DMD Fast Forward. Published on October 16, 2012 as doi:10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

TITLE PAGE

Effect of Solvents on the Time Dependent Inhibition of CYP3A4 and the Biotransformation of AZD3839 in Human Liver Microsomes and Hepatocytes

Jenny Aasa, Yin Hu, Göran Eklund, Anders Lindgren, Pawel Baranczewski, Jonas Malmquist, Dominika Turek, and Tjerk Bueters

DMPK, CNSP iMed Science, AstraZeneca R&D, Innovative Medicines, SE-15185 Södertälje, Sweden (J.A., Y.H., G.E., A.L., P.B., T.B.); Medicinal Chemistry, CNSP iMed Science, AstraZeneca R&D, Innovative Medicines, SE-15185 Södertälje, Sweden (D.T.); and Global DMPK Screening and Profiling, AstraZeneca R&D, SE-15185 Södertälje, Sweden (J.M.)

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Effect of Solvents on the TDI of CYP3A4.

Corresponding author: Jenny Aasa, DMPK CNSP iMed Science, AstraZeneca R&D, Innovative Medicines, phone: +46 (0)8 553 21756, mail: jenny.aasa@astrazeneca.com

Number of text pages:36

Number of tables:2

Number of figures:10

Number of references:27

Number of words in Abstract:250

Number of words in Introduction:667

Number of words in Discussion:1367

Non-standard abbreviations: CYP450, cytochrome P450; TDI, time dependent inhibition; MBI, mechanism based inhibition; DDI, drug-drug interactions; IC_{50}, value where 50% of the maximal inhibition is achieved; KPO_4, potassium phosphate buffer; DMSO, dimethyl sulfoxide; $Fe(CN)_6$, potassium ferricyanide; FDA, U.S. Food and Drug Administration; HLM, human liver microsomes; Hhep, human hepatocytes; MDZ, midazolam; TSN, testosterone; UPLC-MS/MS, ultra pressure liquid chromatography-tandem mass spectrometry; k_{obs}, apparent enzyme inactivation rate constant; K_I, inhibitor concentration that causes half the maximal rate of enzyme inactivation; k_{inact}, maximal rate of enzyme inactivation; K_m, Michaelis-Menten constant, substrate concentration supporting half the maximal rate of an enzyme catalyzed reaction; FTMS, Fourier Transform Mass Spectrometer.

ABSTRACT

Time dependent inhibition (TDI) of cytochrome P450 (CYP) is usually studied in human liver microsomes (HLM), by investigating whether the inhibitory potency is increased with increased incubation times. The presented work was initiated due to a discrepancy observed for the CYP3A4 TDI results for a drug candidate compound (AZD3839) from an early screening method, where no TDI was detected compared to a regulatory method, where TDI was detected. We show here that the different solvents present in the respective studies; DMSO (screening method) *versus* methanol or water (regulatory method), were the reason to the different TDI results. We further demonstrate why DMSO, present at the levels of 0.2% and 0.5% in the incubations, masked the TDI effect. In addition to the TDI experiments performed in HLM, TDI studies with AZD3839 were performed in pooled human hepatocytes (Hhep) from different suppliers, using DMSO, methanol or water. The results from these experiments show no TDI or attenuated TDI effect depending on the supplier. Metabolite identification of the compound dissolved in DMSO, methanol or water, after incubations with the different systems (HLM or Hhep) show different profiles, which may be the reason to the differences in the TDI outcomes. Thorough investigations of the biotransformation of AZD3839 have been performed, in order to find the reactive pathway causing the TDI of CYP3A4, and are presented here. Our findings show that the *in vitro* risk profile for DDI potential of AZD3839 is very much dependent on the chosen test system and the experimental conditions used.

INTRODUCTION

Inhibition of cytochrome P450 (CYP) enzymes may lead to serious drug-drug interactions (DDIs) causing adverse drug effects (Wienkers and Heath, 2005) via either reversible or irreversible reactions (Murray, 1997; Lin and Lu, 1998). Reversible inhibition means that two or more drugs compete at a P450-active site and the perpetrating drug inhibits the interaction of other drugs with the P450 in a concentration-dependent manner. With irreversible inhibition, referred to as mechanism based inhibition (MBI) or time dependent inhibition (TDI), the drug is converted by the P450 to a reactive intermediate that inactivates the function of the P450 in a quasi-irreversible (metabolic intermediate complex to the heme) or irreversible way (Silverman, 1995; Lin and Lu, 1998). MBI/TDI is thought to cause more serious DDIs than reversible inhibition as de novo synthesis of the inactivated protein is needed (Watanabe et al., 2007). For these reasons the potential of new chemical entities to cause DDIs via inhibition of P450 metabolism are studied pre-clinically and clinically during drug development. The most clinically relevant CYP enzyme in this regard is CYP3A4, which comprises about 30% of the total CYP content (Shimada et al., 1994) and is involved in the metabolism of more than 50% of the drugs on the market (Lehmann et al., 1998; Guengerich, 1999). Human liver microsomes (HLM) are the traditional enzyme system used for the study of P450 inhibition. Typically in drug discovery a simpler screening assay is used to identify and remove new chemical entities that cause unacceptable P450 inhibition, but in the development phase a more extensive study is conducted where regulatory authority recommendations are helping in the study design (Bjornsson et al, 2003; EMA draft DDI guideline, 2010; FDA draft DDI guidance, 2012). The outcomes of the preclinical reversible inhibition studies (IC_{50} and K_i values) and TDI studies (K_I and k_{inact} values) are subsequently used for projection of possible DDIs in the clinic (Shardlow et al., 2011). It has been shown

that such simulations based on data obtained from HLM often overpredict the magnitude of DDIs when compared to the actual observed interaction in human (Einolf, 2007; Chen et al., 2011). In the later years several laboratories have developed P450 inhibition methods using human hepatocytes (Hhep), which is a more complete system compared to HLMs, as both phase II enzymes and transporters are present apart from the phase I enzymes in HLM (Hewitt et al., 2007). These differences could potentially alter the biotransformation of a compound, and thus be the reason for different outcome. The literature reports indicate that predictions of *in vivo* DDIs based on Hhep data might be more accurate than using HLM data (Xu et al., 2009; Chen et al., 2011; Kirby et al., 2011).

Different experimental conditions may also be a factor that affects the biotransformation of a compound. It has been described that several organic solvents such as DMSO, methanol and acetonitrile reduce the activity of P450 enzymes, although various enzyme isoforms are differently susceptible to organic solvents. For example, DMSO inhibited CYP3A4-mediated reactions even at low concentrations (0.1-0.2%), whereas methanol did not alter reactions present below 1% . CYP2C8/2C9-mediated conversions, however, were equally affected by DMSO and methanol (Chauret et al., 1998; Busby et al., 1999). It has been recommended to keep the amount of organic solvents to a minimum, and to use methanol or acetonitrile when possible. During screening activities in drug discovery, however, DMSO continues to be frequently used due to solubility considerations and other practicalities.

AZD3839 is an inhibitor of the β-secretase enzyme and is being developed for the treatment of Alzheimer's disease. It has recently entered phase 1 clinical studies. We discovered a discrepancy in the CYP3A4 TDI results for AZD3839, between the discovery screening method - no TDI and a later regulatory method - TDI found. In the present work we elucidate

the reason for the discrepancy in the TDI results using HLM and cryopreserved Hhep. In addition, the biotransformation of AZD3839 has been thoroughly investigated in the different test systems and under different experimental conditions, in order to understand the metabolic pathway and find the reactive metabolite(s) responsible for the observed TDI.

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Chemicals and Reagents. AZD3839 ((*S*)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1*H*-isoindol-3-amine hemifumarate) and the metabolites M1 ((*S*)-5-(3-(3-amino-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1*H*-isoindol-1-yl)phenyl)pyrimidine 1-oxide) and M2 ((*S*)-4-(3-amino-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1*H*-isoindol-1-yl)-2-(difluoromethyl)pyridine 1-oxide) were obtained from the Medicinal Chemistry department at CNSP iMed Science, AstraZeneca R&D, Södertälje, Sweden. ^{14}C-labelled AZD3839 ([U-*phenyl*-^{14}C]-(*S*)-1-(2-(difluoromethyl)pyridin-4-yl)-4-fluoro-1-(3-(pyrimidin-5-yl)phenyl)-1*H*-isoindol-3-amine hemifumarate) (Fig. 1) was obtained from the Isotope Chemistry department at Global DMPK Screening and Profiling, AstraZeneca R&D, Södertälje, Sweden. Midazolam, 1'OH-midazolam, testosterone, 6β-OH-testosterone, 6β-OH-testosterone-D7, mifepristone, verapamil, troleandomycin, NADPH, reduced glutathione, potassium ferricyanide and methoxylamine were all purchased from Sigma-Aldrich (St. Louis, MO, USA). 1'OH-midazolam and testosterone were also purchased from BD Biosciences (Bedford, MA, US) and Steraloids (Newport, RI, US), respectively. $^{13}C_3$-1'OH-midazolam was purchased from Toronto Research Chemicals Inc. (ON, Canada).

Test systems. Pooled human liver microsomes were from an AstraZeneca in house pool (7 donors, mixed gender), from Invitrogen (Life Technologies, Austin, TX) pooled from 87 livers (mixed gender) or purchased from BD Gentest (Woburn, MA, USA) pooled from 33 and 150 donors (mixed gender). Human hepatocytes were purchased from CellzDirect (one male and two female donors) and KaLy-Cell (two male and 2 female donors). Recombinant expressed human enzymes were obtained from BD Gentest (Woburn, MA, USA).

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Determination of enzyme kinetics in HLM. Various concentrations of [^{14}C]AZD3839 between 1 and 100 μM were incubated in triplicate in the presence of 0.25 μg microsomal proteins in 50 mM KPO4 buffer, pH 7.4, at 37°C (final incubation volume 100 μL). The incubations were initiated by the addition of NADPH at a final concentration of 1 mM and terminated after 10 min by addition of 100 μL of acetonitrile. The incubation times were chosen in the linear range in relation to amount of proteins and incubation time (data not shown). The control samples were incubated without NADPH. Precipitated proteins were removed by centrifugation at 3000 rpm for 15 min, at 4°C and acetonitrile was removed under a flow of nitrogen for 5 min, at 37°C. The supernatants were transferred to vials and analyzed using a LC-MS/MS/RAM system.

Reversible CYP3A4 inhibition by AZD3839 and N-oxide metabolites in HLM. The ability of AZD3839 to inhibit the activity of CYP3A4 was assessed using two typical CYP3A4 substrates, MDZ (2 μM) and TSN (50 μM) which were co-incubated with AZD3839 (0.1, 0.3, 1, 3, 10, 30 and 100 μM) in triplicate and human liver microsomes at 0.01 mg/mL (MDZ) or 0.05 mg/mL (TSN) in the presence of 1 mM NADPH, 0.1 M phosphate buffer (pH 7.4), and 5 mM $MgCl_2$ at 37°C. The organic solvent was below 1%. The reactions were terminated by the addition of one volume of ethyl acetate after 3 min (MDZ) or 7 min (TSN), vortexed and centrifuged at 3000 rpm for five min. The supernatants were transferred to new plates, evaporated to dryness under a stream of nitrogen and reconstituted in 75 μL of mobile phase before analysis using LC-MS/MS according to the analytical parameters described in Table 1. The inhibitory potency towards CYP3A4 was also investigated by incubating the AZD3839 N-oxide metabolites M1 and M2, in a competitive manner, at various concentrations; 0.5, 1, 3, 12.5, 30, 50 and 100 μM (0.5% methanol) at 37°C with midazolam (5 μM) or testosterone (50 μM), pooled HLM (0.05 mg/mL (MDZ) or

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

0.1 mg/mL (TSN)) and NADPH (1 mM) for 5 min (MDZ) or 10 min (TSN). The reactions were terminated by the addition of one equal volume of ice-cold acetonitrile containing internal standard ($^{13}C_3$-1'OH-midazolam or 6β-OH-testosterone-D7). After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS (Waters Corp., Milford, MA, USA) according to the analytical parameters described in Table 1.

TDI in HLM

K_I and k_{inact} determination. To determine K_I and k_{inact} for the inactivation of CYP3A4, various concentrations of AZD3839 at 0.5, 0.7, 1, 2.5, 5, 7.5, 12.5, 25, 30, 45, 50, 65, 75, 100 µM (0.5% methanol) or the positive control, verapamil (0.3, 1, 3, 10, 30, 65, 100 µM) were pre-incubated with pooled HLM (0.5 mg/mL) for 10 minutes (at 37°C). The reactions were initiated by NADPH (1 mM) to give a final volume of 100 µL. At the end of each pre-incubation period: 3, 5, 10, 20 and 30 min, an aliquot was transferred to pre-warmed 50 mM KPO_4 containing MDZ (25 µM, corresponding to ca 5 times K_m) and NADPH (1 mM). A pre-incubation for 30 min without NADPH (denoted as 0 min) was also performed. The diluted samples were incubated for further 5 min before the reactions were terminated by the addition of an equal volume of ice-cold acetonitrile containing the internal standard, $^{13}C_3$-1'OH-midazolam. After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS (Waters Corp., Milford, MA, USA) according to the analytical parameters described in Table 1.

Effect of organic solvents on TDI in HLM. To evaluate how the different solvents used for dissolving AZD3839 (DMSO, methanol or water), affected the inactivation of CYP3A4 in TDI experiments, various concentrations of the compound at 0.5, 1, 3, 12.5, 30, 50 and 100 µM (final amount of solvent was 0% (water) 0.2% or 0.5%). were pre-incubated at 37°C for 10 min with pooled HLM at 0.5 mg/mL (MDZ) or 1 mg/mL (TSN) before initiation of the

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

reactions by addition of NADPH (1 mM) or 50 mM KPO_4. At the end of the 30 min pre-incubation period, fractions were diluted (10-fold) into incubation buffer (50 mM KPO_4) containing MDZ (25 µM) or TSN (300 µM, corresponding to 6 times K_m) and NADPH (1 mM). In addition to the 30 min pre-incubations, longer pre-incubations (60, 90 and 120 min) were performed for incubations containing 0.5% DMSO prior to the 10-fold dilution into the substrate incubations (300 µM TSN). The diluted samples were incubated for 5 min (MDZ) or 10 min (TSN) before the reactions were terminated by the addition of an equal volume of ice-cold acetonitrile containing internal standard ($^{13}C_3$-1'OH-midazolam or 6β-OH-testosterone-D7). After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS (Waters Corp., Milford, MA, USA) according to the analytical parameters described in Table 1.

Inhibition of CYP3A4 by AZD3839 metabolites. The metabolites M1 and M2 were also incubated in a time dependent manner, at the same concentrations as described above, with HLM at final concentrations of 1 mg/mL. After an initial pre-incubation for 10 min the reactions were initiated by the addition of NADPH (1 mM) to give a final volume of 100 µL. At the end of each pre-incubation period: 0, 30, 60 min an aliquot of 10 µL was transferred to pre-warmed 50 mM KPO_4, TSN (300 µM) and NADPH (1 mM) (total volume of 100 µL). The diluted samples were incubated for 10 min before the reactions were terminated by the addition of an equal volume of ice-cold acetonitrile containing the internal standards (6β-OH-testosterone-D7). After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS (Waters Corp., Milford, MA, USA) according to the analytical parameters described in Table 1. In addition to the analysis of the probe substrate metabolites the presence of metabolites of M1 and M2 was investigated where liquid chromatography mass spectrometry (LC/MS) was carried out on a LTQ Orbitrap hybrid FTMS (Thermo Scientific) interfaced to an Accela HPLC pump (Thermo Scientific) and a CTC PAL autosampler (CTC

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Analytics). Electrospray in positive ion detection mode was used for ionization. The ion transfer line temperature and spray voltage were set to 300 °C and 4.2 kV, respectively. The mass spectrometer was operated in full scan mode (m/z 150 – 1000) in combination with data dependent product ion scanning of the most intense full scan ions. Normalized collision energy was set to 35. The mass spectrometric resolution was set to 30000 in fullscan mode and 15000 in data dependent product ion scan mode. Chromatography was performed on a YMC Basic column (150 x 4.6 mm, 3 µm particle size). The flow rate was 1 mL/min with a 1:20 split to the ion source and flow scintillation monitor (625 TR, Perkin Elmer Life and Analytical Sciences). The mobile phase consisted of aqueous acetonitrile containing 0.1% formic acid and was programmed for a linear increase from 5% to 27% acetonitrile during a 50 min period, the acetonitrile was thereafter increased to 100% during the next 5 min period and was kept there for another 3 minutes before returning to the initial condition. Scintillation cocktail (Ultima Flo-M, Perkin Elmer Life and Analytical Sciences) was pumped at a flow rate of 4 mL/min to the flow scintillation analyzer which was equipped with a 0.5 mL flow cell.

TDI in Hhep. Pooled Hhep with three (CellzDirect) or four (KaLy-Cell) donors, at the cell density of 1 x 10^6 cells per mL, were used to study the potential for AZD3839 to inhibit CYP3A4 activity. All suspensions were incubated in a humidified atmosphere of CO_2/air (5%/95%) at 37°C. Pre-incubations were started with the addition of AZD3839 at various concentrations (2, 20 and 50 µM for CellzDirect hepatocytes or 0.5, 1, 3, 12.5, 30, 50 and 100 µM for KaLy-Cell hepatocytes) and solvents to pre-warmed hepatocytes. The final amount of solvent was 0.2% (DMSO or methanol) or no solvent present. As positive controls, mifepristone and troleandomycin were used. At the end of each pre-incubation period (0, 10,

30 and 60 min for CellzDirect hepatocytes or 0, 15, 30, 60 and 120 min for KaLy-Cell hepatocytes) a fraction was diluted 5 times into the substrate incubation containing MDZ at 30 µM. The diluted samples were further incubated for 15 min before the reactions were stopped by the addition of one equal volume of ice-cold acetonitrile containing internal standard. After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS.

Mechanism of TDI. To investigate if AZD3839 binds irreversible or quasi-irreversible to HLM proteins, AZD3839 at 50 µM (0.5% DMSO or 0.5% methanol) was incubated in duplicate with pooled HLM (1 mg/mL) with and without NADPH (5 mM) for 30 min prior to a 2-fold dilution to a second incubation (10 min) containing buffer (100 mM KPO_4) or $Fe(CN)_6$. The enzyme activity was measured after another 2-fold dilution to a third incubation (15 min), containing NADPH (5 mM) and midazolam (10 µM). The metabolite 1'OH-midazolam was quantified by LC-MS/MS, running in the positive electrospray ionization and MRM modes. Mifepristone and troleandomycin were used as control compounds, for detection of irreversible (He et al., 1999) and quasi-irreversible inhibition (Lim et al., 2005), respectively.

Metabolic profiling in HLM and Hhep. [^{14}C]AZD3839 dissolved in either DMSO, methanol or water, at the final incubation concentrations of 5, 10 and 50 µM were incubated at 37 °C for 0, 10, 30 and 60 min with HLM (0.5 mg/mL) and NADPH (1 mM) or Hhep (cell density of ca 2 million cells/mL). All suspensions were incubated in a humidified atmosphere of CO_2/air (5%/95%) at 37°C. The final amount of solvent was 0.5% (DMSO or methanol) or no solvent present. The reactions were terminated by the addition of one equal volume of ice-

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

cold acetonitrile and centrifuged at 3800*g* for 10 minutes. The supernatants were transferred to new glass tubes of which 25 µL was transferred to scintillation vials for measurement of total radioactivity. The rest was stored at –70^{o}C until further used. Prior to analysis, the acetonitrile was evaporated from the supernatants from which 25 µL was transferred to scintillation vials for measurement of total radioactivity. The remaining supernatants were transferred to vials for LC/MS and flow scintillation analysis. For the analysis 50 µL was injected.

Influence of trapping agents on the biotransformation and TDI of AZD3839. In order to find the reactive metabolite(s) responsible for the CYP3A4 TDI, [^{14}C]AZD3839 at 50 µM (0.5% DMSO, 0.5% methanol or water) was incubated with pooled HLM (1 mg/mL), NADPH (1 mM) and reduced glutathione (GSH), potassium ferricyanide ($Fe(CN)_6$) or methoxylamine at 5 and 30 mM. The reactions were terminated at 0 min and 30 min by the addition of an equal volume of ice-cold acetonitrile. After centrifugation at 1600 *g* for 10 min the acetonitrile was evaporated and the remaining was analyzed by LC/MS and flow scintillation as described above. The AZD3839 N-oxide metabolites, M1 and M2 at 30 µM (0.5% methanol), were also incubated with pooled HLM (1 mg/mL), NADPH (1 mM) and 30 mM GSH for 0 and 30 min prior to termination by the addition of an equal volume of ice-cold acetonitrile. Sample work-up and analysis were performed as described above. The effect of reduced glutathione on the CYP3A4 TDI effect was also investigated by pre-incubating AZD3839 at 0.5-100 µM (0.2% DMSO or 0.2% methanol) with pooled HLM (1 mg/mL), with and without NADPH (1 mM) and GSH (5 and 30 mM) for 30 min (after an initial pre-incubation period of 10 min prior to addition of NADPH). After the pre-incubation step, fractions were diluted (10-fold) into incubation buffer (50 mM KPO_4) containing

testosterone (300 µM) and NADPH (1 mM). The diluted samples were incubated for 10 min before the reactions were terminated by the addition of an equal volume of ice-cold acetonitrile containing the internal standard (6β-OH-testosterone-D7). After centrifugation at 1600 *g* for 10 min the supernatants were analyzed by UPLC-MS/MS (Waters Corp., Milford, MA, USA) according to the analytical parameters described in Table 1.

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Data processing. *IC_{50} determination.* Percentage of remaining activity was determined as compared to the activity of vehicle controls using equation 1, where V(sample) is the reaction velocity of the sample and Mean V(control) is the mean reaction velocity of the vehicle control.

$$\% \text{ Remaining Activity} = \frac{V_{(sample)}}{\text{Mean } V_{(control)}} \text{ x } 100 \quad (1)$$

K_I and k_{inact}. The apparent enzyme inactivation rate constant (k_{obs}) for each inhibitor concentration was obtained by plotting the logartithm of percent activity remaining versus pre-incubation time for each inhibitor concentration (I). The maximum inactivation rate constant (k_{inact}) and the inactivation constant that produces half the maximum enzyme inactivation rate (K_I) were determined by non-linear regression by using the following equation (eq 2). Data fitting was performed by using the software SigmaPlot for Windows (SigmaPlot 2001 version 7.00, SPSS ASC GmbH, Erkrath, Germany).

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I \times [I]} \quad (2)$$

$Fe(CN)_6$ displacement. The calculations were performed using equation 3. At least 30% inhibition in buffer is required for the compound to be classified as a TDI compound. If % inhibition (buffer) - % inhibition ($Fe(CN)_6$) is greater than 20%, the compound is classified as a quasi-irreversbile inhibitor.

$$\% \text{ inhibition} = 1 - \frac{\% \text{ control activity (NADPH)}}{\% \text{ control activity (no NADPH)}} \times 100 \quad (3)$$

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Half life. The half life ($t_{1/2}$) of AZD3839 was calculated in incubations with HLM where different solvents were present by using equation 4, where k is the slope of the natural logarithm of percent remaining versus incubation time.

$$t_{1/2} = \frac{LN2}{k} \quad (4)$$

RESULTS

Determination of enzyme kinetics in HLM. AZD3839 was metabolized under initial rate conditions in HLM to two metabolites, N-oxidation either in the pyrimidine ring (M1) or in the pyridine ring (M2) (Fig. 1). The $K_{m,app}$ and V_{max} values for the two N-oxides in HLM were obtained using the Michaelis-Menten plot: $K_{m,app}$ and V_{max} were 2.0 μM and 217 pmol/min/mg (CL_{int} = 108 μL/min/mg) for the formation of the metabolite M1, and 4.9 μM and 195 pmol/min/mg (CL_{int} = 40 μL/min/mg), for the formation of the metabolite M2, respectively. Incubations with different recombinant expressed human enzymes indicated that CYP3A4 was the major enzyme responsible for metabolism of the compound. The contribution of CYP3A4 was estimated to be approximately 90 % after compensation for abundance of different CYPs in human liver. The formation of M1 and M2 from 5 μM of AZD3839 in HLM was nearly completely blocked by 1 μM of the specific CYP3A4 inhibitor ketoconazole. Wang et al. 2005 showed that the metabolite retorsin N-oxide could be reduced back to its parent compound in HLM. The N-oxide metabolites, M1 and M2, however, were not converted back to AZD3839 in HLM under the experimental conditions used (data not shown).

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Reversible CYP3A4 inhibition by AZD3839 and N-oxide metabolites in HLM. AZD3839 was characterized as a competitive CYP3A4 inhibitor in HLM using both MDZ and TSN as probe substrates, with the IC_{50} values of 11 µM and 2.8 µM, respectively. Competitive inhibition of CYP3A4 in HLM was also observed by M1 and M2. The individual IC_{50} values are presented in Table 2.

TDI of CYP3A4 by AZD3839 and N-oxide metabolites in HLM. AZD3839 is a potent time dependent inhibitor of CYP3A4 *in vitro*, with K_I and k_{inact} values determined to be 0.58 ± 0.14 µM and 0.025 ± 0.003 min^{-1} (*N*=3) using MDZ as a probe substrate (Fig. 2A). The inhibition parameters for the positive control verapamil (Fig. 2B); K_I = 2.23 ± 0.08 µM and k_{inact} = 0.035 ± 0.001 min^{-1}, were of similar magnitude as reported elsewhere (Wang et al., 2004; Chen et al., 2011).

The influence of organic solvents on TDI of CYP3A4 was examined by comparing IC_{50} curves of AZD3839 at 0 min and 30 min pre-incubation times with DMSO and methanol at 0.2% and 0.5% or only water present (TSN as probe substrate). When AZD3839 was dissolved in 0.2% or 0.5% methanol or in water a >5-fold shift in the IC_{50} curves was detected between 0 min and 30 min pre-incubation times, which is indicative for TDI (Fig. 3A-B). No shift occurred when 0.2% or 0.5% DMSO was present in a similar experimental setup (Fig. 3C). However, when pre-incubation times were prolonged to 60, 90 and 120 min time dependent shifts in the IC_{50} curves were observed in the presence of 0.5% DMSO (Fig. 3D). Similar results were found when using MDZ as CYP3A4 probe substrate (data not shown).

To further elucidate which P450 reaction was responsible for the TDI of CYP3A4, incubations were prepared with M1 and M2 using methanol. Shifts in the IC_{50} curves for both

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

M1 and M2, at the pre-incubation times 0, 30 and 60 min were observed. In Fig. 4 the curves are presented with TSN as CYP3A4 probe substrate. The same pattern was observed with MDZ (data not shown).

TDI in Hhep. In Hhep from CellzDirect a concentration dependent inhibition was observed of the CYP3A4 activity, but no TDI. The results were similar independently of solvent used (Fig 5). However, in the Hhep from KaLy-Cell TDI was detected, with K_I and k_{inact} determined to be 2.81µM and 0.01 min^{-1}, respectively (Fig. 6).

Mechanism of TDI. When 50 µM of AZD3839 was dissolved in 0.5% DMSO only 25% inhibition of CYP3A4 was observed in buffer in the $Fe(CN)_6$ displacement experiment (Fig. 7). This was consistent with the previous HLM experiments where AZD3839 was not identified as a time dependent inhibitor of CYP3A4 in the presence of DMSO. However, when AZD3839 was dissolved in 0.5% methanol, 50% reduction of the CYP3A4 activity was observed, which classified the compound as a time dependent inhibitor. Addition of $Fe(CN)_6$ only reversed 12% of the lost CYP activity, showing that the nature of binding was irreversible. Troleandomycin and mifepristone are characterized as a quasi-irreversible inhibitor and mechanism based inhibitor, respectively (He et al., 1999; Lim et al., 2005;Fontana et al., 2005), and were used as control compounds. The CYP3A4 inhibiting effect of troleandomycin was reversed by the addition of $Fe(CN)_6$ but not for mifepristone, which is in line with the expectations.

Metabolic profiling in HLM and Hhep. The N-oxide metabolites, M1 and M2, were the major metabolites from AZD3839 in HLM. These metabolites were generated independently of solvent used (DMSO, methanol, water). However, a quantitative difference was present:

less M1 and M2 was formed in the presence of DMSO (Fig. 8A) compared to methanol (Fig. 8B) or water (Fig. 8C). The half life for AZD3839 (10 μM) in the incubations containing DMSO was ca 6.5 hours, while in the incubations containing methanol or water the half life was circa 2-2.5 hours.

The metabolic profile of AZD3839 in Hhep was different from HLM. The major metabolite in the Hhep from CellzDirect was M23 (Fig. 1, Fig. 9), which was not present in HLM. There was no quantitative difference depending on solvent present (Fig. 9A-B). M1 and M2 were also present but to a much lesser extent compared to HLM. In Hhep from KaLy-Cell (Fig. 9C) there was very little turnover and M23 was present only as trace (<1%).

Influence of trapping agents on the biotransformation and TDI of AZD3839. The production of M1 and M2 from [^{14}C]AZD3839 in HLM in the presence of 5 mM of GSH, $Fe(CN)_6$ or methoxylamine was diminished. The metabolites totally disappeared in the presence of 30 mM of GSH, $Fe(CN)_6$ or methoxylamine. No adducts could be detected with any of the trapping agents in spite of the demonstrated TDI. The same pattern was observed when M1 and M2 were incubated with GSH (5 and 30 mM). Conversion of M1 to M23 and M2 to M9 decreased in the presence of 5 mM of GSH and disappeared at 30 mM of GSH. The CYP3A4 TDI effect (IC_{50} shift) by AZD3839 (0.2% methanol) in the presence of GSH disappeared (Fig. 10A). Analysis of the formed metabolites from those incubations revealed that M1, M2 and M9 were formed in the absence of GSH, and that M2 decreased and M9 disappeared in the presence of GSH. Consistent with other results, no TDI was observed when AZD3839 was dissolved in DMSO (0.2%), and the GSH did not affect this result (Fig. 10B). From the metabolic profile (MS-data) M1 and M2 were formed but no M9 in the incubations where DMSO was present.

DISCUSSION

The BACE1 inhibitor AZD3839 is a compound that is under development for the treatment of Alzheimers Disease. Because Alzheimer patients are meant to be treated chronically with AZD3839 and are using other co-medication, potential DDIs are important to consider. The assessment of the DDI potential of AZD3839 is rather complex. The high dependency on CYP3A4 leads to a high risk for DDIs with other CYP3A4 drugs. Moreover, AZD3839 and its metabolites M1 and M2 inhibited CYP3A4 in a reversible and an irreversible manner, which could not only affect the metabolism of other CYP3A4 substrates, but also its own metabolism.

Predictions based on the in vitro TDI data generated in HLM using methanol or water indicated that AZD3839 was expected to be a potent *in vivo* inhibitor of CYP3A4 and to have strong interactions with other CYP3A4 substrates such as MDZ or simvastatin (median 9 fold increase in AUC). No interaction with 200 mg of ketoconazole was predicted (median 1.2 fold increase in AUC), because CYP3A4 already was nearly maximally inhibited by AZD3839. Based on the CYP inhibitory data with DMSO, however, the risk profile for AZD3839 with respect to expected DDIs was reversed: no interaction potential with MDZ or simvastatin, but an interaction was predicted with coadministration of 200 mg of ketoconazole (median 2.5 fold increase in AUC).

From the AZD3839 metabolite identification experiments in HLM we observed that there was a quantitative difference in the metabolites formed depending on solvent; less M1 and M2 were formed in DMSO-containing incubations compared to methanol- or water-containing incubations under the same incubation time as a consequence of the reduced CYP3A4 activity. The same phenomenon was observed by Nishiya et al., 2010, who studied TDI of CYP3A4 by diazepam dissolved in methanol, acetonitrile or DMSO. They observed

that the formation of the metabolites of diazepam decreased when the substrate was dissolved in DMSO compared with methanol and acetonitrile and the TDI was attenuated. However, TDI of AZD3839 in 0.5% DMSO was observed when the pre-incubation times for AZD3839 were prolonged to 60, 90 and 120 min. This suggests that that there is a quantitative relationship between the metabolites formed in HLM and the TDI effect observed. DMSO reduced the formation of reactive species of AZD3839 that cause TDI of CYP3A4. This finding is further strengthened by the observation that also trapping agents attenuated the metabolic conversion of AZD3839 and abolished TDI. It is well known that DMSO inhibits CYP3A4 (Chauret et al., 1998; Busby et al., 1999). Although it should be recognized that DMSO is an oxidizable solvent and the amount of 0.2% corresponds to circa 30 mM, which is a high substrate concentration, we had not anticipated to observe this dramatic difference in the TDI results at these amounts that cause approximately 30% reduction in CYP3A4 activity (Chauret et al., 1998; Busby et al., 1999). These results further strengthen that the enzyme inhibiting effect of DMSO, even at low amounts, should be considered when performing metabolism studies, when DMSO-sensitive enzymes are involved. Apart from the discrepancies in TDI results we also discovered that the clearance and half life predictions of AZD3839 from HLM were solvent dependent, with about twice as long half life observed when DMSO was present in the incubations compared to methanol. This could be relevant when predicting human clearance based on *in vitro* test systems. To avoid e.g. false TDI negative results, it is recommended to avoid DMSO in the assessment of DDI compounds in early clinical development.

Lately, laboratories have developed P450 inhibition methods using Hhep as a complement to studies in HLM (Lu et al., 2008; Mao et al., 2011). We observed that the TDI results with AZD3839 in a typical TDI experiment was dependent on the supplier of the hepatocytes. No TDI could be detected from IC_{50} shift experiments performed in Hhep from CellzDirect. In

Hhep from KaLy-Cell TDI was detected although the Ki value was less potent compared to HLM. This attenuated TDI effect in Hhep compared to HLM has been observed elsewhere (Chen et al., 2011), and is probably due to lower intracellular concentration of the inhibitor caused by e.g. non-specific binding, metabolite consumption and/or active transport. Incubations of AZD3839 in HLM were also 2 fold more diluted during the TDI determination than in Hhep incubations. Theoretically, higher remaining concentrations of AZD3839 in Hhep incubations could retain some effects. However, AZD3839 showed no competitive inhibition in Hhep and the magnitude of TDI in Hheps was reduced rather than increased. Therefore, it is unlikely that lesser dilution has had a significant impact on the TDI determination of AZD3893 in Hhep. When the metabolic profiles of AZD3839 were investigated in Hhep it was observed that also the biotransformation of AZD3839 was different depending on supplier of Hhep, but also compared to HLM. In Hhep from CellzDirect the major metabolite was M23 but in Hhep from KaLy-Cell it was only present at trace amount. M23 was not present in HLM at all. M1 and M2 were generated in Hhep but to a lesser extent compared to HLM. Interestingly, there was no quantitative difference in metabolite formation depending on solvent present in the incubations with Hhep from CellzDirect. The different TDI results in the Hhep from the two suppliers may be explained by the different metabolic profiles of AZD3839 between the Hhep batches. We have not found any obvious differences in the CYP3A4 activity between the different Hhep batches.

It has been shown that *in vivo* DDI predictions, using data obtained with Hhep, to the clinical outcome give a better correlation compared to HLM that tend to overpredict (Xu et al., 2009; Chen et al., 2011; Kirby et al., 2011). The differences in the TDI results observed for AZD3839 between the two hepatocyte suppliers make it difficult to conclude which data to use for predictions of *in vivo* DDI. It reveals the uncertainty that is associated with these projections based on relatively simple test systems. If a compound shows different metabolic

pathways between HLM and Hhep it could be useful to use Hhep in parallel to HLM, but additional studies and insight are needed before using Hhep as the first choice of enzyme system for studying TDI, particularly if results are dependent on the supplier.

From a $Fe(CN)_6$ displacement study it was shown that AZD3839 was an irreversible inhibitor. We did not detect any adducts, but the trapping agents affected the biotransformation of AZD3839 in such way that the production of the major metabolites, M1 and M2, decreased in the presence of 5 mM of trapping agent and disappeared totally in the presence of 30 mM. This inhibiting effect of trapping agents was also shown by Zhang et al. (2009) who observed that GSH above 1 mM leveled off the amount of GSH adduct of the imine methine metabolite of 3-methylindole, which could indicate a potential CYP inhibition by GSH. In addition methoxylamine was also shown to be an inhibitor of CYP3A4 at the lower mM-concentrations. The inclusion of trapping agents to the HLM incubations with AZD3839 also affected the TDI results in such way that in the presence of GSH no TDI was observed. This protection against TDI together with the inhibiting effect of the generation of M1 and M2 by GSH strongly suggests that the reactive metabolic pathway causing the CYP3A4 TDI in HLM goes via these two metabolites. The fact that also M1 and M2 themselves are causing TDI may point to a subsequent metabolic conversion of these N-oxides that give rise to reactive species.

In summary, we have discovered that the presence of DMSO in incubations, even at the low amount of 0.2%, masked the TDI of CYP3A4 by AZD3839 in a standard 30 min pre-incubation method in HLM. When prolonging the pre-incubation times to or longer than 60 min, TDI was observed. The reason for lack of TDI at the 30 min pre-incubation is probably due to a CYP3A4-inhibiting effect of DMSO. The major metabolic pathways of AZD3839

via M1 and M2 in HLM, seemed to be responsible for the TDI observed as both metabolites inhibited CYP3A4 in a competitively and time dependently manner and reduction of formation of these metabolites by trapping agents prevented TDI. The sensitivity towards DMSO was not prominent in Hhep, where the amount of metabolites of AZD3839 was equal between incubations with water or DMSO present. However, different metabolic profiles and TDI results were observed from the different batches of Hhep, with no TDI detected in one batch and a less potent TDI, compared to HLM, in another. These findings show that the *in vitro* risk profile for DDI potential is very much dependent on the chosen test system and the experimental conditions. More investigations are required to evaluate the potential of hepatocytes as an *in vitro* system for DDI predictions.

ACKNOWLEDGEMENT

We want to thank Anna-Pia Palmgren who performed the $Fe(CN)_6$ displacement study, Antti Kautiainen who analyzed the metabolite identification experiments and Professor Neal Castagnoli, Jr. for constructive and fruitful discussions around the biotransformation of AZD3839.

Authorship Contributions

Participated in research design: Aasa, Hu, Baranczewski.

Conducted experiments: Aasa, Hu, Baranczewski.

Contributed new reagents or analytic tools: Eklund. Malmquist, Turek

Performed data analysis: Aasa, Hu, Baranczewski, Eklund, Lindgren

Wrote or contributed to the writing of the manuscript: Aasa, Hu, Eklund, Baranczewski, Bueters.

REFERENCES

Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach RS, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J and Wrighton SA (2003) The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *J Clin Pharmacol* **43**:443-469.

Busby WF, Ackerman JM and Crespi CL (1999) Effect of methanol, ethanol, dimethyl sulphoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochrome P-450 *Drug Metab Dispos* **27**:246-249.

Chauret N, Gauthier A and Nicoll-Griffith DA (1998) Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. *Drug Metab Dispos* **26**:1-4.

Chen Y, Liu L, Monshouwer M and Fretler AJ (2011) Determination of time-dependent inactivation of CYP3A4 in cryopreserved human hepatocyte and assessment of human drug-drug interactions. *Drug Metab Dispos* **39**:2085-2092.

Einolf HJ (2007) Comparison of different approaches to predict metabolic drug-drug interactions. *Xenobiotica* **37**:1257-1294.

EMA Draft DDI Guideline (2010) Guideline on the investigation of drug interactions (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf).

FDA Draft DDI Guidance (2012) Drug interaction studies – study design, data analysis, implications for dosing and labeling recommendations (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf).

Fontana E, Dansette PM and Poli SM (2005) Cytochrome P450 enzymes mechanism based inhibitors: common sub-structures and reactivity. *Curr Drug Metab* **6**:413-454.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol* **39**:1-17.

He K, Woolf TF and Hollenberg PF (1999) Mechanism-based inactivation of cytochrome P-450-3A4 by mifepristone (RU486). *J Pharmacol Exp Ther* **288**:791-797.

Hewitt NJ, Lechón MJG, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GMM and Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39**:159-234.

Kirby BJ, Collier AC, Kharasch ED, Whittington D, Thummel KE and Unadkat JD (2011) Complex drug interactions of HIV protease inhibitors 1: inactivation, induction and inhibition of cytochrome P450 3A by ritonavir and nelfinavir. *Drug Metab Dispos* **39**:1070-1078.

Lehmann JM, McKee, DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The Human Orphan Nuclear Receptor PXR Is Activated by Compounds That Regulate CYP3A4 Gene Expression and Cause Drug Interactions. *J. Clin Investig* **102**:1016–1023.

Lim HK, Duczac N, Brougham L, Elliot M, Patel K and Chan K (2005) Automated screening with confirmation of mechanism-based inactivation CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP1A2 in pooled human liver microsomes. *Drug Metab Dispos* **33**:1211-1219.

Lin JH and Lu AY (1998) Inhibition and induction of cytochrome P450 and the clinical implications .*Clin Pharmacokinet* **35**:361-390.

Lu C, Berg C, Prakash SR, Lee FW, and Balani SK (2008) Prediction of pharmacokinetic drug-drug interactions using human hepatocyte suspension in plasma and cytochrome P450 phenotypic data. III. in vitro-in vivo correlation with fluconazole. *Drug Metab Dispos* **36**:1261-1266.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA and Hall SD (2011) Prediction of CYP3A-mediated drug-drug interactions using human hepatocytes suspended in human plasma. *Drug Metab Dispos* **39**:591-602.

Murray M (1997) Drug-mediated inactivation of cytochrome P450. *Clin Exp Pharmacol Physiol* **24**:465-470.

Nishiya Y, Nakamura K, Okudaira N, Abe K, Kobayashi N and Okazaki O (2010) Effects of organic solvents on the time-dependent inhibition of CYP3A4 by diazepam. *Xenobiotica* **40**:1-8.

Shardlow CE, Generaux GT, MacLauchlin CC, Pons N, Skordos KW and Bloomer JC (2011) Utilizing drug-drug interaction prediction tools during drug development: enhanced decision making based on clinical risk. *Drug Metab Dispos* **39:**2076-2084.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270**:414-423.

Silverman RB (1995) Mechanism-based enzyme inactivators. *Methods Enzymol* **249**:240-283.

Wang JH, Jones DR and Hall SD (2004) Prediction of cytochrome P450 3A inhibition by verapamil enantiomers and their metabolites. *Drug Metab Dispos* **32**:259-266.

Wang YP, Yan J, Fu PP and Chou MW (2005) Human liver microsomal reduction of pyrrolizidine alkaloid *N*-oxides to form the corresponding carcinogenic parent alkaloid. *Toxicol Lett* **155**:411-420.

Watanabe A, Nakamura K, Okudaira N, Okazaki O, and Sudo K (2007) Risk assessment for drug-drug interaction caused by metabolism-based inhibition of CYP3A using automated in vitro assay systems and its application in the early drug discovery process. *Drug Metab Dispos* **35:**1232–1238.

Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4:**825–833.

Xu L, Chen Y, Pan Y, Skiles GL and Shou M (2009) Prediction of human drug-drug interactions from time-dependent inactivation of CYP3A4 in primary hepatocytes using a population-based simulator. *Drug Metab Dispos* **37**:2330-2339.

Zhang C, Wong S, Delarosa EM, Kenny JR, Halladay JS, Hop CE and Khojasteh-Bakht SC (2009) Inhibitory properties of trapping agents: glutathione, potassium cyanide, and methoxylamine, against major human cytochrome P450 isoforms. *Drug Metab Lett* **3**:125-129.

LEGENDS OF FIGURES

Figure 1. Biotransformation of AZD3839

Metabolism scheme of AZD3839 from HLM and Hhep incubations.

Figure 2. K_I and k_{inact} in HLM. A) Fitting curves of the k_{obs} *vs* AZD3839 concentration in HLM using MDZ as probe substrate. Circles are mean ± SD (*N=3*). B) Fitting curves of the k_{obs} *vs* verapamil concentration (positive control) in HLM using MDZ as probe substrate. Circles are mean ± SD (*N=3*). The solid lines represent the best fit to the data after non-linear regression.

Figure 3. Effect of solvents on TDI of CYP3A4 by AZD3839 in HLM using TSN as probe subtrate. A) An IC_{50} shift for AZD3839 between 0 min (dotted lines) and 30 min (solid lines) pre-incubation in the presence of 0.2% (triangles) and 0.5% (circles) methanol was found, which points to TDI. The solid lines represent the best fit to the data with non-linear regression. B). An IC_{50} shift was seen between 0 min and 30 min pre-incubation with no solvent present similar to methanol. C) An IC_{50} shift was absent after pre-incubation for 30 min with AZD3839 in the presence of 0.2% (triangles) or 0.5% (circles) DMSO. The solid lines represent the best fit to the data with non-linear regression. D) Prolongation of pre-incubation times with AZD3839 to 60, 90 and 120 min in the presence of 0.5% DMSO showed a time-dependent shift.

Figure 4. TDI of CYP3A4 by AZD3839 in HLM using TSN as probe subtrate. An IC_{50} shift was observed for M1 (A) and M2 (B) between 0 min (closed circles), 30 min (open circles) and 60 min (triangles) pre-incubation times in the presence of 0.5% methanol. The solid lines represent the best fit to the data with non-linear regression. Similar results have been found using MDZ as probe substrate.

Figure 5. TDI of CYP3A4 by AZD3839 in Hhep. No IC_{50}-shift was observed between 0 min and 10, 30 or 60 min pre-incubation times in Hhep from CellzDirect independently of no

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

solvent (A), methanol (B) or DMSO (C) present in the incubations. The solid lines represent the best fit to the data after non-linear regression to mean data of duplicate data points (A and C) or single data points (B).

Figure 6. K_I and k_{inact} in Hhep. Fitting curves of the k_{obs} *vs* AZD3839 concentration in HLM using MDZ as probe substrate. The solid lines represent the best fit to the data after non-linear regression.

Figure 7. Ferricyanide displacement of AZD3839 in HLM. 50 µM AZD3839 was incubated with HLM with or without $Fe(CN)_6$ in the presence of 0.5% methanol or DMSO. Mifepristone and troleandomycin were used as control compounds, for detection of irreversible or quasi-irreversible inhibition, respectively.

Figure 8. Metabolic profiles of AZD3839 in HLM. 50 µM AZD3839 was incubated with pooled HLM for 60 min with 0.5% DMSO (A), 0.5% methanol (B) or no solvent (C) present. M1 and M2 correspond to N-oxide metabolites, M9 corresponds to a double N-oxide, M13 and M24 correspond to hydroxylated metabolites.

Figure 9. Metabolic profile of AZD3839 in Hhep. 50 µM AZD3839 was incubated with Hhep from CellzDirect with 0.5% DMSO (A) or no solvent present (B) or with Hhep from KaLy-Cell (C). M1 and M2 correspond to N-oxide metabolites, M23 corresponds to a pyrimidine-2,4-dione metabolite and M24 and M29 correspond to hydroxylated metabolites and M44 is a metabolite with an unknown structure.

Figure 10. Influence of GSH on TDI of CYP3A4 by AZD3839 in HLM using TSN as probe subtrate. A) An IC_{50} shift was observed for AZD3839 between 0 min (open triangles circles) and 30 min (open circles) in the presence of 0.2% methanol. The IC_{50} shift was abolished when 30 mM GSH was included (closed triangles and circles). B) No TDI was observed in the presence of 0.2% DMSO independent of 30 mM GSH. The dotted and solid lines represent the best fit to the data with non-linear regression.

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

TABLES

Table 1. Instrument properties for the analysis of MDZ and TSN metabolites and internal standards in different assays

Assay	**AZD3839 reversible inhibition**		**AZD3839/M1/M2 TDI, M1/M2 reversible inhibition**	
Instrument	**Micromass LC-MS/MS**		**Micromass TQD UPLC-MS/MS**	
Substrate	**MDZ**	**TSN**	**MDZ**	**TSN**
Analyte	1'OH-MDZ	6β-OH-TSN	1'OH-MDZ	6β-OH-TSN
Internal standard (IS)	α-OH-triazolam	6β-OH-TSN-d_3	$^{13}C_3$-1'OH-MDZ	6β-OH-TSN-*d7*
Mobile phase	72% methanol, 28% water, 0.1% Formic Acid, 0.01% Morpholine	A: 1 mM ammonium acetate buffer, pH 2.5 B: Ethanol	A: 99.9% water, 0.1% glacial acetic acid B: 99.9% acetonitrile, 0.1% glacial acetic acid	A: 99.9% water, 0.1% glacial acetic acid B: 99.9% acetonitrile, 0.1% glacial acetic acid
Flow rate (mL/min)	0.800	0.400	0.500	0.500

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Column	Phenomenex Luna Phenyl-Hexyl, 3 µm, 4.5 x 50 mm	YMC-ODS-AQ, 3 µm, 4.0 x 50 mm		BEH C18, 1.7 µm		BEH C18, 1.7 µm	
Ion source/ mode	ESI+	ESI+		ESI+		ESI+	
Run time	3.5 min	8 min		3 min		5 min	
MRM (Analyte)	342 → 324	305 → 269		342 → 3168		305 → 269	
MRM (IS)	346 → 328	308 → 272		345→ 327		312 → 276	
Gradient	Isocratic	Time (min)	% B	Time (min)	% B	Time (min)	% B
		0.01	37	0	5	0	5
		3	37	0.1	5	0.1	5
		3.1	65	1.5	100	4	5
		5.1	65	2.3	100	4.5	100
		5.2	37	2.4	5	4.6	100
		8	37	3.0	5	5	5

DMD Fast Forward. Published on October 16, 2012 as DOI: 10.1124/dmd.112.047597
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. IC_{50} values for AZD3839, M1 and M2 using testosterone- and midazolam-hydroxylations as CYP3A4 probe reactions.

	IC50 (µM)	
	Testosterone	**Midazolam**
AZD3839	2.8	11
M1	6.1 ± 2.6	13.9 ± 4.8
M2	16.3 ± 4.2	22.2 ± 8.3

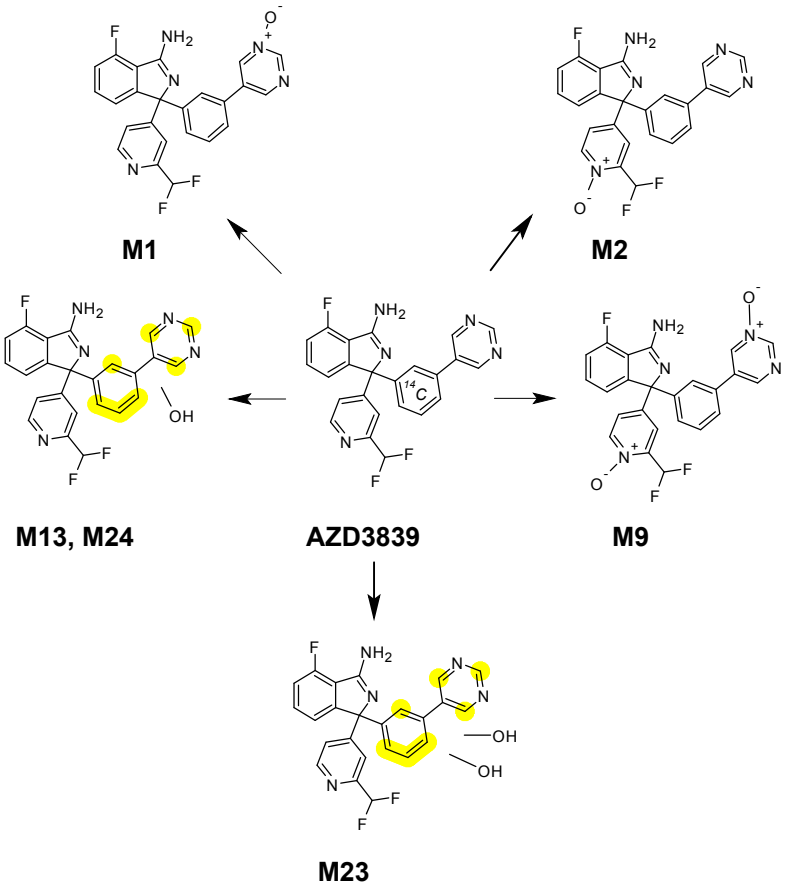


Figure 1

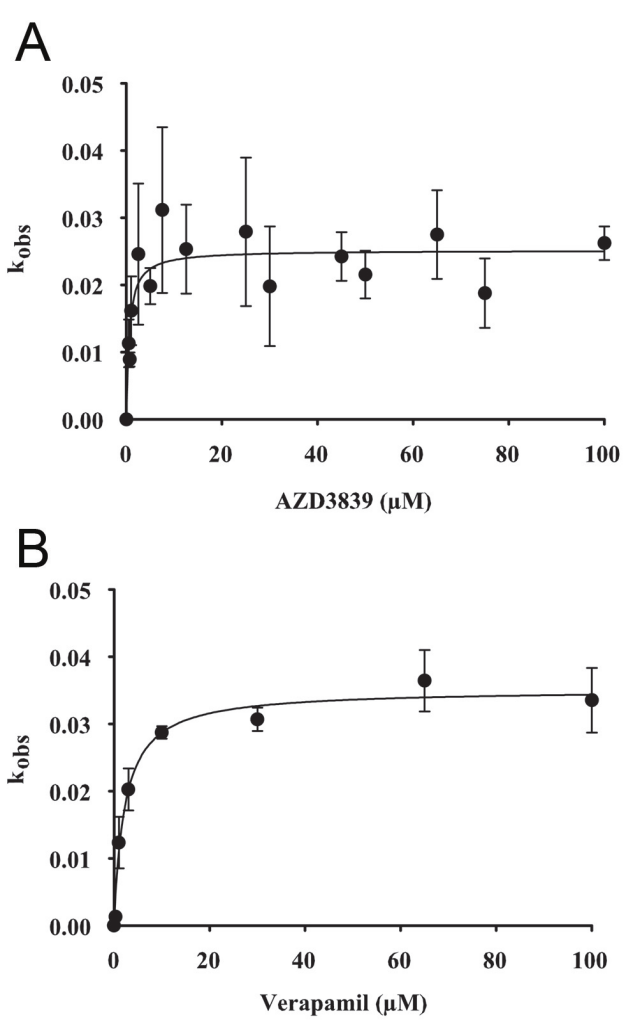

A
0.05
0.04
0.03
0.02
0.01
0.00
k_{obs}
0
20
40
60
80
100
AZD3839 (μM)
B
0.05
0.04
0.03
0.02
0.01
0.00
k_{obs}
0
20
40
60
80
100
Verapamil (μM)

Figure 2

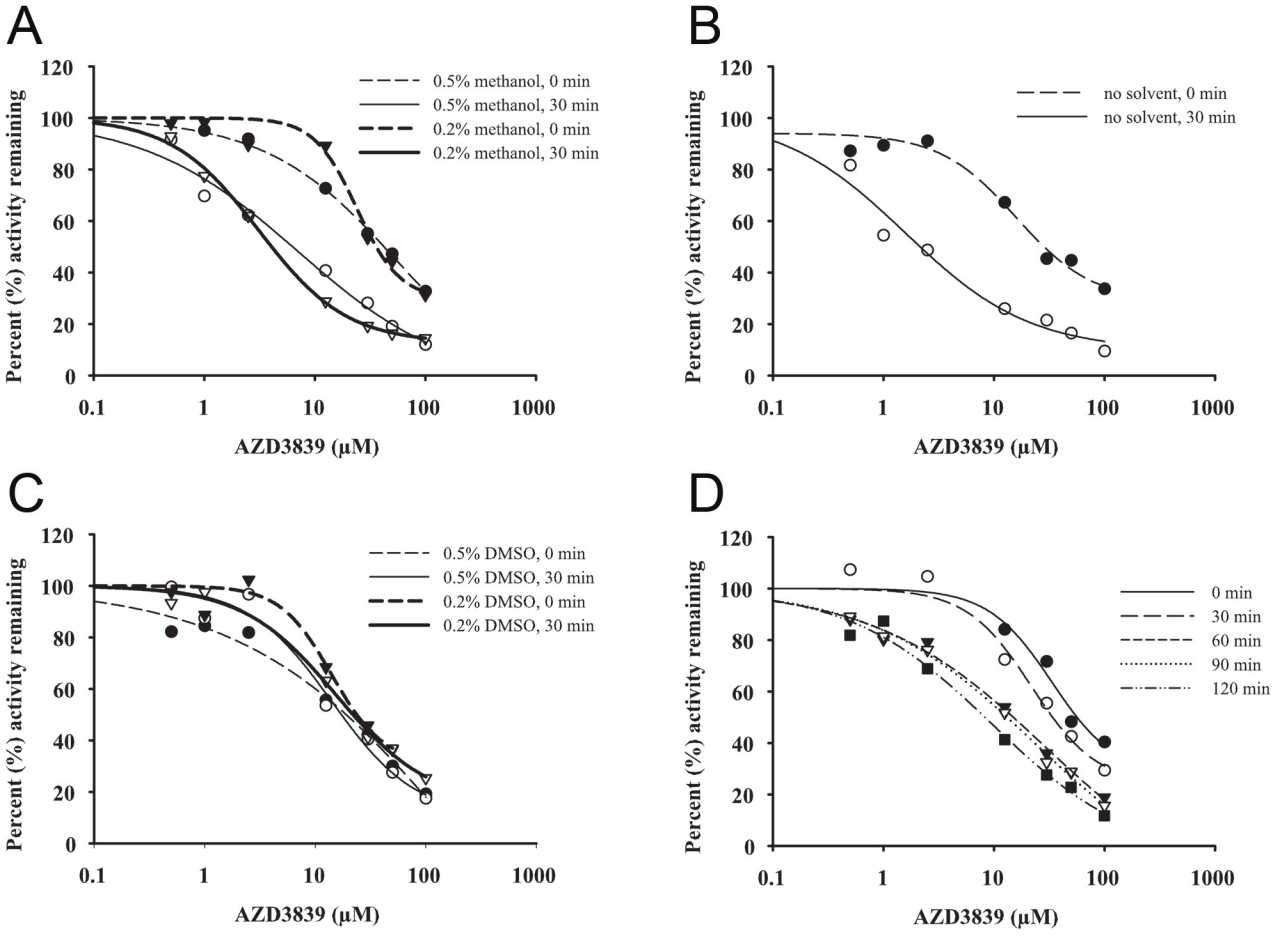
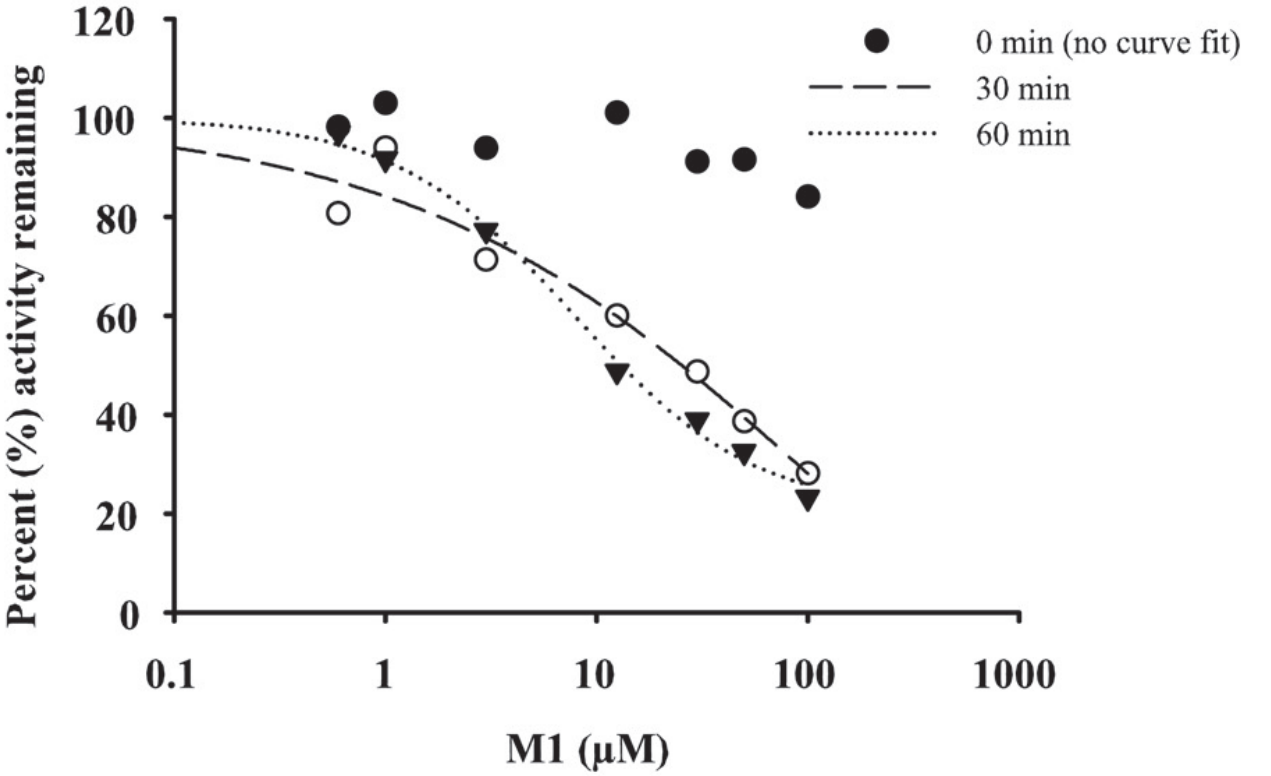

A
Percent (%) activity remaining
AZD3839 (µM)
0.5% methanol, 0 min
0.5% methanol, 30 min
0.2% methanol, 0 min
0.2% methanol, 30 min
B
Percent (%) activity remaining
AZD3839 (µM)
no solvent, 0 min
no solvent, 30 min
C
Percent (%) activity remaining
AZD3839 (µM)
0.5% DMSO, 0 min
0.5% DMSO, 30 min
0.2% DMSO, 0 min
0.2% DMSO, 30 min
D
Percent (%) activity remaining
AZD3839 (µM)
0 min
30 min
60 min
90 min
120 min

Figure 3

A


120
100
80
60
40
20
0
Percent (%) activity remaining
0.1
1
10
100
1000
M1 (μM)
0 min (no curve fit)
30 min
60 min

B

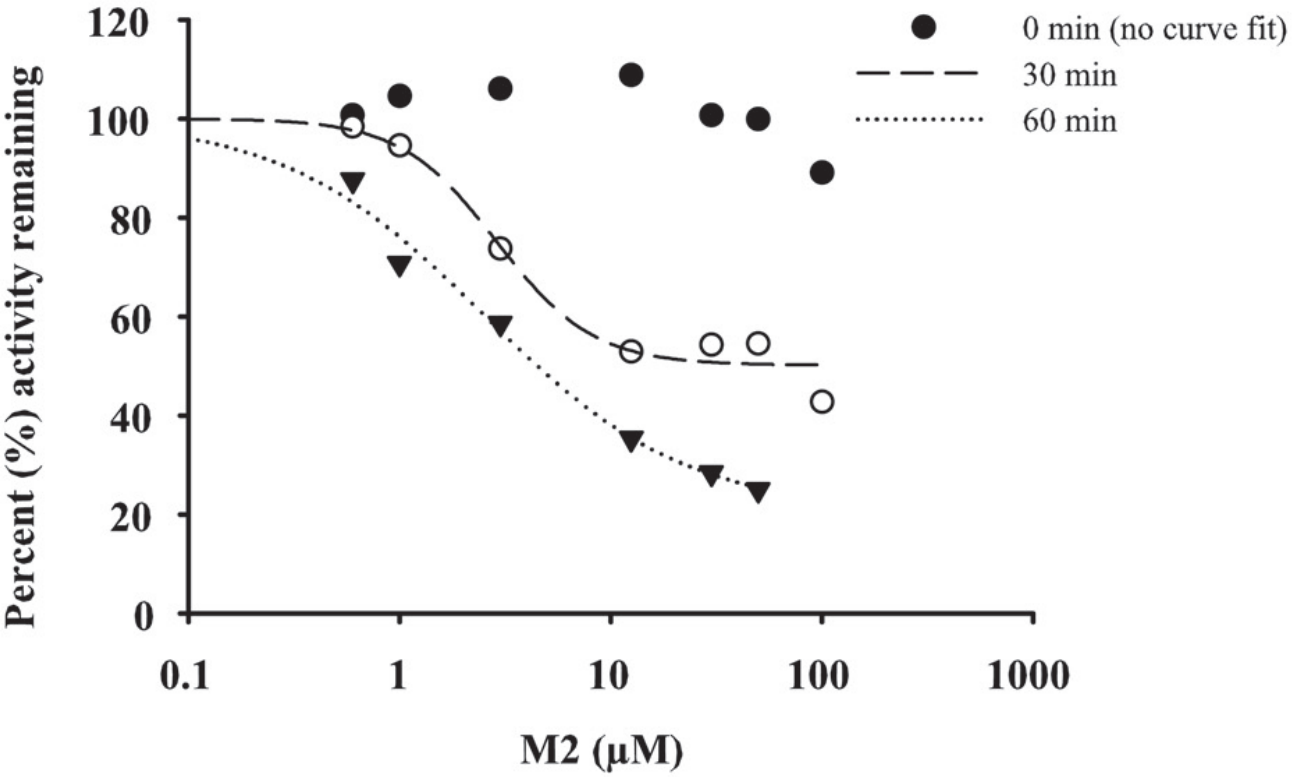

120
100
80
60
40
20
0
Percent (%) activity remaining
0.1
1
10
100
1000
M2 (μM)
0 min (no curve fit)
30 min
60 min

Figure 4

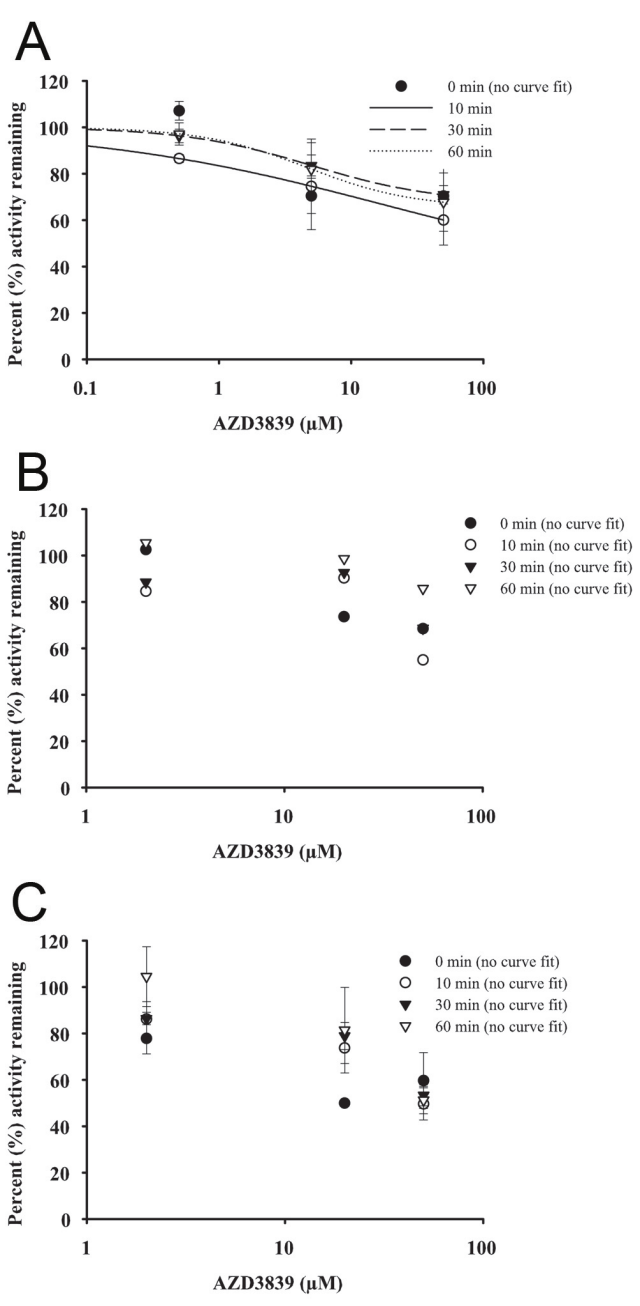

A
120
100
80
60
40
20
0
Percent (%) activity remaining
0.1
1
10
100
AZD3839 (µM)
0 min (no curve fit)
10 min
30 min
60 min
B
120
100
80
60
40
20
0
Percent (%) activity remaining
1
10
100
AZD3839 (µM)
0 min (no curve fit)
10 min (no curve fit)
30 min (no curve fit)
60 min (no curve fit)
C
120
100
80
60
40
20
0
Percent (%) activity remaining
1
10
100
AZD3839 (µM)
0 min (no curve fit)
10 min (no curve fit)
30 min (no curve fit)
60 min (no curve fit)

Figure 5

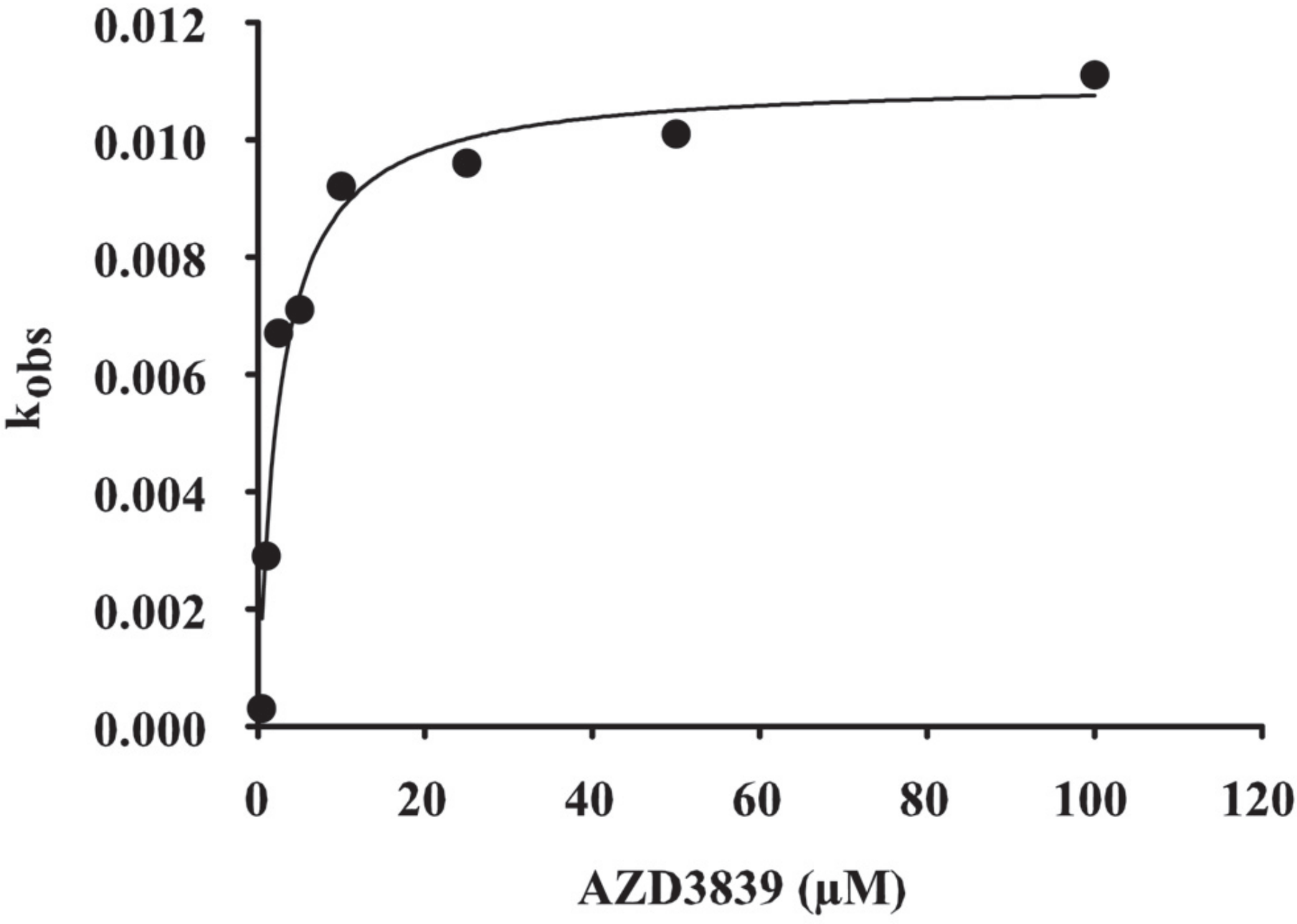

0.012
0.010
0.008
0.006
0.004
0.002
0.000
k_{obs}
0
20
40
60
80
100
120
AZD3839 (μM)

Figure 6

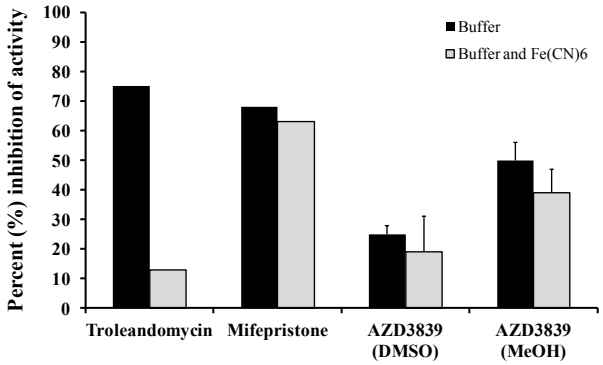


Figure 7

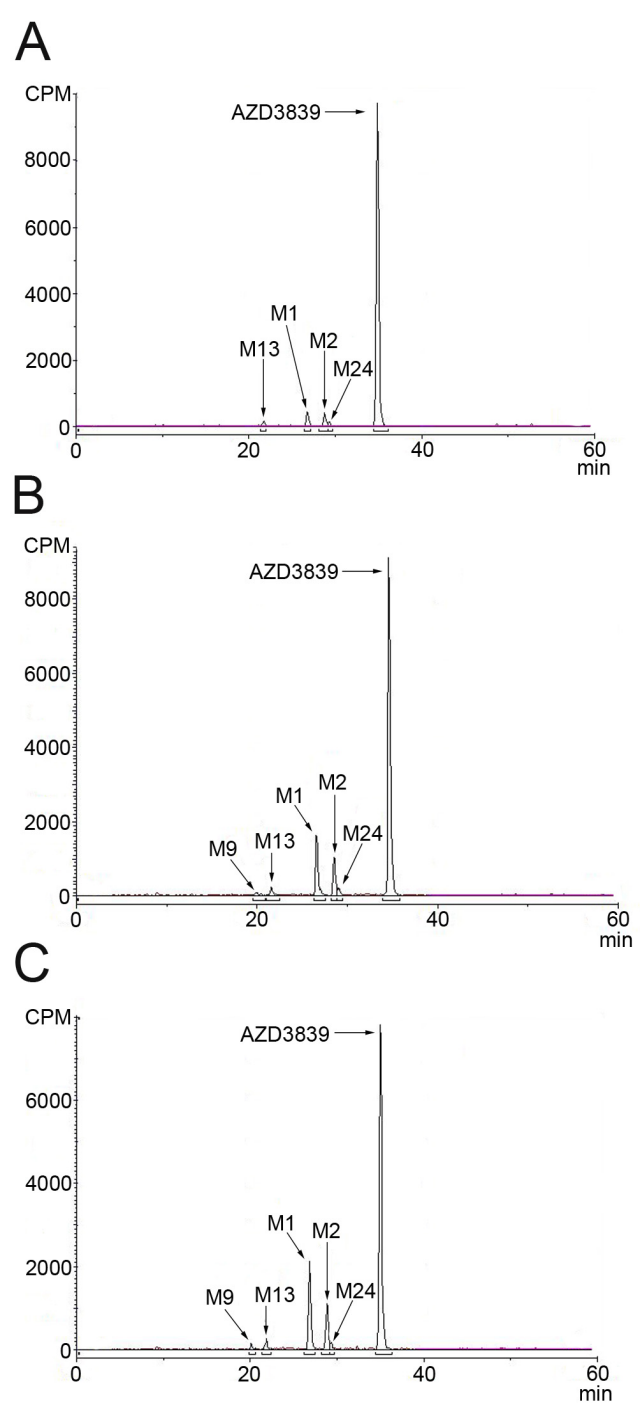

A
CPM
AZD3839
M1
M13
M2
M24
0
2000
4000
6000
8000
0
20
40
60
min
B
CPM
AZD3839
M1
M2
M9
M13
M24
0
2000
4000
6000
8000
0
20
40
60
min
C
CPM
AZD3839
M1
M2
M9
M13
M24
0
2000
4000
6000
0
20
40
60
min

Figure 8

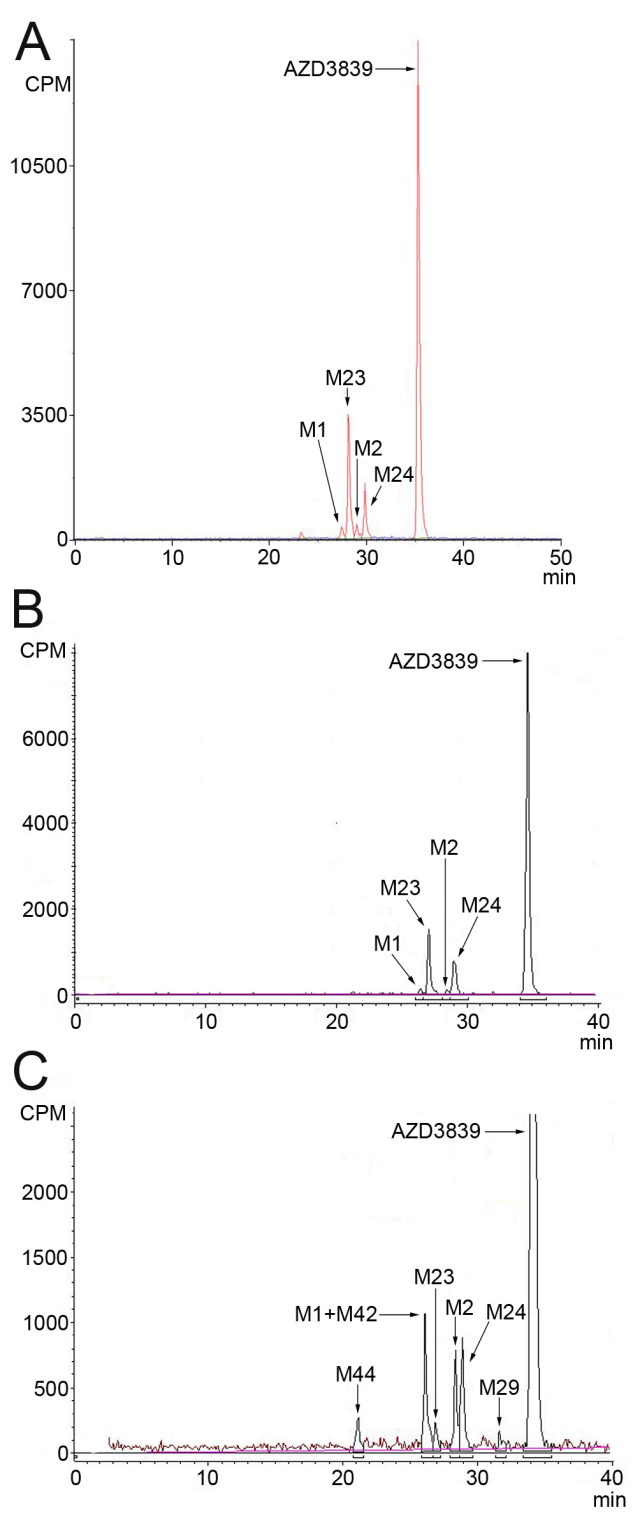
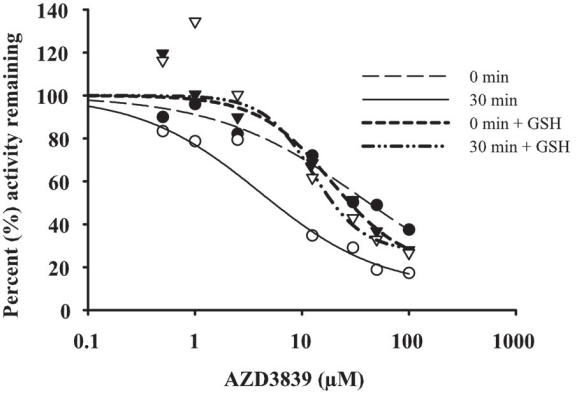

A
CPM
10500
7000
3500
0
AZD3839
M23
M1
M2
M24
0
10
20
30
40
50
min
B
CPM
6000
4000
2000
0
AZD3839
M2
M23
M24
M1
0
10
20
30
40
min
C
CPM
2000
1500
1000
500
0
AZD3839
M23
M1+M42
M2
M24
M44
M29
0
10
20
30
40
min

Figure 9

A


Percent (%) activity remaining
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
AZD3839 (μM)
0 min
30 min
0 min + GSH
30 min + GSH

B

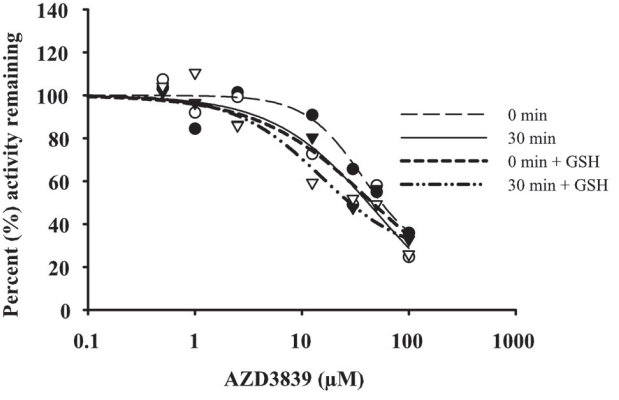

Percent (%) activity remaining
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
AZD3839 (μM)
0 min
30 min
0 min + GSH
30 min + GSH

Figure 10