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PHARMACOKINETICS AND DISPOSITION

Concentration effect relationship of CYP3A inhibition by ritonavir in humans

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Abstract
Purpose To investigate the dose and concentration dependency of CYP3A inhibition by ritonavir using the established limited sampling strategy with midazolam for CYP3A activity.
Methods An open, fixed-sequence study was carried out in 12 healthy subjects. Single ascending doses of ritonavir (0.1–300 mg) were evaluated for CYP3A inhibition in two cohorts using midazolam as a marker substance.
Results Ritonavir administered as a single oral dose produced a dose-dependent CYP3A inhibition with an ID_{50} of 3.4 mg. Using the measured ritonavir concentrations an exposure–inhibition effect curve was established with an IC_{50} of 600 h pmol/L (AUC_{2-4}). Over the ritonavir dose range studied non-linear exposure of ritonavir was observed.
Conclusions Ritonavir shows a dose and concentration effect relationship of CYP3A inhibition. In addition, a proposed auto-inhibition of ritonavir metabolism resulted in a non-linear exposure of ritonavir with sub-proportional concentrations at low doses. A time-dependent CYP3A activity may result when inhibitors of CYP3A with short elimination half-lives are used.

Keywords CYP3A · Ritonavir · Inhibition · Midazolam

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Introduction

Cytochrome P 450 3A (CYP3A) is the most abundant enzyme of cytochromes in the liver and the gut wall responsible for the metabolism of approximately 50 % of drugs being metabolised by cytochromes [1]. CYP3A activity determines the clearance of many drugs with a narrow therapeutic range. Alterations of its activity by co-administered enzyme inducers, inhibitors, or both may require dose modifications to maintain drug concentrations within safe and effective margins.

Inhibition of cytochromes mainly occurs by competitive reversible inhibition and mechanism-based irreversible inhibition. Ritonavir is used to boost exposure of other protease inhibitors that are part of the highly active antiretroviral therapy (HAART). Based on in vitro and in vivo studies, ritonavir is primarily metabolised by CYP3A and to a lesser extent by CYP2D6. It is a very potent irreversible inhibitor of CYP3A [2–5]. Metabolic drug–drug interactions that arise through mechanism-based inactivation of CYP3A could result in marked drug–drug interactions and/or toxicities [6]. Midazolam is frequently used to determine the activity of CYP3A and has become a preferred CYP3A phenotyping probe [7–10].

Standard doses of 100–300 mg of ritonavir are used to facilitate CYP3A inhibition. Quite often multiple dosing is applied, at least during the time interval of observation [11, 12]. Only one study has been carried out with ritonavir doses as low as 20 mg to assess the potential for doses <100 mg to provide boosting of drugs that are substrates of CYP3A [13]. The 20 mg dose resulted in more than 60 % midazolam clearance reduction and CYP3A inhibition was already almost maximal at the 50 mg ritonavir dose. Hence, no clear-cut dose–response (dose–inhibition) relationship was established. Furthermore, in this study elvitegravir, a newer integrase inhibitor, was always co-administered with ritonavir, which may complicate the interpretation of the results.

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We therefore investigated the dose and concentration dependency of CYP3A inhibition by ritonavir using the established limited sampling strategy with midazolam for CYP3A activity.

Materials and methods

The study was approved by the Competent Authority in Germany (EudraCT No: 2011-000516-25) and the Ethics Committee of the Medical Faculty of the University of Heidelberg. It was conducted at the Department of Clinical Pharmacology and Pharmacoepidemiology in accordance with the standards of Good Clinical Practice (as defined in the ICH E6 Guideline for Good Clinical Practice), in agreement with the Declaration of Helsinki, and the specific legal requirements in Germany.

Study population

Twelve healthy non-smoking Caucasian participants (male, n=8; female, n=4) were enrolled in this clinical trial. They were all aged between 19 and 50 years with an average age of 29.8±8.4 years. All participants were mentally and physically healthy as confirmed by the medical history, a physical examination as well as an electrocardiogram and appropriate laboratory analyses. The subjects had to undergo a urine drug screening and women were tested for pregnancy. Before inclusion the investigators affirmed that none of the exclusion criteria was applicable to the volunteers. These included the intake of any continuous medication other than oral contraceptives or any other substance known to interact with drug metabolising enzymes or transport system enzymes. Any condition that could potentially modify absorption, distribution, metabolism or excretion of the study drugs, allergies (except for mild forms of hay fever) or history of hypersensitivity reactions, smoking, excessive alcohol drinking, blood donation or participation in a study within the last 2 months, positive drug screening or known or admitted drug abuse and an inability to communicate well with the investigator would also lead to exclusion. Neither pregnant nor lactating women were included. All participants were required to agree on the use of two appropriate contraception methods and gave their written informed consent before any study measures were carried out.

Study design and blood sampling

The study was carried out as a randomised, open clinical trial with fixed sequence design. Subjects were randomised to two dosing groups with four doses each, both consisting of 6 participants using a web-based randomisation (http://www.randomization.com). There was a total of 6 inpatient study days for each participant. Before the first drug administration women were again tested for pregnancy. Midazolam was used as a biomarker for CYP3A activity. To assess baseline activity each participant received on the first occasion a single oral dose of 3 mg midazolam solution (Dormicum® V 5 mg/5 mL solution for injection, Roche, Grenzach-Wyhlen, Germany). Ritonavir (Norvir® 80 mg/mL solution, Abbott Laboratories Limited, Maidenhead, UK), a known potent inhibitor of CYP3A was given on 4 separate days in rising doses of 0.1, 1, 10, 100 mg (group A) or 0.3, 3, 30, 300 mg (group B) with 2, 4 and 7 days' washout. The fixed sequence design with increasing ritonavir doses was chosen because of the known mechanism-based inhibition. Ritonavir solution was diluted in chocolate milk (Happy Drink Schoko, Hochwald Nahrungsmittelwerke, Thalfang, Germany) as suggested by the manufacturer to achieve the predefined doses [14]. Ritonavir solution was always administered 10 min before intake of midazolam solution to avoid a pharmaceutical interaction.

On each inpatient day 5 blood samples (4.9 mL each) were collected to determine midazolam and ritonavir concentrations. The samples were taken before drug administration and 2, 2.5, 3. and 4 h after midazolam administration. Blood samples were centrifuged at 3,600 U (= 2,500 g) at 4 °C and plasma was distributed into three aliquots, each stored at −20 °C until analysis.

Quantification of midazolam and 1'-hydroxymidazolam in plasma

Midazolam and 1′-hydroxymidazolam (for plausibility checks only) concentrations in plasma were determined by high performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), as described elsewhere [15–17], with a lower limit of quantification for midazolam of 0.525-ng/mL with accuracy/precision of −4.2 %/5.0 % for midazolam and +11.6/4.6 % for 1′-hydroxymidazolam.

Quantification of ritonavir in plasma

Plasma concentrations of ritonavir were determined by solid phase extraction and ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS) using a Waters Acquity UPLC and Waters Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). Plasma (0.25 mL) was extracted using Waters standard procedure for 96-well-based solid phase extraction (Waters μElution MCX). Extracts were chromatographed on a Waters BEH C18 1.7 μm UPLC column with a fast gradient consisting of formic acid, ammonia and acetonitrile. Ritonavir was quantified using a deuterium labelled internal standard and positive electrospray tandem mass spectrometry in the multiple reaction monitoring mode with a lower limit of quantification of 1 pg/mL plasma. The

accuracies were always within 100±15 % with corresponding precision of<15 % of the coefficient of variation (CV). The calibrated concentration range was linear (1–20,000 pg/mL) with correlation coefficients>0.99.

Data analysis

Partial area under the midazolam plasma concentration–time curves 2–4 h after midazolam administration (AUC_{2-4} per milligram of midazolam) was determined using Prism 5.03 (GraphPad Software Inc., La Jolla, CA 92037, USA). Similarly, ritonavir AUC_{2-4} was calculated. Midazolam partial metabolic clearance was calculated using the following established equation, which covers conditions from inhibition to induction [7, 10]:

$$CL_{met}[mL/\min] = \frac{5668}{AUC_{2-4}[h\ \ nmol/L/mg\ \ dose]}$$

Relationships between midazolam AUC or partial metabolic clearance and ritonavir dose or ritonavir AUC were evaluated using the Hill equation with variable slope using Prism 5.03.

Results

Midazolam plasma concentrations and $AUC_{2-4\ h}$ were increased by single oral doses of ritonavir in a dose-dependent fashion (see Fig. 1) with highest exposure after 300 mg of ritonavir. Correspondingly, calculated midazolam partial metabolic clearance decreased exponentially with increasing ritonavir doses (Fig. 2). Maximum inhibition was observed at 300 mg of ritonavir with midazolam clearance being reduced to 10 % of baseline clearance (without ritonavir; see Table 1). With the eight different ritonavir doses used in this study a dose–inhibition effect curve could be constructed with an ID_{50} of 3.4 mg (r^2=0.8763; Fig. 3). Because of a very sensitive analytical method we were able to quantify ritonavir concentrations after a single oral dose of 0.1 mg between 2 and 4 h after administration and partial AUCs were calculated (Table 1). Using these ritonavir AUCs an exposure–inhibition effect curve was established with an IC_{50} of 600 h pmol/L (r^2= 0.8898; Fig. 4). Ritonavir exposure was nonlinear with the dose-corrected AUC_{2-4} of 39,982±14,785 h pmol/L at the 300-mg dose decreasing to 45±24 h pmol/L at the 0.1-mg dose.

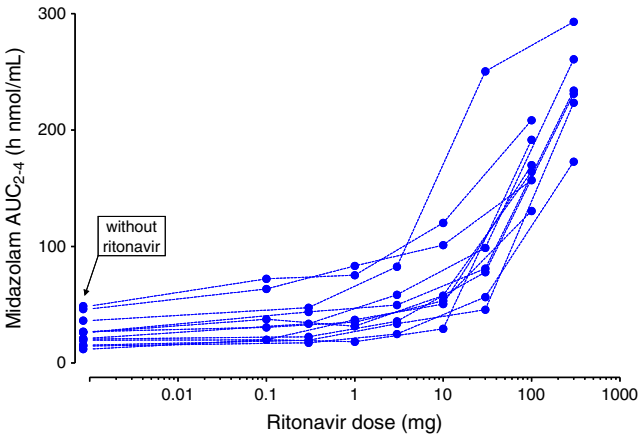

Fig. 1 Individual midazolam partial AUC_{2-4} (normalised to a 1-mg dose) after administration of 3-mg midazolam p.o. and different single oral doses of ritonavir in 12 healthy study participants

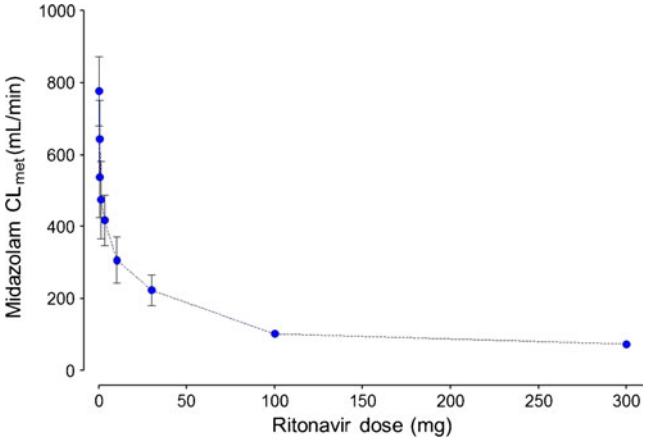

Fig. 2 Mean ± SD partial metabolic clearance of midazolam in relation to concomitantly administered different single oral ritonavir doses ranging from 0.1 to 300 mg

Safety and tolerability

No serious adverse events occurred. All study drugs were well tolerated. Adverse events (AE) occurred in 2 subjects only and were all mild. No actions were taken and all AEs resolved without sequelae.

Discussion

A classical dose-dependent inhibition of in vivo CYP3A activity by the potent inhibitor ritonavir was evaluated using the established midazolam limited sampling strategy [10, 16]. The usual ritonavir dose in drug–drug interaction studies is the established booster dose of 100 mg b.i.d., which guarantees almost maximum inhibition of CYP3A [13]. Under the study conditions (single-dose administration of the inhibitor) the midazolam clearance reduction with the 300-mg ritonavir dose was 90.7 %. This is close to the previously shown maximum midazolam metabolic clearance reduction to 8.4 % after 2 days of ritonavir 300 mg twice daily [10]. We observed a very low dose of 3 mg ritonavir to elicit 50 % of maximum possible inhibition. Using the obtained parameter of the Hill equation a reduction of the partial metabolic

Table 1 Mean±SD pharmacokinetic parameters of midazolam (3 mg p.o.) and ritonavir (0.1–300 mg) using a limited sampling strategy in 12 healthy study participants

Condition	*n*	Midazolam AUC_{2-4} (h nmol/L)	Midazolam CL_{met} (mL/min)	Ritonavir AUC_{2-4} (h pmol/L)
Without ritonavir	12	26.17±12.00	777.0±331.7	–
0.1 mg ritonavir	6	40.60±22.34	537.2±273.2	4.47±2.40
0.3 mg ritonavir	6	30.70±12.93	644.0±263.3	11.5±10.6
1 mg ritonavir	6	46.76±26.23	474.5±264.4	108±51.3
3 mg ritonavir	6	47.52±20.98	417.3±172.6	617±371
10 mg ritonavir	6	68.78±34.49	306.3±156.4	4,446±1,729
30 mg ritonavir	6	101.8±75.2	223.4±104.9	54,738±81,547
100 mg ritonavir	6	170.3±27.2	102.1±17.0	$1.004*10^6$±$0.405*10^6$
300 mg ritonavir	6	235.9±40.1	74.0±13.6	$12.0*10^6$±$4.4*10^6$

clearance of midazolam by 81.4 % is calculated with 100-mg ritonavir. This is probably not the maximum possible effect because we used a single oral dose of ritonavir only, which was administered 10 min before midazolam. In a clinical setting 100 mg of ritonavir twice daily is applied, which may enhance the inhibitory effect owing to the mechanism-based inhibition. However, when 17 clinical pharmacology trials were evaluated systematically there was no clear correlation between the ritonavir dose needed to boost different protease inhibitors [18]. A low ritonavir dose of for example 10 mg or even less may be used in the future to assess the possibility of CYP3A inhibition with fewer or even without any side effects under conditions where maximum inhibition is not required.

Ritonavir is one of the most potent CYP3A inhibitors known. The IC_{50} of ritonavir ranges between 70 and 130 nM in vitro [19, 20]. With the 100 mg dose of ritonavir used for boosting, a partial AUC_{2-4} of 1,004 h nmol/L was the result in our study. This corresponds to an average concentration of about 500 nM during these 2 h. However, in vivo a 50 % inhibition was obtained using a dose of 3 mg of ritonavir, which corresponds to a partial AUC_{2-4} of 617 h pmol/L (Table 1). The calculated average ritonavir concentration is only about 300 pM, which is far below the reported in vitro IC_{50} values. Recently, an IC_{50} of only 3.5 nM has been reported, which was explained by using lower microsomal concentrations, reducing nonspecific microsomal binding [21]. This is much closer to the measured in vivo concentrations, but still an order of magnitude different. This might be due to the oral administration of the probe drug midazolam and the oral administration of ritonavir. Owing to the study design with both midazolam and ritonavir administered orally, the overall CYP3A4 activity is assessed and the inhibitory effect of ritonavir on CYP3A in the gut wall and liver cannot be differentiated. Using a semi-simultaneous intravenous and oral midazolam administration the CYP3A inhibition of oral ketoconazole was more pronounced in the intestinal wall than in the liver [8]. Concentrations in the gut wall and the liver

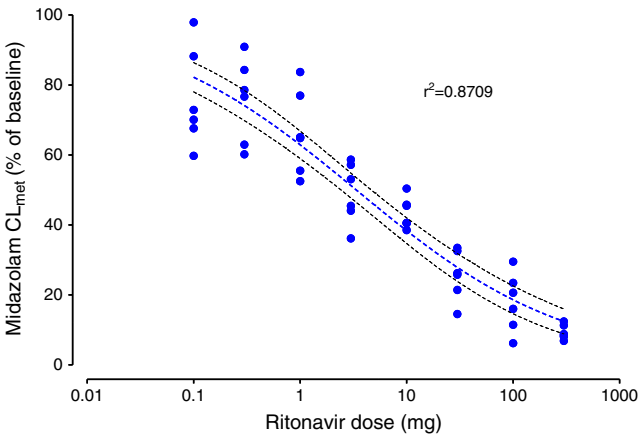

Fig. 3 Partial metabolic clearance of midazolam (percentage of individual baseline clearance) in relation to the ritonavir dose administered. The fitted line (± 95 % confidence intervals) results from a sigmoidal dose–response equation with variable slope (Hill equation)

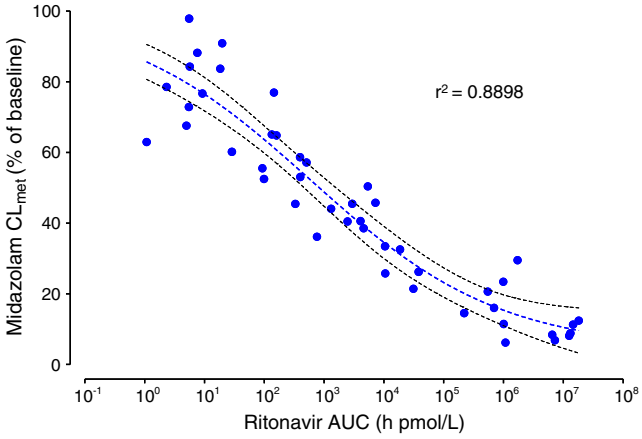

Fig. 4 Partial metabolic clearance of midazolam (percentage of individual baseline clearance) in relation to ritonavir partial AUC_{2-4}. The fitted line (± 95 % confidence intervals) results from a sigmoidal dose–response equation with variable slope (Hill equation)

during first-pass might therefore be much higher than the concentrations measured in the systemic circulation resulting in great inhibition of the first-pass metabolism.

Ritonavir clearly exhibited nonlinear pharmacokinetics when the applied dose was reduced. There was an almost 900-fold decrease in the dose-corrected exposure from a standard 300-mg dose to the lowest dose of 0.1 mg. This must be attributed to the so far neglected mechanism of nonlinearity called auto-inhibition [22]. Drugs like ritonavir that inhibit their own metabolism will be associated with less auto-inhibition at lower doses and consequently with enhanced drug elimination and possibly impaired drug exposure. However, in the case of ritonavir even this largely reduced drug exposure at very low doses did cause CYP3A inhibition, even though only low inhibition or no inhibition would have been anticipated. As a consequence, with a terminal elimination half-life of 5 h a single oral dose of 100 mg of ritonavir should be able to elicit substantial inhibition (50 %) over a period of about 10 half-lives calculated using the exposure data from this study. Even if the elimination half-life is shorter with lower concentrations substantial inhibition will be present for more than 1 day. The long-lasting CYP3A4 inhibition reported in clinical studies [10, 23] (3 days or longer) can be explained by the inhibitory effect at very low concentrations, may be in conjunction with mechanism-based inhibition.

Various wash-out periods were used in this study and the rationale needs to be explained. Single ritonavir doses were used starting with the lowest dose (0.1 or 0.3 mg), 48 h thereafter the next dose (1 or 3 mg) was administered, after a further 96 h 10 or 30 mg ritonavir were given, and the final dose of 100 or 300 mg was given 7 days later. Ritonavir elimination half-life is short (5 h), elimination is almost complete after 24 h. However, mechanism-based inhibition lasts much longer. There have been data published after a 10-day treatment with 400 mg ritonavir daily and triazolam as a CYP3A marker, where 84 h after the last ritonavir dose CYP3A activity was assessed [23]. Triazolam AUC was 20.5±27.8 h ng/mL, which was not significantly higher than the baseline AUC of 13.6±16.3 h ng/mL [23]. Therefore, we were quite confident that 7 days after the single 10 or 30 mg dose of ritonavir no inhibition was present, although this was not proven by an additional activity measurement before each ritonavir dose. With even lower ritonavir doses less inhibition was anticipated and hence a shorter recovery time, explaining the 2-, 4- and 7-day wash-out periods.

In conclusion, this study shows a dose and concentration effect relationship of CYP3A inhibition using the mechanism-based CYP3A inhibitor ritonavir. In addition, a proposed auto-inhibition of ritonavir metabolism resulted in a nonlinear exposure of ritonavir with sub-proportional concentrations at low doses. Time-dependent CYP3A activity may result when inhibitors of CYP3A with short elimination half-lives are used.

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Competing interests None to declare.

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