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Inhibition of oral midazolam clearance by boosting doses of ritonavir, and by 4,4-dimethyl-benziso-(2H)-selenazine (ALT-2074), an experimental catalytic mimic of glutathione oxidase

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The viral protease inhibitor ritonavir is known to inhibit clearance of intravenous midazolam.
- ALT-2074, a catalytic mimic of glutathione oxidase, inhibits human cytochrome P450 3A (CYP3A) isoforms *in vitro*.

WHAT THIS STUDY ADDS

- Short-term administration of low-dose ritonavir increases area under the plasma concentration curve following oral midazolam by a factor of 28.
- Therefore ritonavir is an appropriate positive control inhibitor for clinical drug interaction studies involving CYP3A substrates.
- Midazolam clearance is weakly inhibited by ALT-2074, consistent with its *in vitro* profile.

AIMS

We evaluated whether 'boosting' doses of ritonavir can serve as a positive control inhibitor for pharmacokinetic drug–drug interaction studies involving cytochrome P450 3A (CYP3A). The study also determined whether 4,4-dimethyl-benziso-(2H)-selenazine (ALT-2074), an investigational organoselenium compound that acts as a catalytic mimic of glutathione oxidase, inhibits CYP3A metabolism *in vivo*.

METHODS

Thirteen healthy volunteers received single 3-mg oral doses of midazolam on three occasions: in the control condition, during co-treatment with low-dose ritonavir (three oral doses of 100 mg over 24 h), and during co-treatment with ALT-2074 (three oral doses of 80 mg over 24 h).

RESULTS

Ritonavir increased mean (±SE) total area under the curve (AUC) for midazolam by a factor of 28.4 ± 4.2 ($P < 0.001$), and reduced oral clearance to 4.2 ± 0.5% of control ($P < 0.001$). In contrast, ALT-2074 increased midazolam AUC by 1.25 ± 0.11 ($P < 0.05$), and reduced oral clearance to 88 ± 8% of control.

CONCLUSIONS

Low-dose ritonavir produces extensive CYP3A inhibition exceeding that of ketoconazole (typically 10- to 15-fold midazolam AUC enhancement), and is a suitable positive control index inhibitor for drug–drug interaction studies. ALT-2074 inhibits CYP3A metabolism to a small degree that is of uncertain clinical importance.

Introduction

The benzodiazepine derivative midazolam, a substrate for biotransformation by cytochrome P450 3A (CYP3A) enzymes [1–3], is extensively used in drug development and clinical pharmacology as an index compound to profile the activity of hepatic and enteric CYP3A [4–12]. Under baseline conditions, midazolam undergoes extensive presystemic extraction after oral dosage, with net systemic bioavailability in the range of 30% [13–18]. It is established that incomplete oral bioavailability of midazolam results from a combination of hepatic and enteric CYP3A activity. An enteric-specific CYP3A inhibitor, such as grapefruit juice, has no effect on total area under the plasma concentration curve (AUC) of intravenous midazolam [19, 20], but increases AUC for oral midazolam by a factor of up to twofold [17, 20–23]. In contrast, an inhibitor such as ketoconazole, acting on both hepatic and enteric CYP3A, increases AUC of both intravenous and oral midazolam, but the effect on oral midazolam AUC is substantially greater [14, 18, 24–27].

In the course of drug development, new chemical entities suspected of being CYP3A inhibitors may be evaluated in clinical drug–drug interaction (DDI) studies using midazolam as the *in vivo* CYP3A probe compound [4–12]. The scientific value of such studies is strengthened by inclusion of a 'positive control' arm, intended to depict the 'worst case scenario' DDI. Ketoconazole is a possible choice as a positive control CYP3A inhibitor. However, recent studies suggest that CYP3A inhibition by ritonavir, even at relatively low 'boosting' doses, may produce CYP3A inhibition exceeding that of ketoconazole [28–35].

The present study evaluated low-dose ritonavir as an inhibitor of oral midazolam clearance, in the course of a DDI study of a medication under development. The candidate drug was 4,4-dimethyl-benziso-(2H)-selenazine (ALT-2074; formerly BXT-51072), a low-molecular-weight, orally active, organoselenium catalytic mimic of the enzyme glutathione peroxidase that is being developed for the treatment of inflammatory disorders characterized by the involvement of reactive oxygen species [36–41]. One possible indication is the treatment of acute coronary syndromes.

Previous *in vitro* studies have shown ALT-2074 is an inhibitor of human CYP3A, with an IC_{50} value in the range of 2.0–2.6 μM. This concentration might be achieved within the gastrointestinal tract or in the systemic circulation after oral administration of ALT-2074, raising the possibility that ALT-2074 might produce drug interactions with other CYP3A substrate drugs *in vivo*.

Methods and procedures

Study participants and design

The study protocol and consent form were reviewed and approved by the Western Institutional Review Board (Olympia, WA, USA). All subjects provided written informed consent prior to the study.

Participants were healthy male volunteers aged 18–55 years with no current or prior history of significant medical or psychiatric disease, and receiving no prescription medications. All subjects were within 25% of ideal body weight based on actuarial data incorporating height and frame size. Screening procedures included medical history, physical examination, electrocardiogram, haematology and chemistry screening, and urinalysis.

The DDI study was conducted using a three-way crossover design, with at least a 1-week interval elapsing between trials. After completing a screening period, subjects received each of the following three trial regimens in random sequence:

Trial	Co-treatment	Midazolam
1	Placebo (three doses)	3 mg
2	ALT-2074, 80 mg (three doses)	3 mg
3	Ritonavir, 100 mg (three doses)	3 mg

The dosing schedule for ALT-2074 is consistent with typical therapeutic exposure. The dosage schedule for ritonavir represents exposure consistent with a 'boosting' regimen.

ALT-2074 and placebo (Synvista Therapeutics, Inc., Montvale, NJ, USA) were packaged identically and administered under double-blind conditions. Because of the shape and construction of the oral ritonavir dosage form (Abbott Laboratories, N. Chicago, IL, USA), a placebo to match ritonavir was not available, and ritonavir was given under nonblind conditions.

Subjects were admitted to the Study Unit for the duration of each trial period. Subjects received the first dose of the co-treatment between 16.00 and 18.00 h on the first study day. The second and third co-treatments were administered at 07.30 and 18.00 h on the second study day. Midazolam was prepared as 3 ml of the commercially available parenteral dosage form (1 mg ml^{-1}) mixed with 240 ml of tap water. The midazolam was administered at 08.00 h on the second study day, followed by multiple pharmacokinetic blood samples. Subjects' final blood samples were obtained at 08.00 h on the third study day. Subjects repeated the procedure, being randomized to a different co-treatment at approximately weekly intervals.

Experimental procedures

Subjects were admitted to the study unit on the afternoon prior to each midazolam trial. Between 16.00 and 18.00 h, the first dose of co-treatment was administered (placebo, ALT-2074, or ritonavir). On the following morning, a light breakfast was provided at 07.00 h. At 07.30 h, an indwelling cannula was inserted, and a predose blood sample was taken. The second dose of co-treatment (placebo, ALT-2074, or placebo) was then given. At 08.00 h, a 3-mg oral

dose was administered, followed by blood sampling at 0.25, 0.5, 1.0, 2, 4, 6, 8, 10 and 12 h after dosage. The third dose of co-treatment was given at 18.00 h. The final blood sample was taken at 08.00 h on the following morning, 24 h after midazolam dosage. Subjects were then discharged from the study unit.

Venous blood samples were collected into heparinized tubes and stored on ice until centrifuged. The plasma was separated and frozen at −18°C until assay.

Analysis of samples

Plasma concentrations of midazolam in all samples were determined by liquid chromatography-mass spectroscopy, having a sensitivity limit of 0.5 ng ml^{-1} [15]. All samples from a given subject's set of three trials were extracted and analysed together on the same day using the same calibration standards.

Plasma concentrations of ritonavir during the ritonavir co-treatment trial were determined by high-performance liquid chromatography [42]. Methods were not available for determination of ALT-2074 concentrations.

Pharmacokinetic analysis

The following pharmacokinetic parameters for midazolam were determined using standard model-independent ('noncompartmental') methods: maximum plasma concentration (C_{max}), elimination rate constant (β), elimination half-life ($T_1/_2$), total AUC, and apparent oral clearance (CL).

Statistical analysis

A 50% difference in mean values of midazolam clearance between placebo or ALT-2074 co-treatments, or between placebo and ritonavir co-treatments, was assumed to be of potential clinical importance. Based on prior studies [13, 15, 23], the standard deviation of the difference between mean values was assumed to be 35% of the difference itself. Under these conditions, a sample size of $n = 12$ allows this difference to be detected with $\alpha = 0.05$ and power of at least 0.8.

Arithmetic mean and SD/SE of untransformed pharmacokinetic variables were calculated and presented [43]. Differences among the three co-treatments (placebo, ALT-2074, or ritonavir) were evaluated using analysis of variance (ANOVA) for repeated measures, followed by Dunnett's test to compare ALT-2074 *vs.* placebo and ritonavir *vs.* placebo individually. These analyses were done both without and with rank transformation (nonparametric analysis).

Ratios of pharmacokinetic variables with ALT-2074 or ritonavir co-treatment divided by the placebo value were also calculated. These were aggregated as arithmetic means and standard deviations, or geometric means and 90% confidence intervals (CIs).

Kinetic and statistical analyses were performed using Microsoft Excel or Statistical Analysis Systems (SAS Institute Inc., Cary, NC, USA).

Results

Subjects

Fifteen subjects initiated participation in the study, and completed the first of three trials. Two of these individuals did not complete subsequent trials for administrative reasons. Pharmacokinetic analysis was based on the 13 subjects that completed all three trials. Age ranges were 21–50 years, and weight ranged from 52 to 97 kg. The racial/ethnic distribution was eight White, five African-American.

Studies were completed with no adverse events reported.

Pharmacokinetics of midazolam

Figure 1 shows the mean plasma midazolam concentrations at corresponding times, comparing placebo with ALT-2074 and placebo with ritonavir.

Analysis of variance for repeated measures showed highly significant differences among the three treatments in all pharmacokinetic variables for midazolam (Table 1). Dunnett's test showed significant differences between ritonavir and placebo co-treatments. Ritonavir increased midazolam AUC by a factor of approximately 25, and correspondingly reduced clearance to about 4% of control values. Co-treatment with ALT-2074 increased midazolam AUC by a factor of about 1.25, and reduced

Table 1

Statistical analysis of midazolam pharmacokinetic variables*

	Mean (±SE, n = 13) for treatment conditions:				Dunnett's test	
	Placebo	ALT-2074	Ritonavir	Repeated measures ANOVA	ALT-2074 *vs.* placebo	Ritonavir *vs.* placebo
C_{max} (ng ml^{-1})	9.0 (±1.0)	10.6 (±0.9)	35.6 (±2.8)	F = 81.8, $P < 0.001$	NS	$P < 0.05$
$t_{1/2}$ (h)	2.06 (±0.15)	2.08 (±0.17)	18.07 (±2.25)	F = 49.4, $P < 0.001$	NS	$P < 0.05$
Total AUC (ng ml^{-1} h^{-1})	24.65 (±1.93)	29.87 (±2.62)	651 (±77)	F = 64.9, $P < 0.001$	NS	$P < 0.05$
Clearance (ml min^{-1})	2157 (±142)	1844 (±167)	85.9 (±6.9)	F = 92.2, $P < 0.001$	NS	$P < 0.05$
Clearance (ml min^{-1} kg^{-1})	28.84 (±2.84)	24.15 (±2.38)	1.13 (±0.11)	F = 64.1, $P < 0.001$	NS	$P < 0.05$

*Analysis of actual values without transformation.

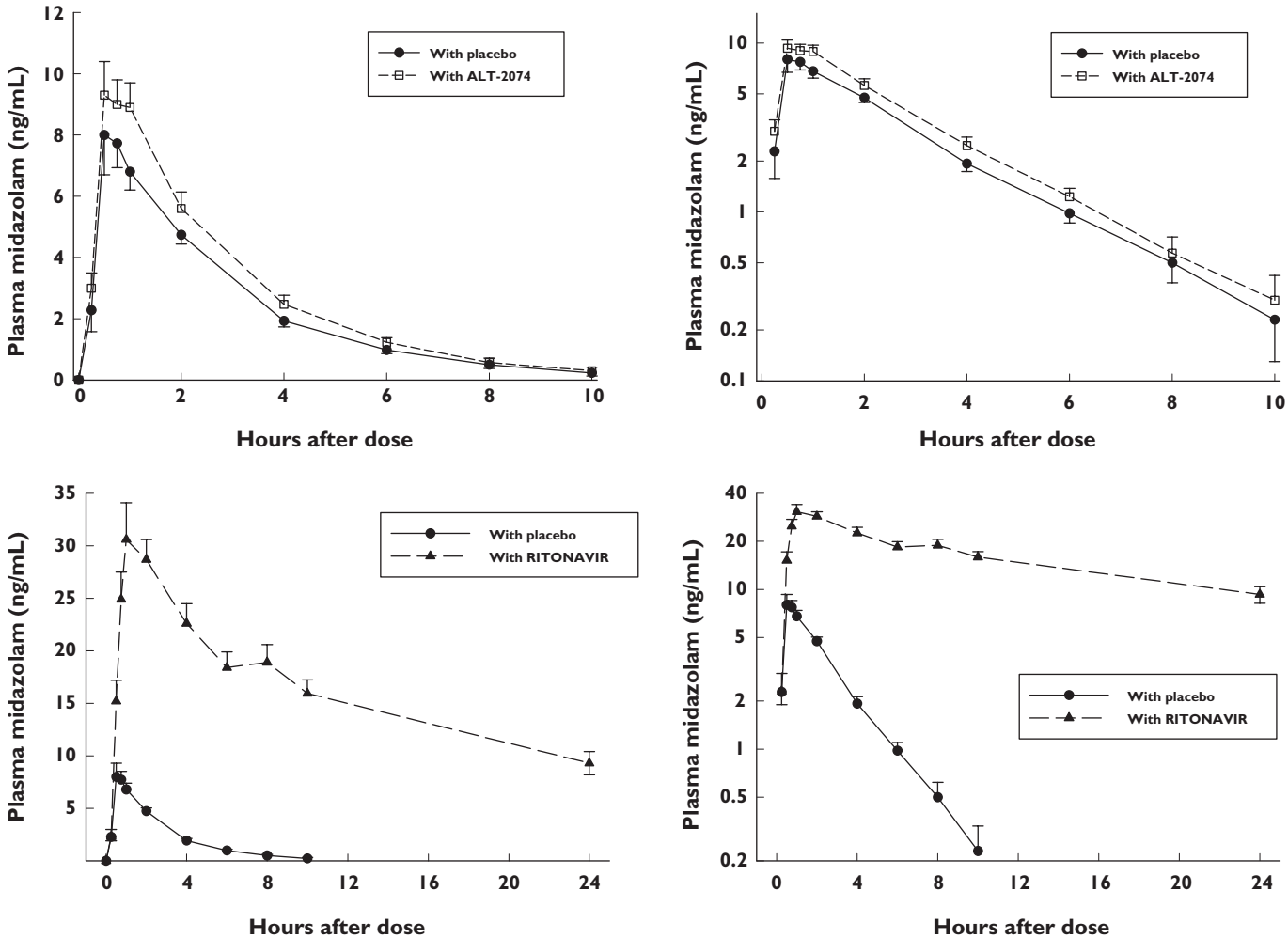

Figure 1

Mean (±SE) plasma midazolam concentrations at corresponding times. Top: placebo and ALT-2074 co-treatments (left: linear scale; right: logarithmic scale). Bottom: placebo and ritonavir co-treatments (left: linear scale; right: logarithmic scale)

clearance to 88% of control. Differences in mean values between ALT-2074 and placebo co-treatments were not significant.

When the ANOVA was done on rank-transformed variables, all conclusions were identical.

Table 2 summarizes the analysis of ratios for the pharmacokinetic variables. Based on untransformed ratios of untransformed values, all ritonavir/placebo ratios were different from 1.0 with a high level of statistical significance. The ALT-2074/placebo ratio for C_{max} and clearance did not differ significantly from 1.0; the AUC ratio was significantly greater than 1.0 ($P < 0.05$, two-tailed test).

Geometric mean ratios underestimated the arithmetic means. For the ritonavir/placebo ratios, the 90% CIs fell entirely outside the arbitrary 80–125% boundary. For ALT-2074/placebo, one extreme of the 90% CI fell outside the 80–125% boundaries.

Plasma ritonavir concentrations

Figure 2 shows mean plasma ritonavir concentrations at specific time points during the ritonavir co-administration trial. The results demonstrate systemic exposure to ritonavir consistent with the ritonavir dosage. However, there was no apparent relationship between the net exposure to ritonavir over the 0–10-h dosage interval (expressed as mean concentration over that interval) and the extent of midazolam clearance impairment relative to the control trial (expressed as midazolam total AUC ratio for Trial 3 divided by Trial 1) (Figure 3).

Discussion

In this study we evaluated the capacity of ALT-2074 and boosting doses of ritonavir to reduce the apparent oral

Table 2

Analysis of ratios for midazolam pharmacokinetic variables

	Ritonavir/placebo ratio			ALT-2074/placebo ratio		
	C_{max}	AUC (total)	Clearance	C_{max}	AUC (total)	Clearance
Arithmetic						
Mean	4.47*	28.4*	0.042*	1.34	1.25**	0.88
SD	2.02	15.3	0.017	0.89	0.39	0.29
SE	0.56	4.2	0.005	0.19	0.11	0.08
Geometric						
Mean	4.10	25.6	0.039	1.21	1.19	0.84
90% CI	3.32, 5.07	20.5, 32.0	0.032, 0.049	0.96, 1.52	1.024, 1.40	0.72, 0.98

Student's *t*-test *vs.* 1.0: *$P < 0.001$; **$P < 0.05$.

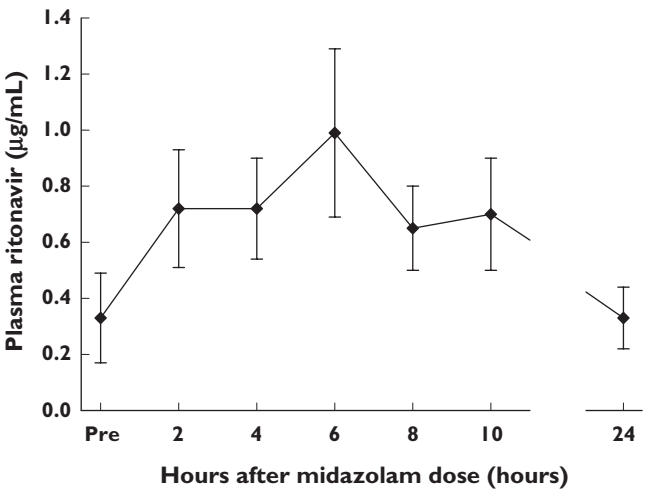

Figure 2

Mean (±SE) plasma ritonavir concentrations at individual time points

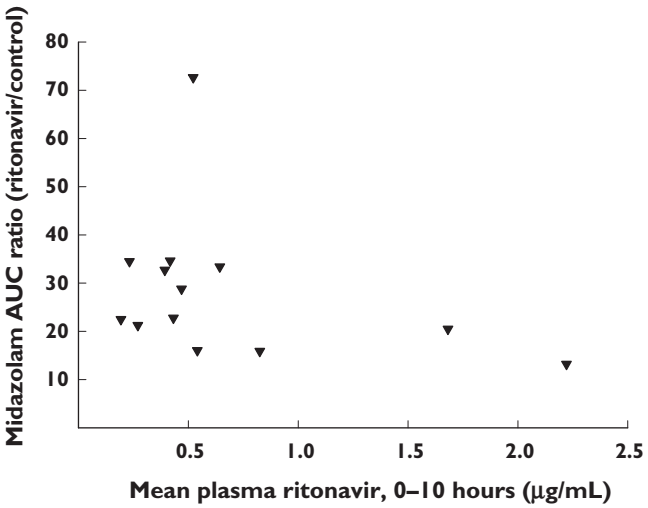

Figure 3

x-axis: Mean plasma ritonavir concentration across the time interval 0–10 h. *y*-axis: Ratio of midazolam total AUC during the ritonavir co-administration condition (Trial 3) divided by the control AUC value (Trial 1). Each point represents an individual subject

clearance and thereby increase the systemic exposure of midazolam in human volunteers. Midazolam is cleared essentially exclusively via biotransformation by CYP3A isoforms (CYP3A4 and CYP3A5) [1–3]. After oral dosage, midazolam clearance is determined by a combination of enteric and hepatic CYP3A activity [13–18] and is considered to be a 'sensitive' CYP3A probe to indicate the capacity of drugs under clinical development to inhibit or induce CYP3A phenotype *in vivo* [4–12]. Although our study subjects were male volunteers, a review of available literature indicates that gender has only a small influence on the kinetics of midazolam and other CYP3A substrate drugs [44]. There is no evidence to indicate that susceptibility to metabolic inhibition is meaningfully influenced by gender [14, 17, 26].

A relatively low dose (100 mg given orally three times over 24 h) of the antiretroviral agent ritonavir was used as a positive control inhibitor. Ritonavir is a highly potent CYP3A inhibitor *in vitro*, with IC_{50} or K_i values in the low nanomolar range [45–48]. Since systemic exposure to ritonavir [I] with usual clinical dosage generally exceeds 1–2 μM, the ratio of [I]/K_i or [I]/IC_{50} will exceed 10.0, thereby predicting a high likelihood of clinical drug interactions involving ritonavir and CYP3A substrates [4–12]. This prediction has been verified in a number of previous clinical studies [28–35, 49–55]. It is also reported that CYP3A inhibition by ritonavir is reversible within a few days after discontinuation of ritonavir [55]. In the present study, a very large ritonavir–midazolam interaction was observed, in which relatively low 'boosting' doses of ritonavir increased midazolam systemic exposure by a factor of about 25. This exceeds the extent of midazolam clearance inhibition generally produced by ketoconazole, which typically increases midazolam exposure by a factor of 10–15 [14, 15, 24–27]. Thus the inclusion of ritonavir as a positive control inhibitor verified the validity of the clinical model, including the identification of midazolam as a 'sensitive' substrate. The fact that impairment of midazolam clearance was independent of systemic exposure to ritonavir indicates that all levels of ritonavir exposure were

sufficient to produce extensive reduction of midazolam clearance [31].

The rationale for the clinical study was based on *in vitro* studies with ALT-2074 indicating an IC_{50} value for CYP3A inhibition (using triazolam hydroxylation as an index reaction) in the range of 2.0–2.6 μM. Assuming maximum systemic exposure to ALT-2074 to be in the range of 500 ng ml^{-1} (2.2 μM), the ratio of $[I]/IC_{50}$ is approximately 1.0, indicating that a clinical drug interaction involving ALT-2074 is 'possible'. However, the results of the clinical study indicated only a modest increase in midazolam AUC in the range of 20–25% with co-administration of ALT-2074. The effect of ALT-2074 was not statistically significant based on ANOVA, with or without rank transformation of the values. Analysis of ratios indicated that the arithmetic mean midazolam AUC ratio (ALT-2074 divided by placebo) of 1.25 was significantly different from 1.0, and the 90% CI (1.02–1.40) for the geometric mean ratio (1.19) fell partially outside the 'default' upper boundary of 1.25 as specified in the Food and Drug Administration guidance. [56] Thus ALT-2074 could be considered a 'weak' CYP3A inhibitor at most. The clinical importance of this inhibition of CYP3A by ALT-2074 remains to be determined.

Competing interests

None to declare.

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