Effects of Ketoconazole on the In Vivo Biotransformation and Hepatobiliary Transport of the Thrombin Inhibitor AZD0837 in Pigs

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ABSTRACT:

Ketoconazole has been shown in clinical trials to increase the plasma exposure of the oral anticoagulant prodrug AZD0837 [(2S)-N-4-[(2-amino(methoxyimino)methyl]benzyl]-1-[(2R)-2-[3-chloro-5-(difluoromethoxy)phenyl]-2-hydroxyethanoyl]-azetidine-2-carboxamide] and its active metabolite, AR-H067637 [(2S)-N-4-[(amino(mino)methyl]benzyl]-1-[(2R)-2-[3-chloro-5-(difluoromethoxy)phenyl]-2-hydroxyethanoyl]-azetidine-2-carboxamide]. To investigate the biotransformation of AZD0837 and the effect of ketoconazole on this process, we used an experimental model in pigs that allows repeated sampling from three blood vessels, the bile duct, and a perfused intestinal segment. The pigs received AZD0837 (500 mg) given enterally either alone (n = 5) or together with single-dose ketoconazole (600 mg) (n = 6). The prodrug (n = 2) and its active metabolite (n = 2) were also administered intravenously to provide reference doses. The plasma data revealed considerable interindividual variation in the exposure of the prodrug, intermediate metabolite, and active metabolite. However, AR-H067637 was detected at very high concentrations in the bile with low variability (Aubile = 53 ± 6% of the enteral dose), showing that the compound had indeed been formed in all of the animals and efficiently transported into the bile canaliculi. Concomitant dosing with ketoconazole increased the area under the plasma concentration-time curve for AZD0837 (by 99%) and for AR-H067637 (by 51%). The effect on the prodrug most likely arose from inhibited CYP3A-mediated metabolism. Reduced metabolism also seemed to explain the increased plasma exposure of the active compound because ketoconazole prolonged the terminal half-life with no apparent effect on the extensive biliary excretion and biliary clearance. These in vivo results were supported by in vitro depletion experiments for AR-H067637 in pig liver microsomes with and without the addition of ketoconazole.

Introduction

Venous thromboembolism is a major cause of cardiovascular-associated death and its high prevalence in the Western world leads to considerable prescribing of oral anticoagulants (Mackman, 2008). In the prevention of venous thrombosis, there is a need for orally available drugs with more predictable pharmacokinetics and improved safety profiles than warfarin, the present first choice for treatment (Weitz and Linkins, 2007; Mackman, 2008). One of the oral anticoagulants in clinical development is AZD0837, a prodrug whose active metabolite, AR-H067637, is a potent and reversible inhibitor of thrombin (Deinum et al., 2007; Lip et al., 2009; Olsson et al., 2010).

The positively charged and polar benzamidine moiety of AR-H067637, essential for binding to the active site of thrombin, prevents the compound from efficiently permeating the intestinal membrane barrier. Thus, use of a prodrug strategy was required to make the drug orally bioavailable without compromising its pharmacodynamic potency. Two metabolic activation steps are necessary to form AR-H067637 from AZD0837 (Fig. 1). The biotransformation starts subsequent to administration of the prodrug; the cytochrome P450 isoenzymes 3A4, 2C9, and 2C19, and the N-hydroxylation reductase enzyme system are involved in the first and second reactions, respectively (Andersson et al., 2005; Johansson et al., 2007).

Ketoconazole was recently observed to elevate the plasma exposure of AZD0837 and AR-H067637 in healthy volunteers by approximately 2-fold (Cullberg et al., 2007). Because CYP3A4 is assumed to be the major cytochrome P450 isoenzyme involved in the demethylation of AZD0837, the increased area under the plasma concentration-time curve (AUC) that was revealed for this compound had been expected (Cullberg et al., 2007; Johansson et al., 2007). However, the effect of ketoconazole on the active metabolite remains more elusive. Because AR-H067637 is believed to primarily be excreted in an unchanged form via the biliary route (data on file, AstraZeneca)
To gain further insight into the mechanisms responsible for the AZD0837-ketoconazole interaction, an advanced pig model previously developed by our group was used (Fig. 2) (Petri et al., 2006; Sjödin et al., 2008). Complementary in vitro depletion experiments in pig liver microsomes were also performed to investigate the potential effect of ketoconazole on the metabolism of the active compound in pigs. The pig, with a gastrointestinal tract anatomically and physiologically resembling that of human, is well suited for bioavailability and pharmacokinetic studies (Krishnan et al., 1994; Martinez et al., 2002). With regard to metabolism, it has been shown to be a suitable model of human CYP3A, displaying enzymatic activity toward prototypical substrates similar to that of the human isoforms (Soucek et al., 2001; Anzenbacherová et al., 2005; Thörn et al., 2009). The literature on transport proteins in pigs is less detailed, but several drug-relevant transporters are known to be expressed in pigs, and drugs dependent on carrier-mediated transport in their clinical disposition have been shown to display similar in vivo pharmacokinetics in pigs and humans (Goh et al., 2002; Török et al., 2003; Sjödin et al., 2008; Bergman et al., 2009). Resolving the causes of the pharmacokinetic interaction between AZD0837 and ketoconazole would lead to improved knowledge about processes that are important for the disposition of AZD0837 and its metabolites and for the prediction of other plausible drug-drug interactions.

Materials and Methods

Interaction Study in Pigs. Animals. The pigs (Hampshire, Yorkshire, and Swedish Landrace) included in the investigation were male, 10 to 12 weeks old, and weighed 25.0 ± 1.5 kg. The study protocol was approved by the local ethics committee for animal experiments (Approval Numbers C 257/6 and C 276/9), and the handling of the animals followed national regulations.

Study design and investigational drugs. Anesthetized pigs received either 500 mg of AZD0837 alone (control group, n = 6) or the same dose given together with 600 mg of ketoconazole (ketoconazole group, n = 6). To ensure that AZD0837 concentrations would be detectable and that ketoconazole would reach levels at which the compounds exerted inhibitory effects on CYP3A, the doses were chosen to be higher than their respective therapeutic dose in humans (normalized to body weight). The drugs were given via a multichannel catheter, which was positioned in the proximal jejunum of the animals (Fig. 2). One of the pigs in the control group was excluded owing to problems encountered when the AZD0837 was administered; hence, five animals treated only with AZD0837 were evaluated in the data analysis. The prodrug (n = 2) and its active metabolite (n = 2) were given as intravenous reference doses in a peripheral ear vein in separate animals.

AZD0837 was dissolved in polyethylene glycol 400-ethanol-water (v/v/v, 20:5:75) with 5 mM citric acid to obtain a final AZD0837 concentration of 10 mg/ml (pH 3.2). The solution was left to adjust to room temperature before the administration of the drug. Ketoconazole was prepared by dispersing tablets (three 200-mg tablets of Fungoral; Janssen-Cilag, Sollentuna, Sweden) in 50 ml of water (37°C) and was given 30 min before AZD0837. After each drug administration, the syringe and catheter used were rinsed with 50 ml of water (37°C). AZD0837 and AR-H067637 were prepared for intravenous dosing as described for AZD0837 above and sterile-filtered. The intravenous doses of AZD0837 and AR-H067637 were 200 mg (10 mg/ml) and 100 mg (5 mg/ml), respectively.

With the intention of detecting any possible leakage of the enteral AZD0837 dose between the site of administration and the intestinal perfusion (Fig. 2), 18.5 kBq of the nonabsorbable marker, [14C]polyethylene glycol 4000 (PEG4000) (GE Healthcare, Little Chalfont, Buckinghamshire, UK), was added to the AZD0837 solution.

Surgical procedures. The study procedure has been described in detail previously (Sjödin et al., 2008) and will, therefore, only be briefly summarized here. The animals were sedated during transport to the experimental site and, shortly after their arrival, they received anesthetics and sedatives. To ensure that the physiological state of the pigs was satisfactory, they were carefully monitored throughout the experiment with respect to body temperature, blood gases, heart rate, arterial and central venous pressures, and electrocardiographic parameters. The pigs were mechanically ventilated with an oxygen-air mix by means of a Servo 900C ventilator (Siemens-Elema, Solna, Sweden). At the end of the study, while they were still under anesthesia, the animals were given a lethal intravenous dose of 20 to 30 mmol of potassium chloride.

For the blood sampling, catheters were inserted into the femoral vein and into the left external jugular vein and advanced under fluoroscopic control to the hepatic vein. The abdominal cavity was opened through a midline incision to enable cannulation of the bile duct and the portal vein and insertion of a multichannel tube (Loc-I-Gut; Synectics Medical, Stockholm, Sweden) into the proximal jejunum of the animals. The Loc-I-Gut catheter was used for both enteral drug administration and intestinal perfusion (Fig. 2). After the surgery
had been completed, the pigs were stabilized 30 min before the experiment was started.

**Intestinal perfusion.** During the 6-h experiment, a 10-cm long jejunal segment, located between the two inflated balloons attached to the Loc-l-Gut tube (Fig. 2), was perfused with phosphate buffer at pH 6.5 (5.4 mM KCl, 48 mM NaCl, 10 mM NaH$_2$PO$_4$, 34 mM Na$_2$HPO$_4$, 1.0 g/l PEG4000, 35 mM mannitol, and 0.2 mg/ml phenol red). The flow rate of the phosphate buffer was set to 2.0 ml/min (syringe pump model 355: Sage Instruments, Orion Research Inc., Cambridge, MA), and the perfusate leaving the segment was continuously collected with a fraction sampling time of 20 min. The samples were stored at –70°C pending analysis.

**Collection of biological samples.** Blood samples were taken from the portal, hepatic, and femoral veins before dosing and 10, 30, 50, 70, 90, 110, 130, 150, 170, 190, 240, 300, and 360 min after the administration of the AZD0837 dose. The blood collection tubes were centrifuged at 4°C for 10 min at a centrifugal force of 1500g (Universal 16R; Hettich, Tuttlingen, Germany), and the plasma obtained was transferred to new vials. Bile was continuously collected from the common bile duct over the same time period using fraction intervals of 20 min. All samples were immediately frozen (plasma at –20°C and bile at –70°C) pending analysis.

**Bioanalysis.** Determination of AZD0837, AR-H069927 [(2S)-N-4-(2-aminophenyl)[2-(hydroxyimino)methyl]-1-[(2R)-2-chloro-5-(difluoromethoxy)phenyl]-2-hydroxyacetyl]azetidine-2-carboxamide], and AR-H067637 in plasma, bile and perfusate samples was performed at AstraZeneca R&D Mölndal (Mölnsdal, Sweden), using liquid chromatography-mass spectrometry (LC-MS/MS). The analytes were isolated from the plasma by solid-phase extraction. The plasma samples (100 μl) were loaded on columns (Isolute C6, 25 mg) together with isotope-labeled internal standards (d4, 50 μl) for all three analytes and 5% methanol in 0.5 M ammonium acetate (250 μl). The columns were washed with 5% methanol in water (450 μl), and the analytes were eluted with 70% acetonitrile in 3.8 mM ammonium acetate and 0.1% acetic acid (400 μl). The eluate was diluted with the ammonium acetate-acetic acid solution (400 μl) before LC-MS/MS analysis of a 5-μl extract. The three analytes were separated on a reverse-phase C18 column (HyperPURITY, 50 × 2.1 mm, 5 μm) at 40°C using a flow rate of 0.20 ml/min. The mobile phases A and B were 20 and 80% acetonitrile, respectively, in 3.8 mM ammonium acetate and 0.1% acetic acid. The analytes were eluted by a linear gradient from 0% to 100% A, which was followed by reequilibration with 100% A. The mass spectrometer, an API 3000 triple quadrupole with electrospray interface, was used in multiple reaction monitoring mode. The linear concentration range was 50 to 50,000 nM for AZD0837 and 20 to 20,000 nM for AR-H069927 and AR-H067637. The bile and perfusate samples (100 μl) were mixed with isotope-labeled internal standards (d4, 50 μl) for all three analytes and 30% methanol in 3.8 mM ammonium acetate and 0.1% acetic acid (600 μl) before LC-MS/MS analysis of a 5-μl extract. Samples with concentrations above the linear concentration range were diluted with perfusate buffer before sample preparation. The chromatographic conditions were the same as those for the plasma samples, with the exception of 30% and 80% methanol being used in the mobile phases A and B, respectively, and a 2-min-long washing step being performed with 100% B before the reequilibration. The linear concentration range was the same as that for the plasma samples.

The ketoconazole concentrations were measured in the samples withdrawn from the portal vein to estimate the concentration of the inhibitor entering the liver. This analysis was performed at Uppsala University in accordance with a previously published method (Vertzoni et al., 2006). The samples were prepared by the addition of internal standard (5 μg/ml 9-actetylanthracene) and acetonitrile (100 μl of plasma-40 μl of internal standard-60 μl of acetonitrile), followed by centrifugation. The clear supernatant was injected directly into an LC system that consisted of an autosampler (Waters 717plus; Waters, Milford, MA) and a pump (LC-10AD; Shimadzu, Kyoto, Japan) operating at a flow rate of 1.0 ml/min. The internal standard and ketoconazole were successfully separated on a C18 column (250 × 4.6 mm, 5 μm), protected by a precolumn (10 × 4.0 mm, 5 μm), with a mobile phase of methanol-water-diethylamine (v/v/v, 70:30:0.1). The linear concentration range used for the calibration curve was 40 to 50,000 nM.

**Pharmacokinetic analysis.** Noncompartmental analysis of the plasma (portal, hepatic, and femoral veins) and bile concentration-time profiles of AZD0837 and its metabolites was performed using WinNonlin 5.2 (Pharsight, Mountain View, CA). The pharmacokinetic parameters determined were the area under the plasma/bile concentration versus time curve (AUC$_{0-\infty}$ and AUC$_{0-\infty}$, the maximum plasma/bile concentration ($C_{\text{max}}$), and the time to reach $C_{\text{max}}$ ($t_{\text{max}}$). The AUC was calculated by means of the linear/logarithmic trapezoidal rule (for the up and down portions of the curve, respectively) to the last measured concentration. Regression analysis of the last three to five data points of the log plasma and bile concentration-time curves was performed to obtain the terminal rate constant, and this parameter was used to calculate the terminal half-life ($t_{\frac{1}{2}}$) and to extrapolate the AUC$_{0-\infty}$ to infinity.

The hepatic extraction ratio ($E_{\text{bile}}$) and hepatic clearance ($CL_{\text{bile}}$) of the prodrug AZD0837 were calculated from the concentrations of AZD0837 in the plasma entering (portal vein) and leaving the liver (hepatic vein) (eqs. 1 and 2):

$$E_{\text{bile}} = \frac{AUC_{0-\infty}, \text{VP}}{AUC_{0-\infty}, \text{VP}}$$  

CL$_{\text{bile}} = \frac{Q_{\text{H}} \cdot E_{\text{bile}} \cdot C_{\text{blood}}}{C_{\text{plasma}}}$

where the values of the hepatic blood flow, $Q_{\text{H}}$, and blood/plasma ratio were 52 ml/min/κg (Nordgren et al., 2002) and 0.6, respectively (data on file, AstraZeneca R&D Mölndal).

The hepatobiliary disposition of AZD0837 and its metabolites was further analyzed by calculating the amount excreted into bile (Ae$_{\text{bile}}$) and the apparent biliary clearance (CL$_{\text{bile}}$) during the 6-h experiment (eq. 3). It should be noted that the apparent CL$_{\text{bile}}$ is a complex term including not only sinusoidal and canalicular transport processes, but also metabolic reactions.

$$CL_{\text{bile}} = \frac{Ae_{\text{bile}}}{AUC_{0-\infty}, \text{VP}}$$

In addition, the biliary excretion data after the enteral and intravenous dosing with AZD0837 were used to approximate the fraction of the dose absorbed ($f_{\text{abs}}$). The $f_{\text{abs}}$ was calculated by dividing the total Ae$_{\text{bile}}$ for each animal after enteral AZD0837 administration (control group) with the mean value of the total Ae$_{\text{bile}}$ after intravenous dosing of the prodrug (eq. 4). The calculation assumes that the fraction of the sum of AZD0837 and its metabolites eliminated via bile remains constant, irrespective of the administration route:

$$f_{\text{abs}} = \frac{\sum Ae_{\text{bile, enteral}} \cdot \text{dose}, \text{enteral}}{\sum Ae_{\text{bile, i.v.}} \cdot \text{dose, i.v.}}$$

To approximate the secretion of the compounds in the entire small intestine, the amounts secrete into the 10-cm segment of the small intestine (Ae$_{\text{intestine}}$) were scaled by a factor of 50, estimating the length of the porcine small intestine to 5 m and assuming no changes in the transport processes along this path (Bergman et al., 2009). These scaled amounts were used to approximate the intestinal clearance (CL$_{\text{intestine}}$) (eq. 5):

$$CL_{\text{intestine}} = \frac{Ae_{\text{intestine}}}{AUC_{0-\infty}, \text{VP}}$$

**Statistical Analysis.** Descriptive statistics were calculated for the pharmacokinetic parameters AUC, $C_{\text{max}}$, $t_{\text{max}}$, $t_{\frac{1}{2}}, CV_t$, CL$_{\text{bile}}$, $Ae_{\text{bile}}$, $Ae_{\text{intestine}}$, and CL$_{\text{intestine}}$. An unpaired Student’s t test was used to evaluate differences between the group of pigs receiving AZD0837 (control group) and that receiving AZD0837 and ketoconazole (ketocozaole group), with the exception of $t_{\text{max}}$ for which the two-sample Mann-Whitney test was used; p < 0.05 was considered to be significant. Owing to the shape of the distribution function, it was decided to log-transform the parameters AUC, $C_{\text{max}}$, $t_{\frac{1}{2}}, CV_t$, $Ae_{\text{bile}}$, $Ae_{\text{intestine}}$, and CL$_{\text{bile}}$ before the statistical analysis was performed.

**In Vitro Metabolism Study in Pig Microsomes.** AR-H067637 was incubated with pig liver microsomes to determine whether the compound was metabolized and, if so, whether this reaction could be inhibited by ketoconazole. The incubations with the microsomes were performed according to the method of multiple depletion curves described in detail elsewhere (Sjögren et al., 2009). The metabolism of AR-H067637 was investigated in triplicate at five concentrations (200, 50, 10, 2, and 0.5 μM), limited by the solubility and the lower limit of detection, with and without addition of the
The concentration of ketoconazole (2 μM) was chosen from the results of our in vivo pig study and corresponded to the mean value of \( C_{\text{max}} \) determined in the portal vein. The incubation buffer consisted of pig liver microsomes (0.5 mg/ml) in a phosphate buffer (80 mM K2HPO4 and 20 mM KH2PO4, pH 7.4), and after a short preincubation with the substrate, conducted either with or without adding the inhibitor, the reaction was started by the addition of NADPH (1 mM). Throughout the experiment, the samples were kept at 37°C. The reaction was terminated at designated time points (0, 15, 30, 45, and 60 min) when 100-μl aliquots were withdrawn from the incubation mixture and added to 300 μl of stop solution (95% methanol). This was followed by centrifugation for 10 min to prepare the samples for chemical analysis; 100 μl of the clear supernatant was removed and mixed with 50 μl of 5% methanol in 50 mM ammonium acetate, 200 μl of 30% methanol, and 50 μl of 0.4 labeled internal standard. The LC-MS/MS analysis was performed at Astrazeneca R&D Mölndal as described for the in vivo plasma samples with a linear concentration range of 20 to 20,000 nM for AR-H067637. Separate experiments verified that AR-H067637 was chemically stable in the incubation buffer (37°C), at least throughout the duration of the depletion experiments.

The resulting concentration-time profiles obtained from all five concentration levels were simultaneously fitted to eq. 6 using nonlinear regression (WinNonlin 5.2):

\[
-\frac{d[C]}{dt} = \frac{v_{\text{max}} \cdot [C]}{K_m + [C]} \tag{6}
\]

where [C] is the substrate concentration, \( v_{\text{max}} \) is the theoretical maximum depletion rate, and \( K_m \) is the substrate concentration at half the theoretical maximum depletion rate. The intrinsic clearance (CLint) was obtained with eq. 7 and normalized to the protein concentration used in the incubation. The fraction of AR-H067637 bound to microsomal proteins or other cellular constituents was not determined, and the calculated values for CLint should thus be interpreted as apparent measures. Nevertheless, these measures fulfill the purpose, that of revealing a possible effect of ketoconazole on the metabolism of AR-H067637.

\[
CL_{\text{int}} = \frac{v_{\text{max}}}{K_m} \tag{7}
\]

### Results

#### Effect of Ketoconazole on the Plasma Pharmacokinetics of AZD0837 and Its Metabolites In Vivo

The fraction of AZD0837 absorbed (\( f_{\text{abs}} \)) after administration of an enteral solution was 76 ± 8% of the dose, as estimated from biliary excretion data. The dominant compound in plasma was the prodrug, whereas the intermediate metabolite, AR-H069927, was present at low concentrations (Table 1; Fig. 3A). The contribution of AR-H067637 to the sum of the plasma exposure (AUC0–6h) for all three compounds ranged from 9 to 43% and from 4 to 48% in the control and ketoconazole groups, respectively (Table 1; Fig. 3B). Ketoconazole seemed to increase the AUC0–6h of AZD0837 (by 99%) and of AR-H067637 (by 51%) (Fig. 3C), but the variability in the plasma pharmacokinetics of AZD0837 and its metabolites made it impossible to determine a statistically significant effect. The AUC0–6h of the intermediate was unaffected by the inhibitor, but its \( C_{\text{max}} \) was somewhat reduced (24%) (Table 1). Ketoconazole prolonged the terminal \( t_{1/2} \) of all three compounds, an effect that was most pronounced for the active compound, whose \( t_{1/2} \) increased from 1.2 ± 0.2 to 2.6 ± 1.4 h (p < 0.05). The treatment ratios presented above and in Table 1 were derived from the peripheral compartment and were of the same magnitude in the portal and the hepatic veins. There was a small and statistically nonsignificant reduction in the AZD0837 hepatic extraction ratio caused by ketoconazole, and, hence, a similar trend was observed for the hepatic clearance of the compound (Table 1). The \( t_{\text{max}} \) and \( C_{\text{max}} \) of ketoconazole reaching the liver (portal vein) after administration of the enteral dispersion were 1.4 h (range 1.0–1.7 h) and 2.03 ± 1.00 μM, respectively.

#### Effect of Ketoconazole on the Bile Pharmacokinetics of AZD0837 and Its Metabolites In Vivo

In pigs, the drug fraction excreted into bile was composed almost exclusively of the active metabolite, which represented 99% of the total biliary exposure, with negligible amounts of the prodrug and intermediate (Table 2). It is noteworthy that this was also the case in the animals that had a low plasma exposure of AR-H067637. Indeed, transport into bile formed a major elimination route for the active compound, as was evident by the fact that approximately 60% of the AR-H067637 intravenous dose was recovered in bile (Table 3). The fraction of the dose detected in bile in the form of the active compound was also extensive after intravenous (−70%) and enteral (−50%) administration of the prodrug AZD0837 (Table 3). With a bile/plasma AUC ratio approaching 1700 (in the control group) (Fig. 4; Table 2), it is likely that the biliary excretion of AR-H067637 was mediated by carrier proteins at the canicular membrane. Ketoconazole did not affect the amount of active metabolite excreted into bile, and the inhibitor did not seem to change the apparent biliary clearance, which was determined to be 34 ± 17 ml/min/kg (for the control group) and 34 ± 29 ml/min/kg (for the group given ketoconazole) (Fig. 4; Table 2). The effects of ketoconazole on the biliary concentration-time curves of the prodrug and metabolites were in accordance with the profiles of the compounds in plasma, with the exception of the changes in the bile AUC and in the \( C_{\text{max}} \) of AR-H067637, which were somewhat smaller than those in plasma (Fig. 4; Tables 1 and 2).

#### Effect of Ketoconazole on the Intestinal Secretion of AZD0837 and Its Metabolites In Vivo

Throughout the experiment, a 10-cm jejunal segment was perfused with a phosphate buffer to determine the

### TABLE 1

Pharmacokinetic parameters for AZD0837 (prodrug), AR-H069927 (intermediate metabolite), and AR-H067637 (active metabolite) derived from plasma data in pigs after enteral dosing of AZD0837 alone (n = 5) and in conjunction with ketoconazole (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>AZD0837</th>
<th>+ Ketoconazole</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0–6h (μmol · h/l)</td>
<td>40.4 ± 22.7</td>
<td>74.4 ± 39.1</td>
<td>1.99</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>2.1 ± 1.5</td>
<td>2.4 ± 1.8</td>
<td>1.08</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>12.5 ± 8.6</td>
<td>193 ± 11.3</td>
<td>15.1</td>
</tr>
<tr>
<td>AUC0–6h (% of the sum of the AUC0–6h)</td>
<td>70.2 ± 15.6</td>
<td>75.5 ± 13.3</td>
<td>1.08</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>4.6 ± 3.7</td>
<td>2.4 ± 1.6</td>
<td>0.76</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>25.1 ± 14.1</td>
<td>22.1 ± 14.4</td>
<td>0.91</td>
</tr>
<tr>
<td>Cmax (μmol/l)</td>
<td>17.8 ± 8.4</td>
<td>26.0 ± 9.5</td>
<td>1.46</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>0.7 ± 0.4</td>
<td>0.6 ± 0.4*</td>
<td>0.76</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>3.8 ± 2.7</td>
<td>4.3 ± 2.6*</td>
<td>1.11</td>
</tr>
<tr>
<td>tmax (h)</td>
<td>AR-H06737</td>
<td>1.2 (0.9–1.5)</td>
<td>1.0 (0.5–1.9)</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>1.5 (0.5–2.5)</td>
<td>0.5 (0.5–2.9)*</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>2.2 (1.9–3.2)</td>
<td>3.2 (2.5–4.0)*</td>
<td>1.0</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>AR-H06737</td>
<td>0.7 ± 0.04</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>1.0 ± 0.2</td>
<td>1.6 ± 0.4*</td>
<td>1.6</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>1.2 ± 0.2</td>
<td>2.6 ± 1.4*</td>
<td>2.6</td>
</tr>
<tr>
<td>Fb (in bile)</td>
<td>AR-H06737</td>
<td>0.41 ± 0.20</td>
<td>0.33 ± 0.20</td>
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<tr>
<td>AR-H069927</td>
<td>13 ± 6</td>
<td>10 ± 6</td>
<td>0.80</td>
</tr>
</tbody>
</table>

* p < 0.05, significantly different from control phase.

1. The geometric mean ratio between the control measurement and the ketoconazole phase.
2. The relative AUC0–6h expressed as a percentage of the summed AUC0–6h values of AZD0837, AR-H069927, and AR-H067637.
3. Pig 16 was excluded from the data analysis.
intestinal secretion because this is a possible elimination pathway after dosing with AZD0837 (Fig. 2). The amounts of the nonabsorbable marker [14C]PEG4000 in the jejunal samples were negligible during the 1st h immediately after AZD0837 dosing, and only 2 of the 47 samples obtained needed correction for leakage of the administered enteral dose. Low concentrations of the prodrug, the intermediate metabolite, and the active metabolite were detected in the perfusate samples leaving the intestinal segment and, after scaling (see Materials and Methods), these amounted to 2.6 ± 3.6, 0.5 ± 0.1, and 1.0 ± 0.6% of the enteral dose, respectively. The scaled amount yielded intestinal clearance values for AZD0837, AR-H069927, and AR-H067637 of 0.4 ± 0.4, 2.8 ± 2.7, and 1.1 ± 0.7 ml/(min · kg), respectively (Table 3). Coadministration of AZD0837 and ketoconazole did not alter the secretion into the perfused segment or the apparent intestinal clearance. Intravenous dosing of AZD0837 and AR-H067637 resulted in lower amounts being excreted into the intestine than after the enteral administration, as shown in Table 3. Because of the absence of a ketoconazole-related effect and the minor concentrations measured in the perfusate samples, intestinal perfusion was not performed in the last four animals included in the interaction study (two pigs in the control group and two pigs in the ketoconazole group).

Effect of Ketoconazole on the Depletion of AR-H067637 in Pig Liver Microsomes. AR-H067637 was chemically stable during the 60-min-long experiment. The compound was slowly metabolized upon incubation with liver microsomes, and the CL_{int} was estimated to be 12.2 ± 2.9 ml/(min · g) protein (Fig. 5). The addition of ketoconazole to the incubation buffer slowed the metabolic depletion of AR-H067637, resulting in a reduction of CL_{int} to 2.4 ± 3.7 ml/(min · g) protein (p < 0.05) (Fig. 5).

Discussion

The prodrug AZD0837 is currently undergoing clinical development for eventual use in the prevention of thromboembolic events. Its active metabolite, AR-H067637, is a potent direct inhibitor of thrombin (Deinum et al., 2009). With the intention of improving understanding of the biotransformation of AZD0837 during its first-pass through the gut and liver and the effect of ketoconazole on this process, we used a pig model enabling intestinal perfusion and simultaneous sampling from the bile duct and three venous blood vessels (the portal, hepatic, and femoral veins).

The prodrug AZD0837 was well absorbed (f_{abs} = 0.76 ± 0.08) from the gut lumen after enteral administration of the compound in solution. However, once AZD0837 was absorbed, the plasma levels of the compound and its intermediate and active metabolites were highly variable. Such a wide range of plasma concentrations has not been observed in humans (e.g., AUC_{AR-H067637}: 21 ± 3% of the sum of the total AUC for AZD0837, AR-H069927, and AR-H067637) and does not pose an issue in clinical use (Olsson et al., 2010). In all of the pigs, a high fraction of the dose was excreted into bile, almost exclusively in the form of the active metabolite, with little variation being observed (AE_{bile}: 53 ± 6% of the dose). Consequently, even the pigs exhibiting low concentrations of the compounds in plasma did indeed excrete amounts of AR-H067637 into bile that were close to those recovered in animals with high plasma concentrations.

The variable plasma concentrations of AZD0837 and its metabolite in the pigs may have several explanations, such as inconsistencies in the absorption of the prodrug and in the formation of the intermediate and active metabolites. However, the f_{abs} estimated in the study showed low variation (coefficient of variation = 11%), and several
pigs displayed relatively low plasma AUC of the metabolites despite having average exposure to the prodrug (Fig. 3B). This suggests that the variability was caused at the level of biotransformation rather than intestinal absorption. For ximelagatran, which exploits the same hydroxylamine reductase system as in the second step of AZD0837 biotransformation, interindividual variation was small in pigs of the same age (10–12 weeks) and breed as the ones included in the present study had a CYP3A hepatic mRNA content ranging from 0.7 to 2.0 when normalized to β-actin (n = 8) (data on file). In addition, the plasma pharmacokinetics of other CYP3A4 substrates investigated in pigs by our group have been shown to vary to the same degree as those of AZD0837 and its metabolites (Thörn et al., 2009).

The excretion of AR-H067637 into bile was very extensive, not only after enteral administration, but also after separate intravenous dosing of the prodrug and the active compound, respectively. To estimate the efficiency of the transport into bile, the total plasma concentration in the portal vein entering the liver was compared with the bile concentration. After enteral prodrug administration, this AUC bile/plasma ratio ranged from 850 to 3170 for AR-H067637, showing that the compound was transported against a concentration gradient through the bile route for AZD0837 (prodrug), AR-H069927 (intermediate metabolite), and AR-H067637 (active metabolite) after enteral administration of AZD0837 (n = 5) and intravenous dosing of AZD0837 (n = 2) and AR-H067637 (n = 2).

**TABLE 2**

Pharmacokinetic parameters for AZD0837 (prodrug), AR-H069927 (intermediate metabolite), and AR-H067637 (active metabolite) derived from bile data in pigs after enteral dosing of AZD0837 alone (n = 5) and together with ketoconazole (n = 6).

Data are mean ± S.D., median (range) for t_{max} or geometric mean ratio (95% CI) for AUC\text{bile}/AUC\text{plasma}.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AZD0837</th>
<th>+ Ketoconazole</th>
<th>Ratio(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC\text{o.a.} (µmol · h/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>136 ± 75</td>
<td>208 ± 107</td>
<td>1.51</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>58 ± 22</td>
<td>60 ± 41</td>
<td>1.01</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>19,620 ± 4567</td>
<td>22,752 ± 5743</td>
<td>1.15</td>
</tr>
<tr>
<td>C_{\text{max}} (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>95 ± 61</td>
<td>102 ± 42</td>
<td>1.14</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>24 ± 8</td>
<td>18 ± 14</td>
<td>0.86</td>
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<tr>
<td>AR-H067637</td>
<td>5756 ± 1432</td>
<td>6114 ± 804</td>
<td>1.08</td>
</tr>
<tr>
<td>t_{\text{max}} (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>1.0 (0.7–1.3)</td>
<td>1.0 (0.7–1.3)</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>0.7 (0.7–1.0)</td>
<td>0.7 (0.7–3.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>1.0 (0.7–1.3)</td>
<td>1.7 (1.3–2.0)</td>
<td>1.7</td>
</tr>
<tr>
<td>t_{1/2b} (h)</td>
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<td></td>
<td></td>
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<tr>
<td>AZD0837</td>
<td>0.9 ± 0.1(^b)</td>
<td>0.9 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>0.9 ± 0.1(^c)</td>
<td>1.2 ± 0.3(^c)</td>
<td>1.3</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>1.6 ± 0.5(^d)</td>
<td>2.8 ± 0.8(^d)</td>
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<tr>
<td>AUC\text{bile}/AUC\text{plasma} (µM)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>2.0 (8.8–4.9)</td>
<td>2.2 (1.6–3.0)</td>
<td>1.1</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>28 (17.4–34)</td>
<td>24 (20.3–30)</td>
<td>1.1</td>
</tr>
<tr>
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<td>1680 (710–3980)</td>
<td>1320 (430–4050)</td>
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</tr>
<tr>
<td>A_{\text{int}} (of dose)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>1.3</td>
</tr>
<tr>
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<td>0.2 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>53.0 ± 5.6</td>
<td>54.8 ± 4.1</td>
<td>1.0</td>
</tr>
<tr>
<td>C_{\text{int}} (µmol/min · kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZD0837</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H069927</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>AR-H067637</td>
<td>33.9 ± 16.9</td>
<td>34.2 ± 29.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Data displayed for each animal (n = 5 and 6).

\(^b\) Data displayed as mean values (n = 3).

\(^c\) The relative AUC\text{o.a.} in bile expressed as a percentage of the summed AUC\text{o.a.} values of AZD0837, AR-H069927, and AR-H067637.

\(^d\) Data displayed for each animal (n = 2).

\(^\) These values were scaled by a factor of 50 to approximate the secretion into the entire small intestine from the 10-cm long jejunal segment (see Results).

\(^\) N.A., not available.

\(^\) Data displayed as mean values (n = 5 and 6).

\(^\) Data displayed as mean values (n = 5 and 6).

\(^\) Data displayed for each animal (n = 5).
across the canalicular membrane in all of the pigs and, consequently, that this transport was mediated by carrier proteins. It also suggests that the canalicular efflux of the active metabolite is a more efficient process than the transport of the compound from the hepatocyte to the blood. Transporters that could be involved in the transport of AR-H067637 across the canalicular membrane include the pig variants of P-glycoprotein (ABC1) and multidrug and toxin extrusion protein 1 (SLC47A1) (AstraZeneca, data on file). The biliary excretion of the prodrug and intermediate was negligible compared with the amounts of AR-H067637 excreted; hence, the metabolite pattern in bile was completely different from that observed in plasma, where AZD0837 dominated. We concluded that the intestine played a minor role in the elimination of AZD0837 and its metabolites, owing to the negligible amounts of the compounds secreted into the perfused intestinal segment after both enteral and intravenous dosing. The CLint, values were in the same range as the CLbile values for the prodrug and intermediate, whereas for the active metabolite these values were 1.1 ± 0.7 and 34 ± 17 ml/(min·kg), respectively.

Ketoconazole elevated the plasma exposure of the prodrug and the active compound by 99 and 51%, respectively. The impact on the prodrug was similar to that observed in clinical ketocanazole-AZD0837 interaction trials in healthy volunteers, whereas the increase in the plasma AUC for AR-H067637 was somewhat smaller than anticipated (115–130% increase in humans) (Cullberg et al., 2007). This finding was explained by the prolonged tmax and t1/2 of the active compound, which shifted the concentration-time curve so that for the group given ketoconazole, a larger part of the AUC0–t was obtained after the last observed concentration at 6 h. The elimination process for the active metabolite seemed to differ from that in humans. This finding was suggested by the considerably shorter terminal t1/2 in pigs (1.2 versus 7.0 h in humans) and by the fact that ketoconazole prolonged the terminal t1/2, an effect that was not observed in humans. Ketoconazole did not affect the amount of AR-H067637 recovered in bile, nor did it seem to change the biliary clearance or the bile/plasma ratio. We speculated that AR-H067637 might be further metabolized in pigs and that ketoconazole could elicit its inhibitory effect via this pathway. Accordingly, AR-H067637 was incubated with and without ketoconazole in pig liver microsomes to test for a possible effect of the inhibitor on this parameter. The active compound was metabolized at a slow rate (CLmic = 12 ± 3 ml/(min·g) protein), and this process was reduced by ketoconazole (p < 0.05) (Fig. 5). This finding suggests that, in addition to biliary excretion, metabolism catalyzed by enzymes susceptible to inhibition by ketocanazole, may also contribute to the elimination of AR-H067637 in pigs.

The maximum concentration of ketoconazole detected in the portal vein was 2.03 ± 1.00 μM. If we assume that the same fraction bound to plasma proteins in pigs as in humans (0.99) (Ito et al., 1998), then the estimated unbound concentration was in the same range as the reported ketoconazole Ks values, obtained from the CYP3A-specific metabolism of midazolam in human liver microsomes (4–15 nM) (von Moltke et al., 1996; Gibbs et al., 1999). The formation of AR-H069927 from AZD0837 is primarily mediated by CYP3A4, and, thus, the increased plasma exposure of the prodrug on coadministration with ketoconazole was expected. Indeed, a small reduction in the hepatic extraction ratio of AZD0837 was observed in association with the elevated plasma AUC. CYP3A activity inhibited at the intestinal level may also have contributed to the ketoconazole effect, which was supported by the increased AZD0837 Cmax and AUC in the portal vein (treatment ratios of 1.31 and 1.63, respectively).

In conclusion, the prodrug AZD0837 was well absorbed and bio-transformed to its active form, the thrombin inhibitor AR-H067637, in pigs. Ketoconazole increased the plasma exposure of the prodrug and active metabolite, presumably because the metabolism of the compounds was inhibited. Biliary excretion formed the major route for the elimination of AR-H067637; the high levels of AR-H067637 in bile in comparison to plasma indicate the involvement of transport proteins in this process.

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Authorship Contributions

Participated in research design: Matsson, Palm, Eriksson, and Lennernäs.
Conducted experiments: Matsson, Bottner, Lundahl, and Knutson.
Performed data analysis: Matsson, Palm, Eriksson, and Lennernäs.
Wrote or contributed to the writing of the manuscript: Matsson, Palm, Eriksson, Bottner, Lundahl, Knutson, and Lennernäs.

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