

A clinical study to assess CYP1A2 and CYP3A4 induction by AZD7325, a selective GABA_A receptor modulator – an *in vitro* and *in vivo* comparison

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- AZD7325 is an orally administered, potent, selective gamma-amino-butyric acid (GABA_A) α _{2,3} receptor modulator intended for the treatment of anxiety.
- The induction effects of AZD7325 on CYP1A2 and CYP3A4 have not been systematically studied.

WHAT THIS STUDY ADDS

- The *in vitro* studies showed that AZD7325 was a moderate CYP1A2 inducer and potent CYP3A4 inducer.
- The follow-up clinical studies in healthy volunteers demonstrated that the expected efficacious daily dose of AZD7325 only weakly induced the pharmacokinetics of the CYP3A4 sensitive substrate, midazolam, and had no effect on the pharmacokinetics of the CYP1A2 substrate, caffeine. There was no apparent change in AZD7325 exposure following co-administration of midazolam or caffeine compared with AZD7325 alone.
- The study demonstrated that clinical exposure of the inducer plays a critical role in the determination of cytochrome P450 induction risk of a drug candidate.

AIM(S)

To investigate the potential of AZD7325 to induce CYP1A2 and CYP3A4 enzyme activities.

METHODS

Induction of CYP1A2 and CYP3A4 by AZD7325 was first evaluated using cultured human hepatocytes. The effect of multiple doses of 10 mg AZD7325 on the pharmacokinetics of midazolam and caffeine was then examined in healthy subjects.

RESULTS

The highest CYP1A2 and CYP3A4 induction responses were observed in human hepatocytes treated with 1 or 10 μ M of AZD7325, in the range of 17.9%–54.9% and 76.9%–85.7% of the positive control responses, respectively. The results triggered the further clinical evaluation of AZD7325 induction potential. AZD7325 reached a plasma C_{\max} of 0.2 μ M after 10 mg daily dosing to steady-state. AZD7325 decreased midazolam geometric mean AUC by 19% (0.81-fold, 90% CI 0.77, 0.87), but had no effect on midazolam C_{\max} (90% CI 0.82, 0.97). The mean CL/F of midazolam increased from 62 l h⁻¹ (midazolam alone) to 76 l h⁻¹ when co-administered with AZD7325. The AUC and C_{\max} of caffeine were not changed after co-administration of AZD7325, with geometric mean ratios (90% CI) of 1.17 (1.12, 1.23) and 0.99 (0.95, 1.03), respectively.

CONCLUSIONS

While AZD7325 appeared to be a potent CYP3A4 inducer and a moderate CYP1A2 inducer from *in vitro* studies, the expected efficacious dose of AZD7325 had no effect on CYP1A2 activity and only a weak inducing effect on CYP3A4 activity. This comparison of *in vitro* and *in vivo* results demonstrates the critical role that clinical exposure plays in evaluating the CYP induction risk of a drug candidate.

Introduction

AZD7325 (Figure 1), 4-amino-8-(2-fluoro-6-methoxyphenyl)-N-propyl-cinnoline-3-carboxamide, is a potent, selective gamma-amino-butyric acid (GABA_A)_{α2,3} receptor modulator intended for the treatment of anxiety [1]. GABA_A receptors are heteropentameric chloride channels, containing α-, β- and γ-subunits, with multiple subtypes [2, 3]. Benzodiazepines are widely used because of their immediate and robust anxiolytic onset, but are also limited because of their side effects. Benzodiazepines have near equipotent affinities at α1, α2, α3 and α5 containing GABA_A receptors and display uniform positive allosteric modulation activity at all these subtype receptors [1, 4]. The anxiolytic effects of benzodiazepines are attributed to GABA_{Aα2,3} receptor modulation, and the sedative effects to GABA_{Aα1,5} receptor modulation [5, 6]. AZD7325, as a selective GABA_{Aα2,3} receptor modulator, is expected to reduce the risk for the benzodiazepine like side effects, such as sedation and cognitive effects.

The cytochrome P450 (CYP) enzymes play an important role in the metabolism of a majority of marketed drugs. CYP3A4 is the predominant human hepatic and intestinal CYP isoform and contributes to the metabolism of approximately 50% of clinically used drugs [7]. CYP1A2 is also one of the major human hepatic CYPs and represents ~13% of total CYP enzymes in liver [8]. Induction of human CYP enzymes may lead to decreased systemic exposure of co-administered drugs and result in inadequate efficacy of the affected drugs [9]. Therefore, it is important to assess CYP induction potential during drug discovery and development to enable planning of appropriate clinical drug–drug interaction studies. CYP induction is generally regulated at the transcriptional level. The pregnane X receptor (PXR) is a transcription factor that is primarily responsible for CYP3A4 and CYP2C9 gene regulation [10, 11], while the aryl hydrocarbon receptor (AhR) is thought to regulate the expression of CYP1A enzymes [12]. CYP

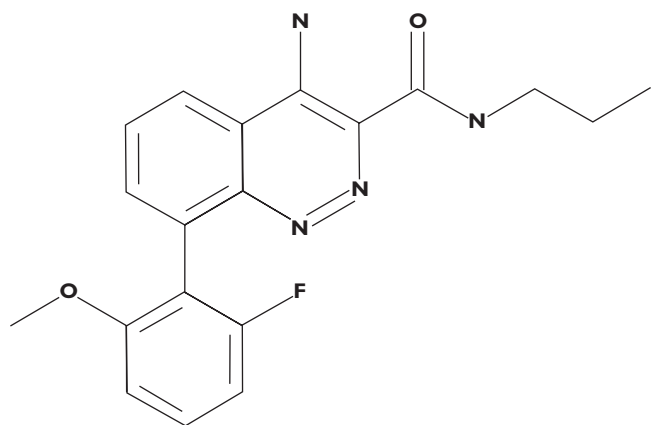


Figure 1

Chemical structure of AZD7325

induction potential can be readily evaluated using a number of *in vitro* test systems, such as nuclear receptor assays, and in cultured hepatocytes. However, CYP induction has demonstrated significant species differences and well controlled experiments in cultured primary human hepatocytes or cell lines that display human hepatocyte-like functions including drug-metabolizing enzymes and nuclear receptors are the preferred *in vitro* model for induction evaluations [13, 14]. In the present study, the induction potential of AZD7325 was first assessed *in vitro* with cultured human hepatocytes by measurement of mRNA concentrations, protein concentration and CYP functional enzyme activity. Based on our *in vitro* results, a clinical drug–drug interaction study was performed in healthy male subjects to investigate the effects of 11–12 days daily oral doses of 10 mg AZD7325, the expected efficacious dose, on the pharmacokinetics of a CYP3A4 substrate, midazolam, and a CYP1A2 substrate, caffeine.

Methods

In vitro CYP Induction by AZD7325

Materials AZD7325 was synthesized at AstraZeneca Pharmaceuticals LP. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO) or BD Biosciences (Woburn, MA). Freshly isolated primary human hepatocytes for induction studies were obtained by BD Gentest from an ethically approved source (Woburn, MA).

Induction evaluation in cultured primary human hepatocytes The potential of AZD7325 to induce the expression of CYP enzymes was investigated in primary cultures of freshly isolated human hepatocytes from one female (HH210) and two male (HH215, HH216) donors. Hepatocytes were treated once daily for 3 consecutive days with AZD7325 (0.01, 0.1, 1, and 10 μM) or positive control inducers, β-naphthoflavone (BNF, 20 μM) and rifampicin (RIF, 20 μM), as well as a solvent vehicle control (0.08% DMSO). After 72 h treatment, the hepatocytes were harvested and homogenized for microsomes preparation [13]. CYP enzyme induction was assessed by microsomal assay using probe substrates phenacetin (200 μM) and testosterone (200 μM) for CYP1A2 and CYP3A4 catalytic activities, respectively. The incubation mixture (0.1 ml) also included 0.2 mg ml⁻¹ microsomes, 0.1 M pH 7.4 potassium phosphate buffer and NADPH re-generating system containing 1.3 mM NADP, 3.3 mM of glucose-6-phosphate and 0.4 units ml⁻¹ of glucose-6-phosphate dehydrogenase. Metabolic reactions were stopped by addition of acetonitrile at 45 and 30 min for CYP1A2 and CYP3A4, respectively. Metabolite formation of the specific probe substrate for each enzyme was analyzed using HPLC with UV absorbance [15]. Fold of induction was calculated as the ratio of activity from AZD7325 or positive control to activity from vehicle control. The extent of enzyme activity induction,

expressed as a percentage of the positive control effect, was calculated as follows:

$$\frac{\text{AZD7325} - \text{vehicle control}}{\text{Positive control} - \text{vehicle control}} \times 100$$

Western blots of CYP1A2 and CYP3A4 CYP1A2 and CYP3A4 protein in microsomes treated with AZD7325 or positive control inducers was determined by electrophoresis under denaturing/reducing conditions and immunoblot analysis. Briefly, microsomal samples (30 µg) were loaded onto the pre-cast Novex mini-gels (Invitrogen, Carlsbad, CA), separated with sodium dodecyl sulphate (SDS) running buffer, and then electrotransferred to nitrocellulose blotting membranes. The relative immunoreactive protein content of CYP1A2 and CYP3A4 present in samples was determined with polyclonal antibodies specific to human CYP1A2 (Xenotech, Lenexa, KS) and CYP3A4 (BD Biosciences, Woburn, MA). The blots were visualized with an anti-rabbit IgG secondary antibody (BD Biosciences, Woburn, MA) biotinylated with streptavidin-horseradish peroxidase. CYP1A2 and CYP3A4 protein was then detected by a chemiluminescence imaging detection reagent (Thermo Scientific, Rockford, IL). The bands were analyzed by densitometry using a Kodak Digital Image Station (Model 440 CF) equipped with 1D Image Analysis Software. Pooled human liver microsomes were used as a reference marker.

TaqMan real-time, reverse transcription (RT)-PCR The mRNA expression for each CYP isoform was determined using TaqMan RT-PCR analysis [16]. Threshold cycle (C_T) values (the fractional cycle number at which the fluorescence passes the fixed threshold) were first determined. Then ΔC_T of each target gene sample was calculated by subtracting the C_T from the corresponding endogenous control, β -actin. The $\Delta\Delta C_T$ was then determined for the positive control inducer or AZD7325 by subtracting the ΔC_T from phosphate-buffered saline. Fold induction was determined by the calculation of $2^{-\Delta\Delta C_T}$. The extent of mRNA induction, expressed as a percentage of the positive control effect, was calculated as follows:

$$\frac{\text{Fold induction from AZD7325} - 1}{\text{Fold induction from positive control} - 1} \times 100$$

In vivo CYP induction by AZD7325

Subjects The drug–drug interaction study was performed in healthy male subjects, aged 18–45 years inclusive with a body mass index of 19–30 kg m⁻². Exclusion criteria included clinically relevant disease or abnormalities, use of any tobacco products within the past 6 months, history of alcohol or drugs of abuse, or use of any prescription medication within 14 days prior to the study. Subjects were not permitted to consume caffeine containing products for 48 h before and after administration of the test dose of

caffeine. Subjects were also asked to refrain from use of any over the counter medications, herbal medications (e.g. St Johns Wort) or foodstuffs (e.g. grapefruit juice) that possibly could interfere with the objectives of this study (e.g. inhibiting or inducing drug metabolizing enzymes), from 14 days prior to the study until the end of the study period. All volunteers provided written informed consent. The protocol was approved by an Independent Ethics Committee, and the study was conducted in accordance with guidelines established by the Declaration of Helsinki and was consistent with International Conference on Harmonization and Good Clinical Practice.

Study design The study was a single centre, open label, fixed sequence, non-randomized study in a single cohort of 24 healthy male subjects to investigate the effects of repeated doses of AZD7325 on the pharmacokinetics of a CYP3A4 substrate (midazolam) and a CYP1A2 substrate (caffeine). All subjects received a single oral dose of 5 mg midazolam (Ratiopharm GmbH, Blaubeuren, Germany) on day –2 and day 11 and a single oral dose of 200 mg caffeine (Pharmapac UK, Bidston, UK) on day –1 and day 12. All subjects received a single daily oral dose of 10 mg AZD7325 as a gelatin capsule (AstraZeneca, Södertälje, Sweden) in the morning on days 1–12. Midazolam and caffeine were administered 1 h after AZD7325 intake to reduce the risk of drug–drug interactions during the absorption phase.

Screening of the healthy volunteers was performed between 30 and 4 days prior to dosing and involved collection of a full medical history and a complete physical examination including electrocardiogram (ECG), vital signs and clinical laboratory tests. Subjects were confined to a clinical research unit from the evening before the study began (day –3) until day 14, 48 h after administration of caffeine on day 12. Subjects returned to the clinical unit for a follow-up visit 7–10 days after completing the study.

Pharmacokinetic sampling and analysis Blood samples for determination of midazolam and its metabolite 1'-hydroxymidazolam plasma concentrations were collected at pre dose, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12 and 24 h post dose after a single midazolam dose (day –2), and after co-administration with AZD7325 (day 11). Blood samples for determination of caffeine and its metabolite, paraxanthine, plasma concentrations were collected at pre dose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12, 18, 24, 36 and 48 h post dose after a single caffeine dose (day –1) and after co-administration with AZD7325 (day 12). Blood samples for determination of AZD7325 plasma concentrations were collected after a single AZD7325 dose on day 1, pre dose, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 h post dose. Trough samples were collected pre-dose on days 4, 6, 8, 9 and 10 and on days 11 and 12 at the same

sample times as when midazolam or caffeine were co-administered. Blood samples were collected and centrifuged at within 30 min of sampling. The plasma samples were stored at or below -70°C until analyzed by BASi (West Lafayette, IN).

Plasma concentrations of midazolam and 1'-hydroxymidazolam were determined using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method after liquid-liquid extraction. The range of quantification was $0.3-300\text{ ng ml}^{-1}$ for both midazolam and 1'-hydroxymidazolam. The accuracy, within-run and between-run precision were $-11.7\% \leq \% \text{bias} \leq 3.8\%$, $\leq 8.4\%$ and $\leq 7.5\%$ for midazolam and $-8.3\% \leq \% \text{bias} \leq 2.0\%$, $\leq 9.9\%$ and $\leq 7.4\%$ for 1'-hydroxymidazolam, respectively. Plasma concentrations of caffeine and paraxanthine were determined using a validated LC-MS/MS method after solid phase extraction. The range of quantification was $25-20\,000\text{ ng ml}^{-1}$ for both caffeine and paraxanthine. The accuracy, within-run and between-run precision were $-12.0\% \leq \% \text{bias} \leq 10.8\%$, $\leq 8.9\%$ and $\leq 12.0\%$ for caffeine and $-13.6\% \leq \% \text{bias} \leq 8.0\%$, $\leq 6.2\%$ and $\leq 11.2\%$ for paraxanthine, respectively. Plasma concentrations of AZD7325 were determined using a validated LC-MS/MS method after solid phase extraction. The range of quantification, accuracy, within-run and between-run precision for AZD7325 were $0.05-40\text{ ng ml}^{-1}$, $-2.7\% \leq \% \text{bias} \leq 14.0\%$, $\leq 11.0\%$ and $\leq 7.4\%$, respectively.

Pharmacokinetic and statistical analysis Plasma concentration-time results were analyzed by standard non-compartmental methods using WinNonlin (Pharsight Corporation, Mountain View, CA). When applicable, the following PK parameters were estimated for midazolam, 1'-hydroxymidazolam, caffeine, paraxanthine and AZD7325: area under the curve extrapolated to infinity (AUC), observed peak concentration (C_{max}), elimination half-life ($t_{1/2}$) and clearance (CL/F).

The natural log-transformed variables AUC and C_{max} were analyzed as dependent variables in a linear mixed effect model with treatment as a fixed effect and subject as a random effect. Estimates of the mean difference (with AZD7325 - without AZD7325) were calculated with 90% confidence intervals (CI). The results were transformed back to the original scale in order to give an estimate of the true ratios (with AZD7325 : without AZD7325) and 90% CIs for these ratios. No clinically relevant effect on the pharmacokinetics of midazolam or caffeine after co-administration of AZD7325 could be claimed if the 90% CIs of the ratio of the geometric means were completely contained in (0.8, 1.25).

Results

In vitro CYP Induction by AZD7325

The potential of AZD7325 to induce CYP1A2 and CYP3A4 expression was evaluated using primary cultures of fresh

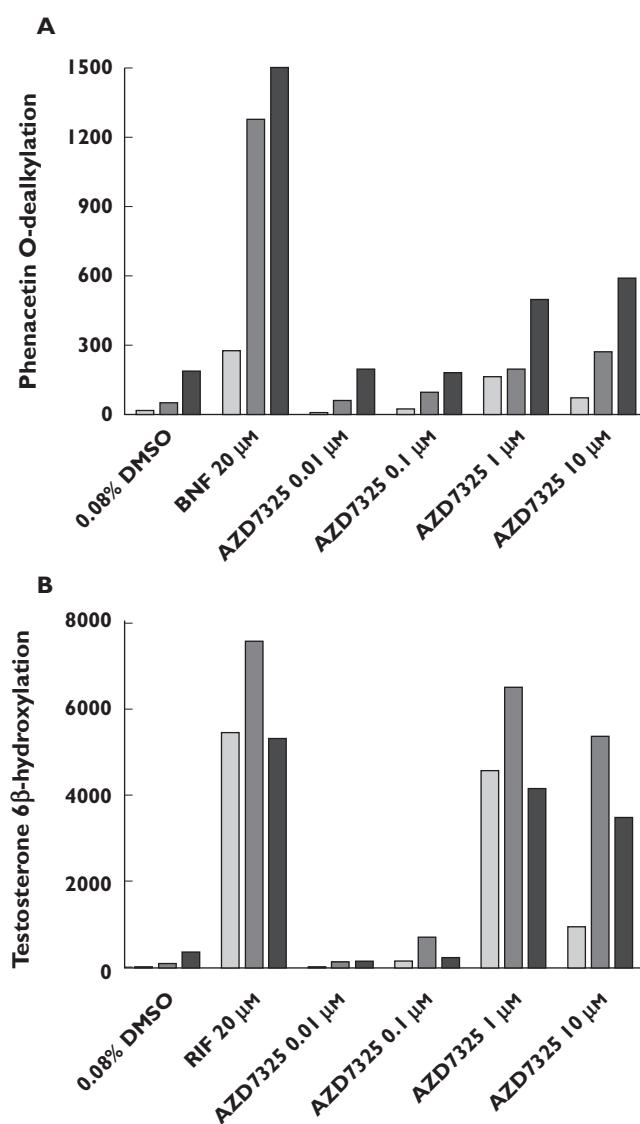


Figure 2

Microsomal enzyme activity ($\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$) of CYP1A2 (A) and CYP3A4 (B) in cultured human hepatocytes treated with AZD7325 (0.01, 0.1, 1, $10\text{ }\mu\text{M}$) or positive control inducers, β -naphthoflavone (BNF) or rifampicin (RIF), from three human donors (HH210, HH215, HH216) using CYP selective probe drugs. Each bar represents the mean activity from duplicate incubations

human hepatocytes. A cytotoxicity assay using the tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium and visual inspection showed no apparent reduction in hepatocyte viability after treatment with AZD7325 at a concentration up to $10\text{ }\mu\text{M}$. Treatment with positive control inducers, β -naphthoflavone or rifampicin, caused marked induction of CYP1A2 activity (8.9- to 24-fold) and CYP3A4 activity (14- to 112-fold), respectively, in hepatocytes from three individual donors (Figure 2). Marked induction of mRNA expression (Figure 3) and protein expression (Figure 4) was also observed with positive control inducer treatment in all three donors.

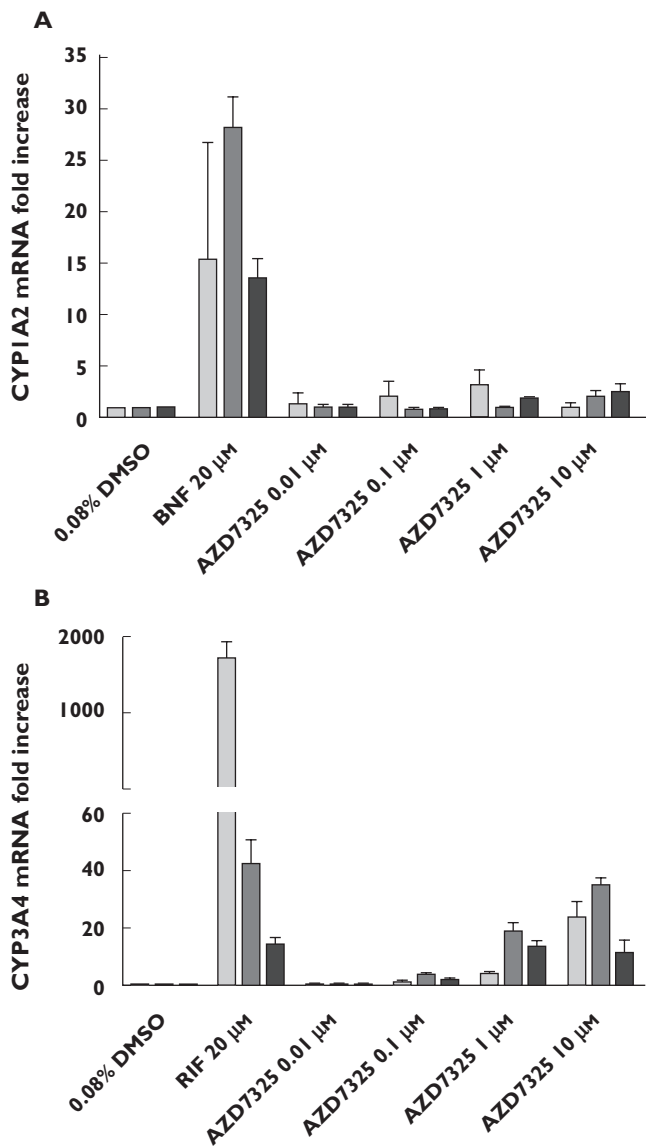


Figure 3

The mRNA expression of CYP1A2 (A) and CYP3A4 (B) in cultured human hepatocyte cultures treated with AZD7325 (0.01, 0.1, 1, 10 μM) or positive control inducers, β-naphthoflavone (BNF) or rifampicin (RIF), from three human donors (□ HH210, ▒ HH215, ■ HH216)

Increase of CYP1A2 activity was observed in all three preparations of hepatocytes treated with 0.01, 0.1, 1 or 10 μM AZD7325 (Figure 2). The maximal induction response (8.9-fold over vehicle controls) was observed at 1 μM in donor HH210, corresponding to 54.9% of the adjusted positive control β-naphthoflavone response. In addition, the maximal induction response of 5.1-fold (17.9% of the positive control response) and 3.1-fold (26.8% of the positive control response) was observed at 10 μM in donor HH215 and donor HH 216, respectively. In general, the results from mRNA assays and Western blots assays were consistent with those observed from CYP1A2 activity assays. AZD7325 at 1 or 10 μM caused a maximal CYP1A2

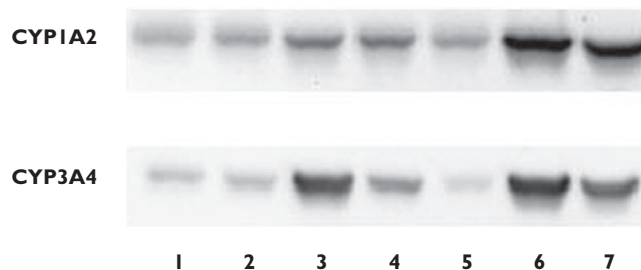


Figure 4

Western blot analysis of CYP1A2 (A) and CYP3A4 (B) protein expression levels in cultured human hepatocytes treated with AZD7325 from a representative donor (HH210). Band 1 to 4 were microsomes (30 μg) from human hepatocytes treated with 0.01, 0.1, 1, 10 μM AZD7325, respectively. Band 5 was microsomes from human hepatocytes treated with 0.08% DMSO that was used as baseline. Band 6 was microsomes from human hepatocytes treated with positive control inducers (20 μM β-naphthoflavone or 20 μM rifampicin). Band 7 was pooled human liver microsomes that were used as a positive control

mRNA expression of 3.2-fold, 2.1-fold and 2.5-fold in donor HH210, HH215 and HH216, respectively (Figure 3). The densitometry and image data from Western blots demonstrated an induction (1.5-fold to 3.5-fold) in CYP1A2 protein expression in hepatocytes from multiple donors after treatment with AZD7325 (Figure 4). Though variability was observed among the three donors, the results from enzyme activity, mRNA and protein expression suggested that AZD7325 was a moderate inducer of CYP1A2 *in vitro*.

A concentration-related increase in CYP3A4 activity was observed with 0.01, 0.1, 1 or 10 μM AZD7325 treatment in all three preparations of primary human hepatocyte cultures (Figure 2). The maximal induction response observed at 1 μM of AZD7325 treatment was 94-fold, 53-fold and 11-fold over vehicle controls in donors HH210, HH215 and HH216, respectively. The CYP3A4 activity increase was in the range of 76.9–85.7% relative to the adjusted positive control rifampicin response, indicating that AZD7325 was a potent CYP3A4 inducer *in vitro*. The increase in testosterone 6β-hydroxylase activity observed in hepatocyte microsomes was similar or lower after exposure to 10 μM than exposure to 1 μM AZD7325. The average induction response observed after incubation with 0.1 μM was only 3.4-fold over vehicle controls in the three donors. Little or no induction of CYP3A4 activity was observed with 0.01 μM AZD7325 treatment in all three donors. Similarly, AZD7325 caused maximal CYP3A4 mRNA expression increases of 24-fold, 35-fold and 12-fold in donor HH210, HH215 and HH216, respectively (Figure 3). The induction of CYP3A4 mRNA expression was similar to the positive control rifampicin (82.4% and 95.6%) in donors HH215 and HH216 but the relative induction was much lower (1.31% of positive control response) in donor HH210, due to the strong observed response to rifampicin in this donor (1744-fold over vehicle control). In the Western blot study, treatment with AZD7325 caused a CYP3A4 protein

expression increase of 1.5-fold to 74-fold in the three donors (Figure 4). The results in general supported the data obtained from microsomal CYP3A4 enzyme activity and mRNA assays.

In vivo CYP induction by AZD7325

Demographics A total of 24 healthy male subjects were enrolled, 23 completed the study, and one withdrew his consent on day -2, prior to dosing with AZD7325 due to a pre dose adverse event of back pain. The subject was therefore withdrawn from the study. The subject received one dose of midazolam 5 mg. Subject demographics and baseline characteristics are summarized in Table 1.

Pharmacokinetics The mean caffeine and its metabolite, paraxanthine, plasma concentration–time profiles after oral administration of 200 mg caffeine before and after co-administration with repeat dose AZD7325 (10 mg) are shown in Figure 5. No significant change in caffeine and paraxanthine exposure was observed with co-administration of repeat doses of AZD7325 compared with caffeine alone. The individual AUCs of caffeine and paraxanthine with and without co-administration of AZD7325 are depicted in Figure 6. The corresponding 90% CIs for AUC and C_{max} of caffeine and paraxanthine were within the pre-defined range of 0.80–1.25 (Table 2). The geometric mean CL/F of caffeine decreased slightly from 5.8 l h⁻¹ (caffeine alone) to 5.0 l h⁻¹ when given during AZD7325 steady-state administration. The median $t_{1/2}$ of caffeine alone or co-administered with AZD7325 (6.3 vs. 7.0 h) and paraxanthine (7.4 vs. 8.0 h) was similar between treatments.

The mean plasma concentration–time profiles of midazolam and its metabolite 1'-hydroxymidazolam after oral administration of 5 mg midazolam alone and after co-administration with repeat doses of AZD7325 (10 mg) are illustrated in Figure 7. The key pharmacokinetic parameters are summarized in Table 3. The AUC geometric mean ratio of midazolam after administration of AZD7325 to

steady-state to midazolam alone was 0.81 (90% CI 0.77, 0.87), which was not contained within the pre-defined range of 0.8, 1.25. The lower limit of the 90% CI was below 0.8 indicating that the AUC of midazolam was 19% lower in combination with the AZD7325 administration. The C_{max} of midazolam decreased with AZD7325 co-administration, but the 90% CI (0.82, 0.97) was within the range of 0.80, 1.25. The corresponding CIs for AUC and C_{max} of the 1'-hydroxymidazolam indicated that AZD7325 had no significant effect on the exposure of 1'-hydroxymidazolam. The individual AUCs of midazolam and 1'-hydroxymidazolam with and without co-administration of AZD7325 are depicted in Figure 8. The geometric mean CL/F of midazolam increased from 62 l h⁻¹ (midazolam alone) to 76 l h⁻¹ when administered during AZD7325 steady-state administration. The median $t_{1/2}$ of midazolam

Table 1

Demographic characteristics

Characteristic	All subjects
<i>n</i>	24
Gender, male, <i>n</i> (%)	24 (100%)
Race:	
White	20 (83%)
Black or African American	1 (4%)
Asian (except Indian/Pakistani)	1 (4%)
Other	2 (8%)
Age (years), mean (range)	27.7 (20–41)
Weight (kg), mean (range)	81.3 (62–96)
Height (cm), mean (range)	177.5 (168–189)
Body mass index (kg m ⁻²), mean (range)	25.8 (21–30)

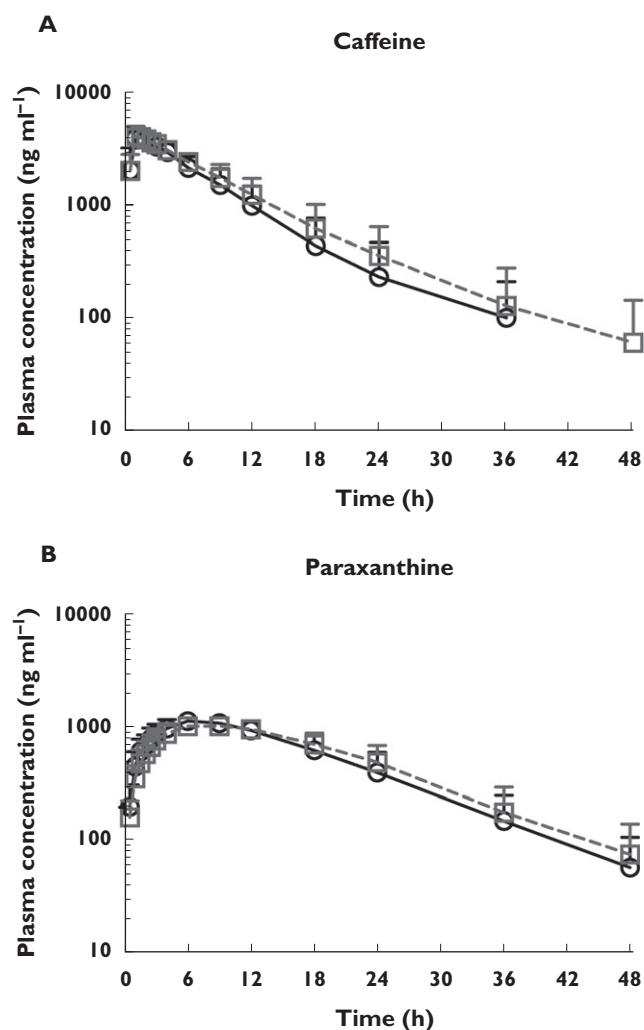


Figure 5

The mean plasma concentration–time profiles of caffeine (A) and its metabolite paraxanthine (B) after oral administration of 200 mg caffeine alone and after co-administration to steady-state with AZD7325 (10 mg) in healthy male volunteers ($n = 23$). Error bars represent SD. Caffeine alone (○), caffeine in the presence of AZD7325 (□)

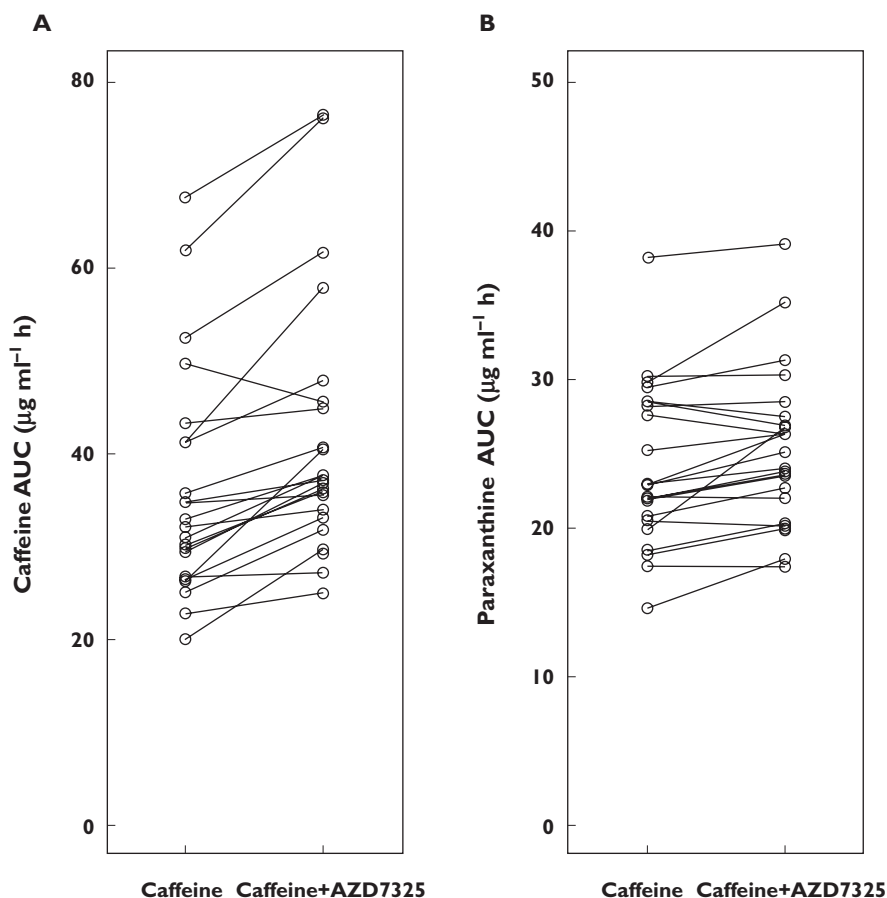


Figure 6

Individual AUC values of caffeine ($n = 22$, A) or paraxanthine ($n = 23$, B) following a 200 mg single dose of caffeine alone or in combination with a 10 mg once daily administration of AZD7325 to steady-state

Table 2

Effect of steady-state treatment of AZD7325 on caffeine pharmacokinetics

Parameter	Caffeine alone ($n = 23$)	Caffeine + AZD7325 ($n = 23$)	Ratio
Caffeine			
AUC ($\mu\text{g ml}^{-1} \text{ h}$)	34.0 (29.7, 38.8)	39.8 (34.9, 45.5)	1.17 (1.12, 1.23)
C_{max} ($\mu\text{g ml}^{-1}$)	4.3 (4.1, 4.6)	4.3 (4.1, 4.5)	0.99 (0.95, 1.03)
Paraxanthine			
AUC ($\mu\text{g ml}^{-1} \text{ h}$)	23.5 (21.4, 25.7)	24.9 (22.8, 27.3)	1.06 (1.03, 1.10)
C_{max} ($\mu\text{g ml}^{-1}$)	1.2 (1.12, 1.28)	1.1 (1.02, 1.17)	0.92 (0.90, 0.93)

AUC and C_{max} values are reported as geometric mean with 95% confident interval (CI). Ratio is expressed as geometric mean ratio with 90% CI.

decreased from 5.2 h (midazolam alone) to 3.6 h (midazolam with AZD7325) while the median $t_{1/2}$ of 1'-hydroxymidazolam after midazolam administration alone or when co-administered with AZD7325 (2.3 vs. 2.4 h) was similar between treatments.

The mean AZD7325 plasma concentration–time profiles on day 1, day 11 and day 12 after single daily oral administration of 10 mg AZD7325 are shown in Figure 9. Plots of individual C_{trough} values on day 1 to day 12 indi-

cated that steady-state was reached on day 6 for most subjects. The geometric mean C_{max} and AUC of AZD7325 were 57 ng ml^{-1} and 261 $\text{ng ml}^{-1} \text{ h}$, respectively on day 1. The geometric mean C_{max} was 69 and 72 ng ml^{-1} on day 11 (in combination of midazolam) and day 12 (in combination with caffeine), respectively, indicating the C_{max} of AZD7325 at steady-state was about 0.2 μM . The geometric mean AUC(0,24 h) on day 11 and day 12 was 285 and 327 $\text{ng ml}^{-1} \text{ h}$, respectively. The geometric mean CL/F of

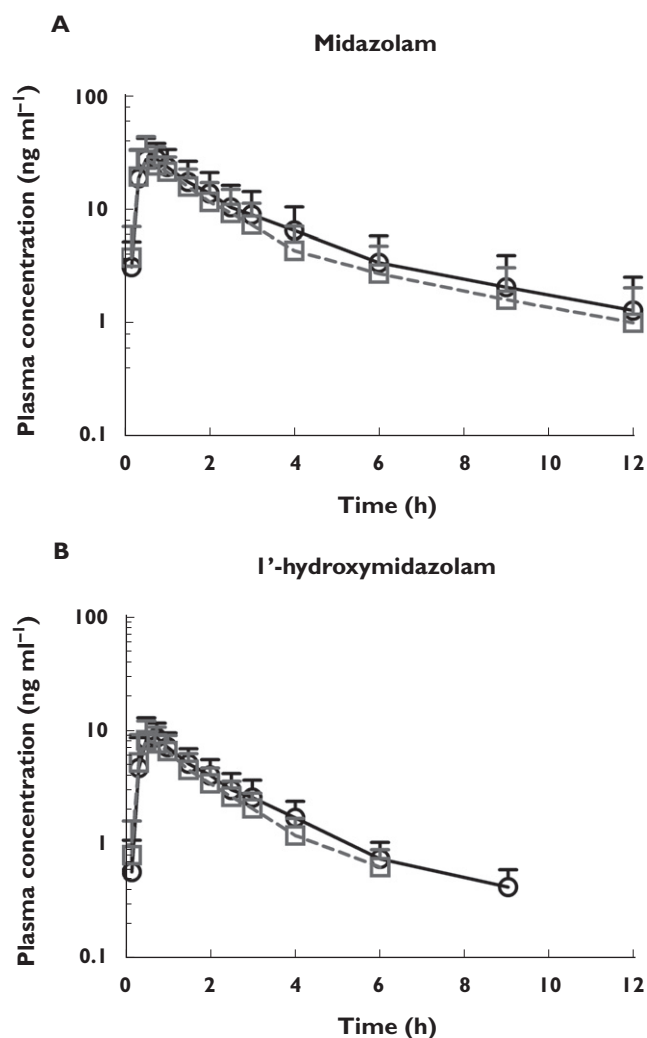


Figure 7

The mean plasma concentration–time profiles of midazolam (A) and its metabolite 1'-hydroxymidazolam (B) after oral administration of 5 mg midazolam alone and after co-administration with AZD7325 (10 mg) to steady-state in healthy male volunteers ($n = 23$). Error bars represent SD. Midazolam alone (○), midazolam in the presence of AZD7325 (□)

AZD7325 was 38 l h^{-1} , 35 l h^{-1} and 31 l h^{-1} on day 1, day 11 and day 12, respectively. There was no apparent change in AZD7325 exposure following co-administration of midazolam or caffeine compared with AZD7325 alone.

Safety AZD7325 administered in combination with caffeine and midazolam was well tolerated by subjects. There were no significant adverse events or discontinuations due to adverse events noted. A total of 68 treatment emergent adverse events (TEAEs) were reported by 20/24 (83%) subjects. Somnolence was predominantly reported in those subjects taking midazolam. The TEAE most commonly reported by subjects receiving AZD7325 was dizziness. The majority of TEAEs (44/68) were considered to be related to AZD7325, 10 of the 68 were considered to be related to

midazolam and 5 of the 68 were considered to be related to caffeine. There were no clinically significant changes in laboratory values, vital signs or ECGs during the study.

Discussion

AZD7325 is a potent, selective GABA_{A α 2,3} receptor modulator intended for the treatment of anxiety. The purpose of this study was to assess the potential of AZD7325 to induce CYP1A2 and CYP3A4 *in vivo* following on from results observed *in vitro*. AZD7325 appeared to be a potent inducer of CYP3A4 and a moderate inducer of CYP1A2 in fresh human hepatocytes. AZD7325 caused a maximal *in vitro* induction response in CYP1A2 activity in the range of 17.9% to 54.9% of the positive control response in the three donors. At $1 \mu\text{M}$, AZD7325 caused a maximal induction response (76.9% to 85.7% of the positive control response) in CYP3A4 activity and the induction of CYP3A4 was lower after exposure to $10 \mu\text{M}$ AZD7325. The decreased induction effect at higher concentrations cannot be attributed to time-dependent inhibition as *in vitro* studies demonstrated that AZD7325 was not a time-dependent inhibitor of CYP3A4/5 (data on file). Both Western blot and PCR analysis for CYP1A2 and CYP3A4 in general supported the results obtained from the microsomal activity assays. Though the *in vitro* results demonstrated AZD7325 to be a potent CYP3A4 inducer, the CYP3A4 induction effect was much lower at lower AZD7325 concentrations, indicating that the clinical dose–exposure relationship of AZD7325 would play a critical role in determining the effect on CYP3A4 expression *in vivo*. Therefore, the clinical evaluation of the impact of this compound on the CYP3A4 substrate midazolam and the CYP1A2 substrate caffeine was performed at the expected efficacious dose of AZD7325 (10 mg). In addition, it has been suggested that *in vivo* evaluation would be warranted if an *in vitro* induction of at least 40% of the positive control was observed [17]. Since a response of more than 40% of the positive control inducer was observed for CYP1A2 in one of three donors and in all three donors for CYP3A4 activity, *in vivo* induction studies were performed using midazolam and caffeine as selective probe drugs for CYP3A4 and CYP1A2, respectively.

The exposure of caffeine and paraxanthine by AUC or C_{max} was unchanged by AZD7325 co-administration, with the 90% CI within the default no effect boundary of 0.8, 1.25. In addition, the CL/F of caffeine and the $t_{1/2}$ of caffeine and paraxanthine were similar between treatments, indicating that at steady-state 10 mg AZD7325 exhibited no effect on CYP1A2 activity. Midazolam AUC decreased statistically significantly by 19% after oral dosing of 10 mg AZD7325 to steady-state. In accordance with this an increase in midazolam CL/F and a slight decrease in midazolam $t_{1/2}$ were also observed. The C_{max} of midazolam also decreased with AZD7325 co-administration, but the 90%

Table 3

Effect of steady-state treatment of AZD7325 on midazolam pharmacokinetics

Parameter	Midazolam alone (n = 24)	Midazolam + AZD7325 (n = 23)	Ratio
Midazolam			
AUC (ng ml ⁻¹ h)	80.9 (66.6, 98.2)	65.8 (54.2, 79.9)	0.81 (0.77, 0.87)
C _{max} (ng ml ⁻¹)	32.2 (27.7, 37.5)	28.6 (24.6, 33.4)	0.89 (0.82, 0.97)
1'-hydroxymidazolam			
AUC (ng ml ⁻¹ h)	21.2 (18.0, 25.0)	18.1 (15.4, 21.3)	0.85 (0.81, 0.90)
C _{max} (ng ml ⁻¹)	10.0 (8.6, 11.8)	9.4 (8.0, 11.0)	0.93 (0.82, 1.06)

AUC and C_{max} values are reported as geometric mean with 95% confident interval (CI). Ratio is expressed as geometric mean ratio with 90% CI.

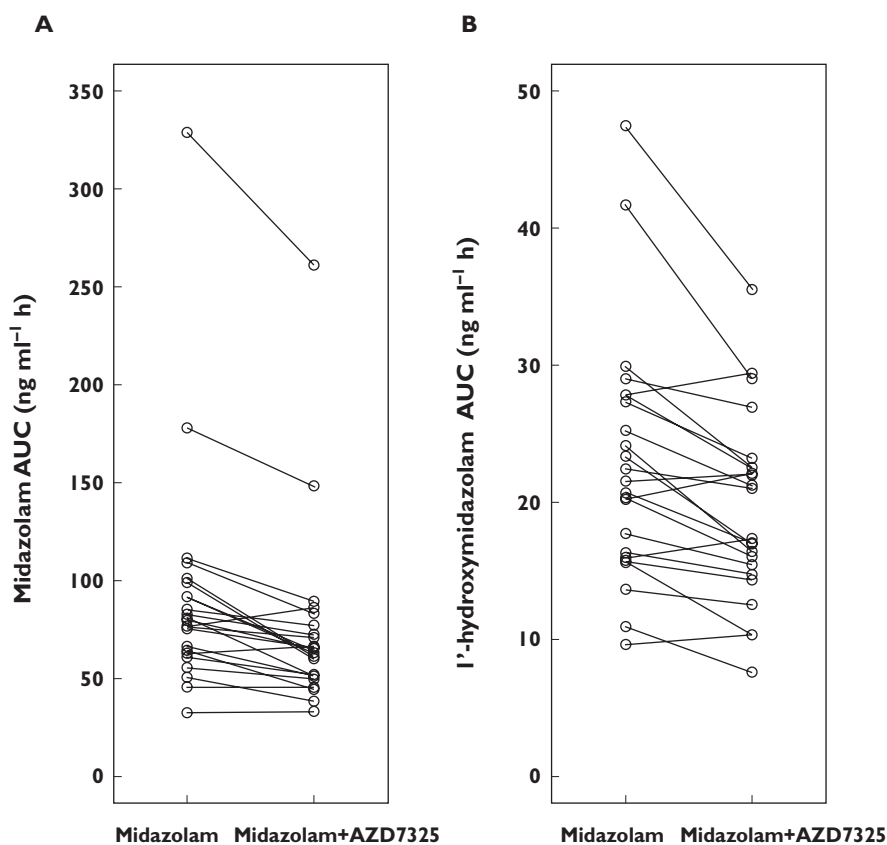


Figure 8

Individual AUC values of midazolam (A) or 1'-hydroxymidazolam (B) following a 5 mg single dose of midazolam alone or in combination with a 10 mg once daily administration of AZD7325 to steady-state (n = 23)

CI was within the default no effect boundary of 0.8, 1.25. The results indicated a weak inducing effect of AZD7325 on CYP3A4 activity, which was not likely to be clinically meaningful at this dose. *In vitro* studies have demonstrated that AZD7325 was primarily metabolized by CYP3A4/5 and CYP2C19 (data on file). The trough concentration of AZD7325 was similar after six doses indicating no evidence of AZD7325 auto-induction after multiple doses, which also indicated that the CYP3A4 induction effect would be minimal at this dose of AZD7325.

Though midazolam exposure decreased with AZD7325 co-administration, there was no notable effect on the 1'-hydroxymidazolam exposure parameters, AUC or C_{max}. It has been demonstrated that 1'-hydroxymidazolam can be rapidly converted to its glucuronide conjugate and about 60% to 80% of the midazolam dose is excreted in the urine as 1'-hydroxymidazolam glucuronide within 24 h [18]. Link *et al.* [19] also demonstrated that glucuronidation plays a major role in the kinetics of the hydroxymidazolam metabolites and proposed that the ratio (1'-

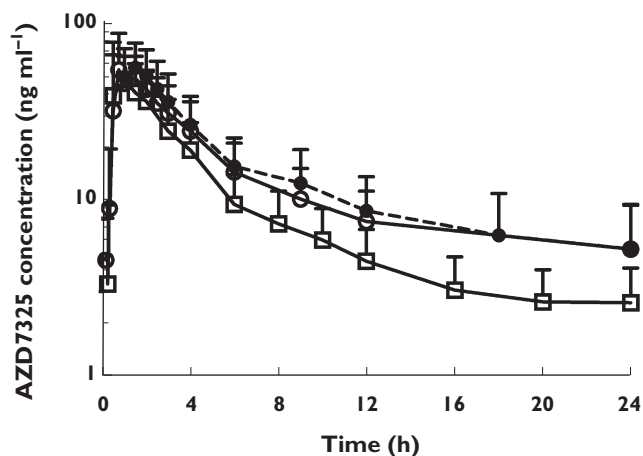


Figure 9

The mean plasma concentration–time profiles of AZD7325 on day 1 (□), day 11 (○) and day 12 (●) after an oral daily dose of 10 mg AZD7325 in healthy male volunteers ($n = 23$). Error bars represent SD

hydroxymidazolam + 1'-hydroxymidazolam glucuronide) vs. midazolam rather than 1'-hydroxymidazolam vs. midazolam may be best used to assess induction of CYP3A4 activity in plasma. This may explain the observed statistically significant decrease of midazolam exposure while no change was observed in 1'-hydroxymidazolam exposure. However, the plasma concentrations of the 1'-hydroxymidazolam glucuronide were not determined in current study.

Extrapolation of the magnitude of *in vivo* induction of CYP inducers from *in vitro* results is quite complicated. A number of mathematical approaches and correlation approaches, including relative induction score (RIS) and AUC/ F_2 approaches, have been proposed and validated with various drugs in different clinical trials [9]. All these approaches incorporated *in vivo* plasma exposure (C_{max} , AUC) of the inducer in addition to the observed *in vitro* induction parameters (EC_{50} , E_{max}) as clinical exposure of the inducer is a critical determinant to characterize appropriately induction risk. Pioglitazone, rosiglitazone and troglitazone exhibited similar CYP3A4 induction potency evaluated in cryopreserved human hepatocytes [20]. However, the daily doses of 8 mg, 45 mg and 600 mg for rosiglitazone, pioglitazone and troglitazone resulted in free plasma concentrations of 0.0028, 0.028 and 0.063 μM , respectively. In accordance with this, troglitazone and pioglitazone clinically decreased midazolam AUC by 67% and 26%, respectively, while rosiglitazone demonstrated no significant effect on the pharmacokinetics of another CYP3A4 probe drug, nifedipine [20]. On the contrary, carbamazepine, phenobarbital and phenytoin only exhibited weak CYP3A4 induction in *in vitro* studies. However, all three drugs caused significant induction in good agreement with predictions when high free plasma concentrations were considered [20]. After repeated oral dosing of

AZD7325 10 mg, the total C_{max} of AZD7325 at steady-state was about 0.2 μM . By considering the human plasma protein binding of 92.4%, the free plasma concentration of AZD7325 at steady-state was only about 0.015 μM . Therefore, it was quite reasonable to expect to observe only weak CYP3A4 induction of midazolam after multiple 10 mg daily doses of AZD7325. Our study provides a valuable additional comparison of *in vitro* and *in vivo* induction results and further demonstrates that the physiologically relevant exposure level of a compound plays a critical role when estimating the *in vivo* impact of an inducer identified through *in vitro* evaluations.

In conclusion, the hepatocyte studies revealed that AZD7325 was a moderate CYP1A2 inducer and a potent CYP3A4 inducer *in vitro*. Follow-up clinical studies demonstrated that a 10 mg daily dose of AZD7325 only weakly induced the pharmacokinetics of the CYP3A4 sensitive substrate, midazolam, and did not significantly affect the pharmacokinetics of CYP1A2 substrate, caffeine. As the induction effect was quite weak, dose adjustments would likely not be necessary for drugs substantially metabolized by CYP3A4 when co-administered with AZD7325 at its predicted efficacious dose. This study also demonstrates, like that shown for other drugs, that the clinical exposure of a CYP inducing drug is a key determinant in predicting clinical drug interaction risk and highlights again the importance in improving the predictive modelling of *in vitro* results to reduce the necessity for clinical drug-drug interaction trials.

Competing Interests

All authors were employees of AstraZeneca at the time of study conduct.

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