Biotransformation of two β-secretase Inhibitors Including Ring Opening and contraction of a Pyrimidine Ring

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Non-standard abbreviations: (S)-25, (S)-1-pyridin-4-yl-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine; AZD3839, (S)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine hemifumarate; CID, collision-induced dissociation; CYP450, cytochrome P450; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; M1, (S)-5-(3-(3-amino-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1H-isoindol-1-yl)phenyl)pyrimidine 1-oxide; M2, (S)-4-(3-amino-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-1-yl)-2-(difluoromethyl)pyridine 1-oxide; MeCN, acetonitrile; MDF, mass defect filtered; MS, mass spectrometry; NMR, nuclear magnetic resonance; UDPGA, uridine 5'-diphospho-glucuronic acid
Abstract

Recently, the discovery of the aminoisooindoles as potent and selective inhibitors of β-secretase was reported [Swahn et al., J. Med. Chem. 2012. DOI: 10.1021/jm3009025] including the close structural analogues compound (S)-25 and AZD3839, the latter being recently progressed into the clinic. The biotransformation of (S)-25 was investigated in vitro and in vivo in rat, rabbit and human and compared to AZD3839 to further understand the metabolic fate of these compounds. In vitro, CYP3A4 was the major responsible enzyme and metabolized both compounds to a large extent in the commonly shared pyridine and pyrimidine rings. The main proposed metabolic pathways in various in vitro systems were \(\text{N}\)-oxidation of the pyridine and/or pyrimidine ring as well as conversion to 4-pyrimidone and pyrimidine-2,4-dione. Both compounds were extensively metabolized and more than 90% was excreted in feces following i.v. dosing of radiolabeled compound to the rat. Here, the main pathways were \(\text{N}\)-oxidation of the pyridine and/or pyrimidine ring, and a ring contraction of the pyrimidine ring into an imidazole ring. Ring-contracted metabolites accounted for 25% of the total metabolism in the rat for (S)-25, whereas the contribution was much smaller for AZD3839. This metabolic pathway was not foreseen based on the obtained in vitro data.

In conclusion, we have discovered an unusual metabolic pathway of aryl-pyrimidine-containing compounds by a ring-opening reaction followed by elimination of a carbon atom and a ring closure to form an imidazole ring.
Introduction

β-secretase (Beta-site amyloid precursor protein cleaving enzyme 1; BACE1) is an aspartyl protease responsible for cleaving the amyloid precursor protein to shorter Aβ peptide fragments (Sinha et al., 1999; Vassar et al., 1999). These fragments form oligomers that deposit in the cerebrum as neurotoxic amyloid plaques (Hardy and Selkoe, 2002). Amyloid pathology is one of the major hallmarks of pathology in Alzheimer’s disease (Hardy and Allsop 1991; Lee et al., 2001). Reduction of Aβ formation through β-secretase inhibition is considered a promising approach to halt or to slow the progression of the disease. The effectiveness of orally administered β-secretase inhibitors in decreasing Aβ levels in the CNS has been demonstrated in mice (May et al., 2011; Swahn et al., 2012a; Lu et al., 2012a), rats (Cumming et al., 2012; Weiss et al., 2012; Wood et al., 2012), guinea pig (Gravenfors et al., 2012; Truong et al., 2010), dog (May et al., 2011), monkey (Sankaranarayanan et al., 2009; Lu et al., 2012b) and human (May et al., 2011; Forman et al., 2012; Lai et al., 2012). It remains to be proven, however, if β-secretase inhibition ultimately leads to improvement of a clinically meaningful endpoint such as attenuated cognitive decline in patients suffering from Alzheimer’s disease.

Recently, the discovery of the aminoisooindoles as β-secretase inhibitors was reported including an early lead compound (S)-25, (S)-1-pyridin-4-yl-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isooindol-3-amine, and AZD3839, (S)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isooindol-3-amine hemifumarate, both potent and selective inhibitor of β-secretase with Ki values in the nanomolar range (Jeppsson et al., 2012; Swahn et al., 2012b). (S)-25 and AZD3839 produced robust efficacy on Aβ biomarkers in plasma and CNS in mice, guinea pig and monkey. AZD3839 was recently progressed for clinical testing in volunteers (clinical trial gov id: NCT01348737).

Initial metabolic profiling of AZD3839 was recently published (Aasa et al., 2013). AZD3839 was mainly metabolized by CYP3A4, and in addition, it was also characterized as a time-dependent inhibitor of CYP3A4, even though this effect was dependent on the choice of solvent and test system. The main
observed metabolites were different $N$-oxidation products of the pyrimidine and pyridine rings. When we did some preliminary in vivo metabolic characterization of (S)-25, however, we noticed some unexpected products that we had not detected during our initial characterization of AZD3839 in spite of these compounds being chemically quite similar. To further understand the metabolic fate of the analogues (S)-25 and AZD3839, the biotransformation of these compounds was further evaluated in several in vitro test systems (rat, rabbit, human) and in intact rat. We have focused our efforts on the metabolism of (S)-25, and have used AZD3839 as a comparator.
Materials and Methods

Chemicals. Compounds (S)-25 [(S)-1-pyridin-4-yl-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine] and AZD3839 [(S)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine hemifumarate] (Table 1 and 2) were synthesized at the Medicinal Chemistry Department at CNSP iMed Science, AstraZeneca R&D, Södertälje, Sweden as described previously (Swahn et al., 2012b). ³H-labelled (S)-25 [(³H)-(1S)-4-fluoro-1-(pyridin-4-yl)-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine] and ¹⁴C-labelled AZD3839 [(U-phenyl-¹⁴C)-(S)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-3-amine hemifumarate], were synthesized at the Isotope Chemistry Department at Global DMPK Screening and Profiling, AstraZeneca R&D Södertälje, Sweden (Supplemental Scheme 1 and 2, and Supplemental Figure 1). Metabolite M1 [(S)-5-(3-(3-amino-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1H-isoindol-1-yl)phenyl)pyrimidine 1-oxide] and M2 [(S)-4-(3-amino-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1H-isoindol-1-yl)-2-(difluoromethyl)pyridine 1-oxide] were synthesized at the Medicinal Chemistry Department (Supplemental Methods). β-glucuronidase from Helix Pomatia was purchased from ICN Biomedicals Inc. (Irvine, CA, USA) and NADPH and GSH were obtained from SigmaAldrich Chemical Co. (St. Louis, MO, USA).

Hepatocytes and liver subcellular fractions. Cryopreserved hepatocytes from a pool of Sprague Dawley rats (male, 11 donors, Lot: JIL), New Zealand White rabbit (male, Lot: NXW ((S)-25), or female (AZD3839)) and Human (mixed gender, 10 donor Lot: GST) were supplied by Celcis IVT (Neuss, Germany). Incubations were handled as previously been described (Floby et al., 2009; Sohlenius-Sternbeck et al., 2010). Fresh rat hepatocytes for metabolite profiling experiments were isolated from male Sprague Dawley rats as previously described (Floby et al., 2009; Sohlenius-Sternbeck et al., 2010). The viability of the hepatocytes was optically determined after staining with Trypan Blue and was found to be between 54 and 92% for all species. Human microsomes pooled from
33 donors, mixed gender, were purchased from BD Gentest (Woburn, MA, USA), while rat microsomes were isolated from Sprague Dawley (mixed gender) rats as previously described (Axelsson et al., 2003). Rat liver S9 fraction homogenate, prepared from the livers of male Sprague Dawley rats pre-treated with Aroclor 1254, was purchased from Molecular Toxicology Inc. (Boone, NC, USA). Its metabolic capacity was checked in-house using key enzyme assays. Microsomes from baculovirus infected insect cells (BTI-TN-5B1-4) over-expressed human cytochrome P450’s: 1A2 (Cat no 456203/lot 21667), 2C8 (Cat no 456252/lot 83493), 2C19 (Cat no 456259/lot 80456), 2C9*1 (Cat no 456258/lot 11293), 2D6 (Cat no 456217/lot 69059), and 3A4 (Cat no 456202/lot 96395) were obtained from BD Gentest.

**Metabolic profiling incubations.** Hepatocyte, microsome and S9 fraction incubations were generally conducted as previously described (Floby et al., 2009; Sohlenius-Sternbeck et al., 2010). Briefly, hepatocyte suspensions were prepared in Williams medium E (1×10^6 hepatocytes/mL), while microsomes and S9 were used at concentrations 0.5-1.5 mg protein/mL. Incubations were supplemented with NADPH (1 mM) and GSH (5 mM) was added to S9 incubations. All incubations were performed at 37°C with gentle shaking in a 5% CO₂ atmosphere. Substrate concentrations for metabolite identification were normally either 1 µM or 5 µM. Hepatocytes were incubated for 2 h and microsomes for 30 min. Positive controls for microsomes and hepatocytes were conducted with cocktail substrates as previously described (Floby et al., 2009).

**Incubations with Human cDNA-Expressed P450.** Incubations of compound (S)-25 (2 µM) with CYP 1A2, 2C8, 2C9, 2C19, 2D6 and 3A4 were done at 50 pmol/mL protein concentration for 30 min at 37°C.

**Reactive metabolite detection.** Detection of reactive metabolites was performed by incubation with microsomes in presence of a mixture of GSH and labeled-GSH [¹⁵N, ¹³C₂] at a ratio of 1:1. The final
concentration of GSH was 2.5 mM. All incubations were stopped by the addition of two volumes of ice-cold MeCN and were then centrifuged prior to LC-MS analysis.

**Determination of number of exchangeable protons.** The number of exchangeable protons was determined by using deuterium-oxide instead of water in the mobile phase.

**Animals.** Adult male Wistar and Sprague Dawley rats were purchased from Taconic M&B A/S, Denmark. The animals were randomly distributed among the cages upon arrival and acclimatized for at least one week before start of the experiment. All animal studies were approved by the Stockholm Södra Animal Research Ethical Board and Animal conducted in compliance with national legislation, which is based on European Community directive 86/609/EEC.

**Surgery.** Surgery for a bile cannulation study with $[^3]$H-(S)-25 was performed two days prior dose administration using aseptic techniques. Rats were anaesthetized with isoflurane in oxygen. The bile duct and duodenum were exposed and catheterized with the same cannula to enable a bile flow to the duodenum under the recovery period. The loop was drawn to the neck. Analgesics were administrated during the operation and post-surgery if signs of pain were noticed.

Surgery was also performed in a rat mass balance study of $[^{14}]$C-AZD3839 conducted at Covance Laboratories Limited (Harrogate, UK). In this study the surgery was performed similarly as described above, but it was started 5 days prior to dosing and two separate cannulas were used for the bile duct and duodenum. Therefore, artificial bile salts were infused into the duodenum during the recovery period.

**Plasma, bile, and urine sampling following single oral and intravenous dose of $[^3]$H-(S)-25 to rat.** Male Sprague Dawley rats received an i.v. or oral dose of 30 µmol/kg, 50 MBq/kg $[^3]$H-(S)-25. Urine and feces was collected from rats in metabolism cages 0-24 h after administration. Bile and plasma was
collected from a bile duct cannulated rat. At the start of the bile collection, the loop was cut and the cannula connected to swivel device, designed to allow free movement of bile duct cannulated rats. Bile was collected for 0-6 h after administration and plasma at 6 h after dose by heart puncture.

The radioactivity in the samples were analysed with a Tri-carb 1900TR liquid scintillation analyzer (Perkin Elmer, Boston, MA). Urine and bile samples were diluted 1:1 and 1:10 with mobile phase A, respectively, centrifuged, and analyzed using LC-MS. Plasma was protein precipitated with addition of cold MeCN (1:4 v/v) followed by centrifugation. The MeCN in the supernatants was evaporated under a flow of nitrogen gas prior to LC-MS analysis.

Glucuronides in bile samples collected 3-4 h after administration, were hydrolyzed with β-glucuronidase. Aliquots of 100 µL were diluted 1:9 with water and incubated together with β-glucuronidase (50 µL) 2 h at 37 °C with gentle shaking. The aliquots were then mixed with 2 volumes of cold MeCN to precipitate proteins. After centrifugation the MeCN in the supernatants was evaporated under a flow of nitrogen at 37 °C. Controls without β-glucuronidase were also prepared.

**Plasma, bile, and urine sampling following single oral dose of [14C]-AZD3839 to rat.** Male Han Wistar rats (n=3) received 5.8 (i.v.) or 58 µmol/kg (p.o.), 9.25 MBq/kg, of [14C]-AZD3839. Plasma, urine and bile samples were pooled from each time point to yield a representative sample for each time, matrix and sex. Plasma sampled at 1, 3 and 8 h and urine 0-24 h and bile 0-48 h were selected for metabolite characterization and processed similarly as described above.

**LC-MS analysis.** Liquid chromatography for metabolite profiling was performed with a Rheos Allegro binary pump (Flux instruments, Basel, Switzerland) and a CTC HTC pal autosampler (CTC Analytics, Zwingen, Switzerland). For [14C]-AZD3839 an Accela HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) was used. The separations were performed on a Waters Atlantis T3 column (50 or 100 x 2.1 mm ID, 3 µm particle size) at a flow rate of 0.25 mL/min. The mobile phase was a binary
mixture of 0.1% formic acid in water/acetonitrile (98:2), v/v, (solvent A) and 0.1% formic acid in water/acetonitrile (20:80), v/v, (solvent B). For the [14C]-labeled compounds the separations were performed on a Waters Atlantis T3 column or YMCBasic (AZD3839) (150 x 4.6 mm, 3 µm particle size) at a flow rate of 1.0 mL/min. The mobile phase was a binary mixture of water, 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid, (solvent B). The column outlet-flow was split 1:20 using an Accurate split (LC Packings, Amsterdam, The Netherlands) with the major part split to the radioactivity detector and 5% (50 µL/min) of the effluent to the MS. MS was performed on a LTQ Orbitrap (Thermo Electron Corporation, San Jose, CA, USA). An electrospray interface in the positive ion mode was used in all experiments. Nitrogen was used as sheath gas at a flow rate of 25 (arbitrary units). The heated capillary temperature and the spray voltages were set to 300°C and 4.2 kV, respectively. Resolution varied between 7500 and 60000. A MS scan ranging from m/z 150-1000 that triggered the following collision-induced dissociation (CID) product ion spectra was used: 1: Product-ion scan (MS²) of predefined ion or the most intense ion from survey scan. 2: Product-ion scan (MS³) of the most intense product ion from MS² scan. 3: Product ion scan (MS³) of the second most intense product ion from MS² scan. Alternatively the last scan was a MS⁴ scan of the most intense ion in the MS³ scan. The softwares Xcalibur 2.0 and Metworks (Thermo Electron Corporation) was used for data acquisition, processing and control of the mass spectrometer.

Radioactivity monitoring. The detection of radio-labeled metabolites and determination of their relative amounts was carried out on a Radiomatic 625TR radioactivity detector (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The instrument was equipped with a 500 µL cell and operated in the homogenous liquid scintillation mode with the addition of 3.0 mL/min of Ultima Flow M scintillation liquid (Perkin Elmer) to the LC effluent (ratio of 1:3.16). The software package FloOne (Perkin Elmer) (\textbf{(S)-25}) or Laura 4.1 (LabLogic, UK) (AZD3839) was used for the radio-chromatographic peak integration and control of the radioactivity detector.
Results

The metabolism of compound (S)-25 was investigated in liver microsomes, freshly prepared rat hepatocytes, cryopreserved human and rabbit hepatocytes, and liver S9 fraction from Sprague Dawley rats pre-treated with Aroclor 1254 and compared to the metabolism of AZD3839. All metabolites of compound (S)-25 are denoted Mxa and metabolites from AZD3839 Mx where x is the number of the metabolite.

In vitro metabolic profiling of (S)-25. Incubating 1 µM of (S)-25 in human or rat liver microsomes led to one dominant metabolite, M1a, and a somewhat smaller metabolite M2a, while in human and rat hepatocytes, an additional metabolite M3a was formed in larger amounts. In rabbit hepatocytes, (S)-25 was more rapidly metabolized to mainly M3a. Figure 1 shows the LC-MS chromatograms of the metabolites formed in human, rat and rabbit hepatocytes after 120 min incubation with 5 µM of (S)-25. Incubations of (S)-25 with human cDNA-expressed P450 enzymes showed that CYP3A4 majorly contributed to the metabolism of (S)-25 in microsomes. Also CYP2C19 and CYP2D6 produced the same metabolites as CYP3A4, but to a much lower extent. The metabolites (Figure 2; Table 1) were putatively identified by accurate mass of the molecular ion, interpretation of product ion spectra and determination of number of exchangeable protons with H/D exchange. Trapping experiments of (S)-25 (10 µM) with 2.5 mM glutathion (GSH) were performed in human and rat microsome incubations. In human no GSH conjugate was detected, but in rat a conjugate was formed: substitution of the fluorine in the aminoisoindol by GSH was detected (data not shown). This metabolite was also formed in incubation of (S)-25 (100 µM) with aroclor-1254 induced S9 fractions. The main metabolites in S9, however, were M2a (data not shown). Note that these incubations were performed at 100 µM substrate concentration to support toxicology studies.

In vivo metabolic profiling of (S)-25. (S)-25 was extensively metabolized in vivo. After an i.v. dose of [³H]-(S)-25 (30 µmol/kg) to rat over 90 % of the radioactive dose was excreted in feces after 48 h, and 55% in bile after 6 h using a bile cannulated rat (Supplemental Table 1). Urinary excretion accounted
for less than 10 %, and metabolites excreted in urine are therefore not presented, also in view of that urinary metabolites were found in other excreta. Figure 3 shows the mass defect-filtered (MDF) chromatograms of plasma and bile collected after p.o. administration of (S)-25 to rat together with the corresponding radio-chromatogram of bile. The biotransformation of (S)-25 appeared different compared to the in vitro studies. Similar to hepatocyte incubations the dominant metabolite in rat plasma was M1a, but additionally, a di-N-oxide M8a was observed. The amount of M2a was too little to become visible in the MDF chromatogram of plasma or bile, and the di-oxidized metabolite M3a was not detected. A new metabolite, M4a, was detected in bile with an exact mass of m/z 386 indicating a loss of one C and gain of one O atom and was putatively identified as a ring contracted product (Figure 2; Table 1). Together with M6a, a combination of M1a with M4a, and their corresponding glucuronides M5a and M7a, respectively, this metabolic path contributed to over 25% of the excreted radioactivity. The radiochromatogram of bile collected after intravenous dose was similar to that after oral dose (not shown).

**Metabolic profiling of AZD3839.** AZD3839 is structurally a close analog of (S)-25. In liver microsomes AZD3839 (1 µM) was mainly metabolized into M1 and M2 (Table 2). These metabolites were identified as pyrimidine and pyridine N-oxides by mass spectrometry and by comparison with synthesized standards of M1 and M2 using LC-MS (Supplemental Figure 2). Metabolic profiling of AZD3839 (100 µM) in S9 incubations revealed an oxidized product M24 as main metabolite next to M1 and M2. Following 120 min incubation of 5 µM of [14C]-AZD3839 with hepatocytes, more than 20 metabolites were detected (Figure 4), of which the main metabolites are depicted in Figure 5. In addition to the two N-oxides formed in microsomes, a di-oxidized metabolite M9 was formed in hepatocytes. M9 was oxidized on both the pyrimidine and the pyridine nitrogen. In contrast to the N-oxides that were dominant in human and rat hepatocytes, another di-oxidized metabolite M23 contributed to over 80% of the radioactivity in rabbit hepatocytes. Additional metabolites (M16 and M31) had been formed by opening of the pyrimidine ring (Table 2).
After administration of $[^{14}\text{C}]-\text{AZD3839}$ (5.8 µmol/kg i.v.) to rat over 90% of the radioactive dose was excreted in feces and less than 1% of $[^{14}\text{C}]-\text{AZD3839}$ was excreted unchanged in urine (Supplemental Table 2). The biotransformation of AZD3839 in vivo resembled the metabolic pattern previously seen in vitro, in contrast to (S)-25. AZD3839 was mainly eliminated via N-oxidation of the pyrimidine and pyridine rings in rat. In the radiochromatograms of plasma and bile following oral administration of $[^{14}\text{C}]-\text{AZD3839}$, also peaks of two di-oxidized products M12 and M23, and a tri-oxidized product M11 were present (Figure 6; Table 2). The glucuronide conjugate of the ring-contracted metabolite, M31, was also detected with AZD3839 (Figure 5; Table 2), but to a lower extent than M5a from (S)-25. In bile also the putative aglycone to M31, M34 was observed together with some minor glucuronides M17, M26 and M35.

**Metabolite characterization.** The collision-induced dissociation (CID) product ion spectra of the protonated molecular [M+H]$^+$ ions of (S)-25 is shown in Figure 7. The most abundant fragments are formed by loss of pyridine, aryl-pyrimidine moiety or NH$_3$. The neutral loss of m/z 148 (C$_8$H$_5$N$_2$F; the fluoroisoindole) is also observed and is a characteristic fragmentation for the entire series. The CID fragmentation of metabolites formed from (S)-25 is summarized in Table 1 including accurate masses. All accurate masses were in the range ± 0.5 mDa from the theoretical mass, which made it possible to determine the molecular formula. A primary fragmentation of the two metabolites M1a, and M8a, with molecular ions at m/z 398 and 414 corresponding to oxidation and di-oxidation, respectively, was a loss of OH$^+$ that is a characteristic neutral loss for N-oxides and N-hydroxyls (Kuffel et al., 2002; Sridhar et al., 2011). Based on this observation and the finding of CID product ions at m/z 242, corresponding to loss of the aryl-pyrimidine moiety, and m/z 303, corresponding to loss of pyridine plus one O atom, M1a was proposed to be the pyridine N-oxide. Likewise, M8a was proposed to be oxidized in both the pyrimidine and pyridine ring based on the product ions at m/z 242 and 319 (303+16). The metabolite M2a with a molecular ion at m/z 398, indicative for mono-oxidation, produced a CID fragment at m/z 381 after loss of NH$_3$. No loss of water or OH$^+$ was observed. A fragment at m/z 226 formed after loss of
the aryl-pyrimidine moiety plus O, indicated oxidation in the aryl ring or pyrimidine ring. Two other fragments at \(m/z\) 354 and 337 were formed after loss of NH\(_3\) from the isoindole and then HCN or CH\(_4\)N\(_2\) from the pyrimidine ring. The oxygen was probably attached in stable position since no loss of oxygen was observed. This was consistent with an oxidation in the aryl ring, or the 4-pyrimidone. M3a, a di-oxidized metabolite with a molecular ion at \(m/z\) 414, was only detected in hepatocytes. The main CID fragment (\(m/z\) 397) produced by this ion was formed after loss of NH\(_3\), which gave no information about the structure as it can be from the aminoisoindole moiety. However, an ion at \(m/z\) 226 formed after loss of the aryl-pyrimidine moiety plus 2 O atoms indicated that the oxidation was in the aryl-pyrimidine moiety. The neutral loss of HNCO and HNC\(_2\)O\(_2\) that produced ions of \(m/z\) 371 and 343 strongly suggested oxidation of the pyrimidine ring, and hence M3a was proposed to be a pyrimidine-2,4-dione. The neutral loss of HNCO has previously been reported to occur from Uracil derivatives (Nelson et al., 1994) and another pyrimidine-2,4-dione containing compound (Morrison et al., 2012) M10a was detected at trace levels in incubation with S9 and trapping experiment with GSH. It was proposed to be a GS conjugate, formed after substitution with F, based on the molecular weight, the isotopic pattern from the labeled GS, and the neutral loss of Glu resulting in an ion at \(m/z\) 540.179 (Data not shown).

The remaining metabolites were only detected in bile from rat following administration with (S)-25. Metabolite M4a produced a molecular ion at \(m/z\) 386 indicating a loss of one C and gain of one O atom (Figure 7). H/D exchange experiments established that the number of exchangeable protons was increased with 1 compared to the parent compound. Combined with the observation of a main fragment in the CID spectrum at \(m/z\) 369.1386 indicating a neutral loss of OH\(^+\), it was concluded that M4a was an N-hydroxyl or N-oxide. The remaining fragments at \(m/z\) 226 (intact isoindol-pyridine moiety), \(m/z\) 342 (loss of H\(_2\)NCO) and \(m/z\) 349 (loss of HCN) showed that the metabolism occurred in the pyrimidine ring. Theoretically, pyrazole, imidazole and ring-opened \(N\)-hydroxylated structures match this pattern. However, the additional observation of a fragment of \(m/z\) 331.1358, in accordance to a loss of C\(_2\)HNO was difficult to explain from a pyrazole or ring-opened structure, and led to the proposal that M4a is an imidazole \(N\)-hydroxyl or \(N\)-oxide. M6a produced a molecular ion at \(m/z\) 402, indicating 1 additional O
atom compared with M4a. It fragmented in the same pattern as M4a and ions at $m/z$ 242 (loss of pyridine isoindole moiety plus O) and $m/z$ 307 (loss of pyridine plus O) indicated that the oxidation occurred in the pyridine ring. As the H/D exchange experiment showed that M6a had 1 extra exchangeable proton compared to (S)-25, M6a was proposed to be a pyridine N-oxide of M4a. After treatment of rat bile with β-glucuronidase, M5a ($m/z$ 562) and M7a ($m/z$ 578) were shown to be glucuronides of M4a and M6a, respectively (Figure 8). Two minor metabolites, M11a and M12a that produced molecular ions at $m/z$ 348.1143 (corresponding to C$_{21}$H$_{18}$O$_3$N$_4$F) and $m/z$ 393.1359 (corresponding to C$_{20}$H$_{15}$O$_2$N$_3$F) were proposed to be products formed after ring opening and further oxidation of the pyrimidine ring. They were not further characterized with MSMS.

The CID fragmentation of AZD3839 resembled that of (S)-25, but in addition, loss of HF was common. The proposed structures of the most abundant metabolites and their CID fragmentation are shown in Table 2. The main metabolites M1 and M2 with molecular ions of $m/z$ 448, indicative of 1 additional O atom compared to the parent molecule, both produced a major product ion formed by neutral loss of OH$^-$. The product ion spectra were very similar but key ions at $m/z$ 276 and 292 (276 plus one oxygen) indicate that oxidation occurred in the pyrimidine or pyridine ring. These two N-oxides were synthesized and compared with the authentic metabolites using LC-MS/MS showing that both the retention time and product ion spectra matched (Supplemental Figure 2). Metabolite M9 produced a molecular ion at $m/z$ 464 that generated a product ion at $m/z$ 430 formed by a double neutral loss of OH$^-$ suggesting that it was a di-N-oxide. This was confirmed by the H/D exchange experiment that shows that M9 had no extra exchangeable protons compared to AZD3839. Two ions at $m/z$ 319 and 303 formed by neutral loss of the pyridine plus one O atom strongly suggested that specifically the pyridine was oxidized. As M9 was shown to be formed from both M1 and M2, it was proposed to be a di-N-oxide oxidized in the pyridine and pyrimidine ring. Two other mono-oxidized metabolites (M13, M24 at $m/z$ 248) were detected in human and rat hepatocytes. They were both shown to be oxidized in the aryl-pyrimidine moiety. The primary fragmentation of M13 was neutral loss of HF and NH$_3$. No loss of O
was observed but an ion at \( m/z \) 354, probably formed after loss of the pyrimidine ring plus the O atom via inductive cleavage, pointed to hydroxylation in the pyrimidine ring. As seen in Table 2, metabolite M24 also fragmented by neutral loss of HF and NH\(_3\), but additionally loss of CO was observed. Thus, in line with M13, M24 were also proposed to be oxidized in the pyrimidine ring, but to the pyrimidine-4-one or pyrimidine-2-one. The fragment at \( m/z \) 384.0942 probably formed after loss of HF and HN=CH-NH\(_2\) from M24 indicated that M24 is the pyrimidine-4-one. The retention times of M13 and M24 at 6.2 and 8.2 min further supported this interpretation. M23 that showed a molecular ion at \( m/z \) 464 (+O\(_2\)), produced key fragment ions at \( m/z \) 421 (loss of HNCO) and 276 indicating oxidation in the aryl-pyrimidine moiety. A possible structure that probably will produce a neutral loss of HNCO is the pyrimidine-2,4-dione, a proposal also in accordance to the number of exchangeable protons. However, there are other structures that may be consistent with the data for example pyrimidine 2- or 4-one also oxidized in the aryl ring.
Discussion

The two $\beta$-secretase inhibitors (S)-25 and AZD3839 belonging to the aminoisouindolseries (Swahn et al., 2012b) were evaluated regarding their biotransformation in rat and human. The main focus is on the biotransformation of (S)-25, but AZD3839 is also discussed as it showed a different metabolic pattern despite its similar structure. A noteworthy finding in this study was the discovery of an uncommon metabolic conversion of the pyrimidine ring into an imidazole ring via a ring-opened intermediate. A related reaction has been shown with vicriviroc, which contains a pyrimidine-4,6-methyl moiety and forms a pyrazyl-hydroxylamine (Ghosal et al., 2007).

In incubations with rat and human hepatocytes (S)-25 were oxidized to a pyridine $N$-oxide. The second most abundant metabolite produced from (S)-25 was M2a, a mono-oxidized metabolite that is suspected to be the 4-pyrimidone, although investigations with NMR are required for a definitive structure determination. M2a was ruled out to be an $N$-oxide as it had one extra exchangeable proton and did not lose a hydroxyl radical in the CID MS/MS experiment. The third main metabolite of (S)-25 identified in vitro, M3a, was proposed to be the pyrimidine-2,4-dione. This was the single dominant metabolite in rabbit hepatocytes. P450 CYP3A4 contributed to the formation of both M1a and M2a, but did not form M3a. The fact that M2a is formed to a higher extent in S9 and hepatocytes than in microsomes may suggest that other enzymes than P450 such as aldehyde oxidase or xanthine oxidase may be involved. 4-pyrimidone and pyrimidine-2,4-dione metabolites have been shown to be the principle metabolic products from VU0409106, a pyrimidine-containing compound, by human and rat hepatocytes (Morrison et al., 2012). For VU0409106 it was demonstrated that both aldehyde oxidase and xanthine oxidase contributed to that reaction. Aldehyde and xanthine oxidase have been shown to catalyze the reaction of iminium ions, formed from aza-heterocycles, into lactams (Garattini and Terao, 2012). The rabbit hepatocytes were included in this report as they give a complete different metabolism than other tested system and are an interesting example of species differences in drug metabolism Substrate-specific species differences of aldehyde oxidase activity is demonstrated (Fukiya et al., 2010; Garattini
et al., 2008) and may be the reason for the complete different metabolic pattern in rabbit compared with rat and man. Although we did not demonstrate involvement of aldehyde oxidase in the metabolism of (S)-25, it is probable that this enzyme is involved also for this compound. Experiments using selective aldehyde or xanthine oxidase inhibitors could be used to address this, but it has not been performed in this study. Thus, the exact mechanism or enzyme responsible for the formation of M3a remains to be elucidated.

The in vivo metabolism of (S)-25 in rat differed significantly from the metabolism observed in vitro. Relatively large amounts of the di-N-oxide M8a was observed in bile, where the greater part of metabolites was excreted. Here, we observed an interesting metabolic pathway of the pyrimidine ring. Metabolite M4a and M6a were formed by a ring-opening reaction followed by elimination of a carbon atom and a ring closure. These ring-contracted metabolites were not detected in any of the in vitro systems tested. The loss of a hydroxyl radical in CID-MS/MS, together with a H/D exchange experiment showing that M4a has one extra exchangeable proton compared with (S)-25, strongly suggested that it is a N-hydroxylated or N-oxidated imidazole. Vicriviroc, which contains a pyrimidine-4,6-methyl moiety formed a ring-contracted metabolite pyrazyl-hydroxylamine using CYP3A4 supersomes (Ghosal et al., 2007). M4a was accompanied by its corresponding glucuronic acid conjugate and the analogous metabolites also oxidized in the pyridine ring. N-glucuronidation of an C4-substituted imidazole, 4-(4’-methyl[1,1’-biphenyl]-2-yl)-1H-imidazole, has been investigated previously (Huskey et al., 1994) and for this compound both nitrogens of the imidazole were susceptible to glucuronidation. In contrast to M5a, which was easily hydrolyzed by β-glucuronidase, these glucuronides were resistant to hydrolysis. This may indicate that the glucuronic acid in M5a is attached to a hydroxyl group. The mechanism of the formation of M4a or M6a is not known at present. It is far from trivial to identify the molecular mechanism and responsible enzyme(s), because the metabolite only is formed in vivo.

In contrast to (S)-25, AZD3839 was excreted in vivo mainly as N-oxides (M1, M2 and M9) in rat in agreement with its in vitro profile. The analogs (M31 and M34) to the ring-contracted metabolites of
(S)-25 were observed, demonstrating that this mechanism also occurred with AZD3839, but to a much lower extent. The 4-pyrimidone and pyrimidine-2,4-dione metabolites M24 and M23 were formed in vivo, but not in the same quantities as was found in the hepatocyte incubations. It is an interesting observation that commonly used in vitro systems such as hepatocytes were quite predictive for AZD3839, but not for the close analogue (S)-25. One speculation could be that the 4-pyrimidone and pyrimidine-2,4-dione metabolites formed in vitro are in fact metabolized to the ring-contracted products in vivo. These metabolites were produced from (S)-25 in much larger quantities. In order to be able to select the best compounds to progress, the involved reactions need to be further understood to effectively use hepatocytes to screen pyrimidine-containing compounds.

In conclusion, we have shown that (S)-25 is metabolized by CYP3A4 to pyridine N-oxide in vitro. In hepatocytes, it also produce 4-pyrimidone and pyrimidine-2,4-dione metabolites to varying extent. In the intact rat, however the pyrimidine ring of compound (S)-25 was mainly metabolized by oxidative reactions including an uncommon ring contraction of the pyrimidine. The analogue to (S)-25, AZD3839 was mainly eliminated via N-oxidation in the intact rat reflecting the pattern observed in rat hepatocytes. Also, the different metabolic pattern in vivo compared to hepatocytes for these close analogues indicates that the involved enzymes and reactions must be further characterized in order to effectively use hepatocytes to screen and predict metabolism of pyrimidine-containing compounds.
Acknowledgments

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Authorship Contributions

*Participated in research design:* Lindgren, Eklund.

*Conducted experiments:* Lindgren, Eklund.

*Contributed new reagents or analytic tools:* Malmquist, Turek, Swahn, Holenz, Von Berg, Karlström.

*Performed data analysis:* Lindgren, Eklund.

*Wrote or contributed to the writing of the manuscript:* Lindgren, Bueters.
References


Figure Legends

Figure 1. Metabolic profiling of compound (S)-25 in hepatocytes. A) Human cryopreserved hepatocytes. B) Rat freshly prepared rat hepatocytes. C) Rabbit cryopreserved hepatocytes. LC-MS chromatograms are showing the MDF scans. Incubations were conducted at 5 µM substrate concentration for 120 min. Proposed structures of identified metabolites are depicted in Figure 2 and Table 1. Relative abundance values are presented for each individual panel.

Figure 2. Proposed metabolites of compound (S)-25. The yellow color indicates atoms where the bond can be attach (markush).

Figure 3. Chromatograms of plasma (6 h) and bile (0-6 h) after oral administration of 30 µmol/kg, 50 MBq/kg of [3H]-(S)-25 to rat. A) LC-MS chromatogram of plasma showing the MDF scan. B) LC-MS chromatogram of bile showing the MDF scan. C) LC-radioactivity chromatogram of bile. Proposed structures of identified metabolites are depicted in Figure 2 and Table 1. Relative abundance or intensity values are presented for each individual panel.

Figure 4. Radiochromatograms of hepatocyte incubations with [14C]-AZD3839. A) Human cryopreserved hepatocytes. B) Rat fresh hepatocytes. C) Rabbit cryopreserved hepatocytes. Incubations were conducted at 5 µM substrate concentration for 120 min. Proposed structures of identified metabolites are depicted in Figure 8 and Table 2.
Figure 5. Proposed metabolites of AZD3839. The yellow color indicates atoms where the bond can be attach (markush).

Figure 6. Radiochromatograms of plasma and bile collected after oral administration of 58μmol/kg, 9.25 MBq/kg of [14C]-AZD3839 to rat. A) plasma 8 h B) Bile 0-48 h Proposed structures of identified metabolites are shown in Figure 5 and Table 2.

Figure 7. CID product ion spectra. A) protonated base [(S)-25 + H] (m/z 382). B) [M4a +H] (m/z 386). C) MS3 of m/z 359.13 from [M4a +H] (m/z 386). Proposed ion fragmentation is shown in the inserted molecular structure. M4a was from bile collected 0-6 h after oral administration of 30 μmol/kg, 50 MBq/kg of [3H]-(S)-25 to rat.

Figure 8. MDF filtered LC-MS chromatograms of bile collected 3-4 h after administration of 30μmol/kg of [3H]-(S)-25 to rat. A) Untreated bile sample. B) Bile sample treated with β-glucuronidase. Proposed structures of identified metabolites are depicted in Figure 2 and Table 1. Relative abundance values are presented for each individual panel.
Table 1. LC-MS/MS CID fragmentation of compound (S)-25 and observed metabolites.

<table>
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<tr>
<th>Metabolite</th>
<th>Structure</th>
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<th>Product Ions m/z</th>
<th>Number of exchangeable protons</th>
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<th>Structure</th>
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Table 2. LC-MS/MS CID fragmentation of AZD3839 and metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Structure</th>
<th>[M+H]^+ m/z</th>
<th>Product Ions m/z</th>
<th>Number of exchangeable protons</th>
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| AZD3839    | ![Structure](image) | 432.1428 | 415,1163 (-NH3)  
395,1101 (415 – HF)  
392.1306 (- 2 x HF)  
304.1117  
284.0992 (- isoxindole)  
276.0743  
256.0681 (276 – HF) | 3 |
| M1         | ![Structure](image) | 448.1377 | 431,1347 (- OH)  
411,1286 (- HF)  
380,1306 (431 – 2xHF)  
276.0742 | 3 |
| M2         | ![Structure](image) | 448.1377 | 431,1353 (- OH)  
380,1310 (431 – 2xHF)  
292.0693 | 3 |
| M3         | ![Structure](image) | No molecular ion detected | No molecular ion detected |  |
| M4         | ![Structure](image) | No molecular ion detected | No molecular ion detected |  |
| M5         | ![Structure](image) | 464.133 | No MS² |  |
| M7         | ![Structure](image) | 787 | No MS² |  |
| M9         | ![Structure](image) | 464.1330 | 447,1297 (- OH)  
444,1263 (- HF)  
430,1269 (- 2 x OH)  
427,1240 (447 - HF)  
407,1171 (427—HF)  
379,1231 (-CHF3CO2)  
319,0993  
303,1039 | 3 |
| -M11       | ![Structure](image) | 480.13 | 463.12 (–OH)  
352  
335  
276 | 5 |
| M12        | ![Structure](image) | 464.13 | 447.13 (- OH)  
427 (- HF)  
427 (447 - HF)  
319  
276 | 4 |
| M13        | ![Structure](image) | 448.1375 | 431,1112 (- NH3)  
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</table>
Figure 1

A) Human

B) Rat

C) Rabbit
Figure 2
Figure 3
Figure 4
FIGURE 5
Figure 6
Figure 7
Figure 8