N+-glucuronides. It should be taken into account in future studies of conjugated hydroxylamines that unsymmetrically substituted amines will afford diastereomeric conjugates.

There is also indirect evidence for the structure of 4 based on different theoretical structures. Especially, differentiating between 3 and 4 was deemed difficult; therefore, a structurally unambiguous synthesis of 3 was developed. The strategy was to incorporate the N-O hydroxylamine moiety as early as possible from starting materials known from the published literature. Therefore, phthalimide 10 was chosen as the starting material (Mitchell and
Whitcombe, 2000). The phthalimide hydroxylamine-protecting group was removed via brief exposure to hydrazine in MeOH and immediately transferred to the next reaction due to its inherent instability under the reaction conditions. The free hydroxylamine 9 was coupled with di-aldehyde 8, using sodium cyanoborohydride, thereby affording 11 via a double reductive amination. The major product of the reaction was the hydroxylamine 2, which was used in the biosynthesis of 4. Several conditions were screened in order to oxidize diol 6 to the corresponding di-aldehyde 8. Surprisingly, when the oxidation was attempted using Parikh-Doering conditions (i.e. SO₃·Pyr, DMSO, DIPEA) (Parikh and Doering, 1967) lactone 7 was afforded instead of the desired di-aldehyde 8. This problem was overcome by using a Swern oxidation (Tidwell, 1990). One could possibly envision epimerization at the anomeric position under the slightly acidic conditions during the reductive amination, but due to anchimeric assistance from the neighbouring acetyl moiety, the β-anomer is favored. Coupling product 11 was then deprotected using a 2-step protocol affording the N-O glucuronide 3. The synthesis unambiguously afforded the N-O glucuronide, thus providing direct identification of metabolite 3 as well as indirect identification of metabolite 4.

One could speculate that 4 might be a ‘‘3-carboxylic ester alternative’’. In this case, the carboxylic moiety of the glucuronic acid would be linked to the N-hydroxylamine moiety. A distance correlation would not have been observed between the anomeric proton of the glucuronide and the piperazine ring protons in this structure. But a correlation is observed and the structure can thus be rejected.

A second alternative structure could theoretically have been the α-anomer of 3. However, in this case the coupling constant of the anomeric proton in the glucuronide moiety would be only 2-3 Hz (Smith and Benet, 1986) and the actual observed coupling constant is 7-8 Hz, indicative of the β-anomer, together with the fact that strong distance correlations from H-1’ to H-3’ and H-5’ are seen.
A third alternative structure is the N-1 piperazine glucuronide instead of the observed N-4 substituted version. If this were the case, the resulting quaternary N would result in a downfield shift of adjacent carbons of the piperazine ring (Miller et al., 2004). This is not seen. Also distance correlations between the glucuronide protons (anomeric proton), piperazine protons H-2" and/or H-6", and an adjacent aromatic ring would have been observed if 4 had an N-1 glucuronide structure. None of this was observed.

The last speculated alternative structure is the N-1 oxide N-4 glucuronide but the shift considerations of the third alternative also apply here.

During the biosynthesis, 1 was observed in the reaction mixture, suggesting that either the substrate 2 or one of the products 3 or 4 is hydrolysed to give 1. It is most likely to arise from the non-enzymatic hydrolysis of either the N-O bond in the glucuronide metabolite 3 or the C-O bond in 2 in the reaction mixture (pH 6.8) or during the acidic HPLC-MS conditions (Turgeon et al., 1992; and Yan et al., 2006). Enzymatic and chemical hydrolysis of quaternary ammonium-linked glucuronide metabolites, such as 4, is generally reported to be mediated only by E. coli (pH 6.5 -7.4) and non-enzymatically only at extreme pH (Kowalczyk et al., 2000), in line with the suggestion above that the parent compound 1 originates from hydrolysis of 2 or 3.

In conclusion, it is worth noting that during the analysis of samples from humans dosed with 1 and from the biosynthesis previously described, both the N-O-glucuronide 3 and the N-oxide/N-glucuronide 4 were formed. Furthermore, the N-oxide/N-glucuronide 4 is a new class of metabolites that will possibly be observed in future metabolism studies of other drugs.
Acknowledgements

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Authorship contributions

Participated in research design: Uldam, Juhl and Dalgaard

Conducted experiments: Uldam, Juhl and Pedersen

Performed data analysis: Uldam, Juhl and Pedersen

Wrote or contributed to the writing of the manuscript: Uldam, Juhl, Pedersen and Dalgaard
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structural characterization by spectroscopic methods of two glucuronide metabolites of
mexiletine after N-oxidation and deamination Drug Metab Dispos 20: 762-769

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Legends for Figures.

Fig 1.

Fig 1. Structures of aryl piperazine antidepressant agent 1, hydroxylamine 2, O-glucuronide of the hydroxylamine 3 and N-glucuronide of the N-oxide 4.

Fig 2.

Fig 2. First synthesis of N-O-glucuronide metabolite 3. Conditions: (a) 1. BrCH$_2$CO$_2$Et, KI, NMP; 2. LiBH$_4$, THF (56%, two steps) (b) SO$_3$pyr, DMSO, CH$_2$Cl$_2$ (c) (CO)$_2$Cl$_2$, DMSO, NEt$_3$, CH$_2$Cl$_2$ (d) N$_2$H$_4$, MeOH (e) NaBH$_3$CN, THF, AcOH (11: 8%; 2: 31%, 2 steps from 6) (f) 1. NaOMe, MeOH; 2. NaOH, acetone, THF (42%, 2 steps)

Fig 3.

Fig 3. Human urine from a subject dosed with 1. Metabolite 3 and 4 observed in chromatogram with m/z values of 491.

Fig 4.

Fig 4. IonTrap MS$^2$ spectra of metabolite 3 and 4.

Fig 5.

Fig 5. $^1$H NMR spectrum of hydroxyl amine 2.

Fig 6.

Fig 6. $^{13}$C NMR spectrum of hydroxyl amine 2.
Fig 7.
Fig 7. $^1$H NMR spectrum of N-O-glucuronide metabolite 3.

Fig 8.
Fig 8. $^{13}$C NMR spectrum of N-O-glucuronide metabolite 3.

Fig 9.
Fig 9. Biosynthesis of 4, incubating 2 (200 μM) with human liver microsomes (0.5 mg protein/ml) and UDPGA (2.5 mM) at 37 °C for 4 h.

Fig 10.
Fig 10. Product 4 after purification using semi-preparative HPLC-MS.

Fig 11.
Fig 11. $^1$H NMR spectrum of N-oxide, N-glucuronide metabolite 4.

Fig 12.
Fig 12. $^{13}$C NMR spectrum of N-oxide, N-glucuronide metabolite 4.

Fig 13.
Fig 13. Slice of ROESY spectrum of 3 showing distance correlations between anomeric proton (H-1’) at δ 4.57 ppm and H-3’ (δ 3.22 ppm), H-5’ (δ 3.65 ppm) and H-2’ (δ 3.04 ppm)
Fig 14.

Fig 14. Slice of ROESY spectrum of 4 showing distance correlations between anomeric proton (H-1’) at δ 4.93 ppm and H-3’ (δ 3.44 ppm), H-5’ (δ 4.02 ppm) and piperazine proton H-3”ax (δ 3.68 ppm) (lower) and normal ¹H spectrum (upper).
### Table 1

\( ^1 \)H NMR chemical shift assignments of 2, 3 and 4 (\( \delta \), multiplicity, integration)\(^a,b\).

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**Piperazine ring**
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* $J_{H-1', H-2'}$ of 8.5 Hz

** $J_{H-1', H-2'}$ of 6.6 Hz

*a: Solvent mixture of D$_2$O/CD$_3$CN/DMSO-d$_6$ 2/3.5/6 used due to low solubility of 4*

*b: Assignment of H and C-atoms based on $^1$H, decoupled $^{13}$C, TOCSY, ROESY, HSQC and HMBC experiments*
Table 2

\(^{13}\)C NMR chemical shift assignments of 2, 3 and 4 (\(\delta^{a, b}\)).

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**Piperazine ring**

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*, ** or reverse

*a: Solvent mixture of D$_2$O/CD$_3$CN/DMSO-d$_6$ 2/3.5/6 used due to low solubility of 4

*b: Assignment of H and C-atoms based on $^1$H, decoupled $^{13}$C, TOCSY, ROESY, HSQC and HMBC experiments*
Figure 1

Oxidation (in vitro and in vivo)

Human liver microsomes + UDPGA
Figure 4

N-oxide/N-glucuronide 4

N-O-glucuronide 3
Figure 11