Is cyclosporin A an inhibitor of drug metabolism?

G. LI¹, G. TREIBER¹, J. MEINSHAUSEN², J. WOLF³, J. WERRINGLOER³ & U. KLOTZ¹

¹Dr Margarete Fischer-Bosch-Institute of Clinical Pharmacology, ²Robert Bosch Hospital, D-7000 Stuttgart and

³Institute of Toxicology, Eberhard-Karls-University, D-7400 Tübingen, FRG

1 The potential for a drug interaction between cyclosporin A and midazolam was investigated since both compounds appear to be metabolized by the same cytochrome P-450 isoenzyme.

2 *In vitro* evaluation of the binding of cyclosporin A to rat microsomal cytochrome P-450 indicated a K_s -value of 0.4 μM . In further studies with rat liver microsomes IC_{50} -values of 6, 8 and 70 μM cyclosporin A were determined for the inhibition of the metabolism of midazolam to its α -OH-, 4-OH- and di-OH-metabolites, respectively.

3 Comparative studies with human liver microsomes indicated IC_{50} -values of approximately 300 μM for the formation of α -OH-midazolam and of 65 μM for the formation of 4-OH-midazolam.

4 The pharmacokinetics of a single intravenous dose of midazolam (0.075 mg kg^{-1}) was studied in nine patients receiving cyclosporin A to prevent rejection of their transplanted kidneys. The average steady state blood concentrations of cyclosporin A, measured by r.i.a. using a specific monoclonal antibody, varied during a dosing interval between 175 and 600 ng ml^{-1} .

5 In these patients the hepatic elimination of midazolam was characterized by a mean $t_{1/2}$ ($\pm$ s.d.) of $2.3 \pm 1.2 \text{ h}$ and a plasma clearance (CL) of $414 \pm 95 \text{ ml min}^{-1}$. These values were not different from those of normal human subjects ($t_{1/2} = 1.5$ to 4 h , CL = 350 to 700 ml min^{-1}).

6 From the results of the *in vitro* experiments it is concluded that cyclosporin A may potentially inhibit drug metabolism. However, therapeutic blood concentrations *in vivo* do not appear to be sufficient to result in an effective impairment of the hepatic elimination of midazolam when given concomitantly.

Keywords cyclosporin A midazolam drug interactions drug metabolism microsomes

Introduction

Cyclosporin A, a potent immunosuppressive agent used widely to prevent rejection of transplanted organs, has a narrow therapeutic index and its toxic effects are mostly concentration-dependent (Rodighiero, 1989). It is metabolized almost completely in the liver by cytochrome P-450 catalyzed reactions (Maurer, 1985) and a cytochrome P-450 isoenzyme in rabbit liver has

been characterized that is involved in the metabolism of cyclosporin A (Bertault-Peres *et al.*, 1987). In human liver a related cytochrome P-450 isoenzyme has been identified recently that catalyzes the three initial steps of cyclosporin A metabolism (Kronbach *et al.*, 1988). Since this isoenzyme is also involved in the metabolism of a variety of other drugs it was concluded that

Correspondence: Professor Dr Ulrich Klotz, Dr Margarete Fischer-Bosch-Institut für klinische Pharmakologie, Auerbachstrasse 112, D-7000 Stuttgart 50, FRG

this might explain why certain drugs (e.g. phenytoin, barbiturates, rifampicin, ketoconazole, methylprednisolone, erythromycin, danazol, norethindrone, diltiazem, nifedipine, verapamil, sulphamethysine) have the potential of altering cyclosporin A blood concentrations (Kronbach *et al.*, 1988; Rodighiero, 1989). Recently a rifampicin-inducible cytochrome P-450 involved in the metabolism of cyclosporin A was purified to electrophoretic homogeneity from human liver microsomes and was found to be 100% homologous with human nifedipine oxidase (Combalbert *et al.*, 1989). In addition, *in vitro* experiments with microsomal fractions prepared from erythromycin-treated rabbits have demonstrated that the macrolide antibiotic inducible cytochrome P-450 LM_{3c} is involved in the metabolism of midazolam and that cyclosporin A inhibits the biotransformation of [¹⁴C]-midazolam in a concentration-dependent manner (Fabre *et al.*, 1988). This would suggest that cyclosporin A may serve indeed as an inhibitor of drug metabolism *in vivo*. We have investigated, therefore, the effect of cyclosporin A on the metabolism of midazolam using human and rat liver microsomes. In addition, a clinical study was performed in order to evaluate whether cyclosporin A impairs drug metabolism in man and the mechanism(s) involved.

Methods

Chemicals

Midazolam and its metabolites α -OH-midazolam 4-OH-midazolam and di-OH-midazolam were generously provided by Dr Heizmann of Hoffmann-La Roche (Grenzach, FRG). Cyclosporin A was a gift from Sandoz Ltd (Basle, Switzerland).

Preparations of liver microsomes

Microsomes from untreated male Wistar rats (200–250 g) and from one human liver sample (taken from a previously healthy victim of a traffic accident who became a kidney donor) were prepared according to standard ultracentrifugation procedures (Dayer *et al.*, 1984). Protein concentrations were measured by the method of Lowry *et al.* (1951).

Spectrophotometric analysis of microsomal cytochrome P-450

Difference spectra of microsomal suspensions were recorded at 20° C using a Sigma ZWS-11

spectrophotometer equipped with a split beam accessory. Total cytochrome P-450 concentrations were measured according to Omura & Sato (1964) and measurements of cyclosporin A binding were carried out as described by Jefcoate (1978) using rat liver microsomes at a concentration corresponding to 1 μ M cytochrome P-450. K_s -values were estimated graphically from Lineweaver-Burk plots.

Assay conditions and h.p.l.c analysis of midazolam metabolism

In a final volume of 250 μ l 50 mM tris-buffer (pH 7.4) liver microsomes from rats (0.5 mg protein = 0.25 nmol cytochrome P-450) or humans (0.16 mg protein = 0.035 nmol cytochrome P-450; only a limited amount of protein was available) were incubated with 1 mg NADPH, 0.3 mg MgCl₂ and 20 nmol midazolam at 37° C for 1 h. The reaction was stopped by extracting this mixture for 5 min with 5 ml benzyl alcohol. After separation the organic phase was evaporated under N₂ and the residue was dissolved in 150 μ l mobile phase (see below); the injection volume was 50 μ l.

Separation of unchanged midazolam (t_r = 20 min), α -OH-midazolam (t_r = 10.5 min), 4-OH-midazolam (t_r = 8.8 min) and di-OH-midazolam (t_r = 5.8 min) was achieved at room temperature on a nucleosil C₁₈-column (25 $\times$ 0.4 cm; particle size 5 μ m) with a mobile phase (flow rate 1 ml min⁻¹) consisting of 400 ml 10 mM phosphate buffer (pH 8.7), 210 ml methanol, 220 ml acetonitrile and 10 ml tetrahydrofuran. U.v.-absorbance at 262 nm was monitored by a Shimadzu SPD-2A photometer and recorded on a Shimadzu C-R3A integrator. The four substances were identified according to their retention times in comparison with those of standards.

Clinical study

After written informed consent was obtained nine outpatients (demographic data, Table 1) participated in this trial which was approved by the ethics committee of our hospital. According to their laboratory values the patients had normal liver and kidney function. They were treated orally for several weeks with cyclosporin A (Sandimmun®) to prevent rejection of a previously transplanted kidney and were regularly seen in our clinic. As indicated clinically subjects received various comedications including urapidil (n = 2), frusemide (n = 2), diltiazem (n = 2), metoprolol (n = 3), low dose prednisolone (n = 8) and azathioprine (n = 5). All individuals received a single intravenous

Table 1 Clinical data of patients studied

Subject (gender)	Age (years)	Weight (kg)	Serum creatinine (mg dl ⁻¹)	Dose of cyclosporin A (mg day ⁻¹)	Blood concentrations of cyclosporin A (ng ml ⁻¹)			
					monoclonal Ab.		polyclonal Ab.	
					C _{ss,min}	C _{ss,max}	C _{ss,min}	C _{ss,max}
1 (F) S	34	105	1.4	280	86	370	360	680
2 (M) NS	52	91	1.5	440	350	600	900	1200
3 (F) NS	49	61	1.6	340	420	1100*	1200	2000
4 (F) NS	65	76	1.5	320	145	860	410	1500
5 (M) S	41	77	1.0	340	170	540	430	1100
6 (M) NS	60	86	1.0	220	190	660	640	1400
7 (M) NS	50	67	1.2	200	130	350	410	740
8 (M) NS	55	75	1.1	360	160	820	600	1600
9 (M) NS	54	65	1.0	440	240	700	520	940
Mean	51	78	1.3	327	210	667	608	1240
± s.d.	9	14	0.3	84	109	239	278	429

F = female, M = male, NS = nonsmoker, S = smoker

Ab. = antibody used for RIA (kit from Sandoz AG., Nuremberg, FRG)

C_{ss,min} = minimal (trough) steady state concentration (prior to next dose)

C_{ss,max} = maximal (peak) steady state concentration (observed after 1 to 2 h following a maintenance dose)

* corresponds to 0.913 µM

dose of 0.075 mg kg⁻¹ of midazolam. Prior to this dose and at 0.17, 0.33, 0.67, 1, 2, 4, 6 and 8 h blood samples (10 ml) were drawn. In six blood samples (0, 1, 2, 4, 6, 8 h) cyclosporin A was measured by r.i.a. using specific monoclonal and unspecific polyclonal antibodies (kit from Sandoz AG, Nürnberg/FRG). In all plasma samples (stored frozen at -20° C until analysis) midazolam was measured by a specific g.l.c.-assay with sensitive EC-detection as described elsewhere (Allonen *et al.*, 1981; Klotz & Ziegler, 1982).

The sedative-hypnotic effect of midazolam was estimated using five visual analogue scales (length each 10 cm, maximal sedative score 50 cm) where the subjects had to rate their subjective state of vigilance (e.g. tired vs awake) and mood (e.g. active vs passive) all the time of blood sampling. From the sum of the five scales a sedation index was formed by subtracting the prestudy values.

Plasma concentrations of midazolam data were analysed individually by noncompartmental linear pharmacokinetic methods. From the slope of the terminal (log linear) phase (fitted by linear regression) λ_z and t_{1/2} were calculated. The AUC was estimated by the linear trapezoidal rule from time zero to the last measurable plasma concentration (C_t) with extrapolation to infinity using the value of C_t/λ_z. Plasma clearance (CL) was calculated from D/AUC and the apparent volume of dis-

tribution V_{ss} from CL × mean residence time (MRT).

Results

In vitro experiments

The addition of cyclosporin A (0.18 to 3 µM) to rat liver microsomal suspensions resulted in reverse-type I spectral changes with a ΔA_{max} (390–420 nm) of 0.017 (µM P-450)⁻¹ and a K_s of 0.4 µM, suggesting a strong ligand interaction of cyclosporin A with the microsomal haeme-protein. During an incubation period of 1 h midazolam (20 nmol) was metabolized extensively to α-OH-midazolam (15–20%), 4-OH-midazolam (20–30%) and di-OH-midazolam (10%) using native rat liver microsomes; no metabolism occurred after heat-inactivation of the microsomal suspension. The formation of all three metabolites was found to be inhibited by cyclosporin A in a concentration-dependent manner. The initial monohydroxylation reactions proved to be more susceptible to inhibition (IC₅₀-values 6.1 µM and 7.8 µM, respectively) than the subsequent transformation to di-OH-midazolam (IC₅₀ 70 µM; see Table 2).

Although human liver microsomes under similar incubation conditions (cf. **Methods**) converted only 4.2% and 2.4% of the added substrate to α-OH- and 4-OH-midazolam, respect-

Table 2 Inhibition of microsomal metabolism of midazolam (mid) by cyclosporin A

	<i>IC₅₀-values of cyclosporin A (μM) for the formation of</i>		
	<i>α-OH-mid</i>	<i>4-OH-mid</i>	<i>di-OH-mid</i>
Rat liver ^a	6	8	70
Human liver ^b	~300	65	not detectable under control conditions

^a means of three separate experiments with the same microsomal preparation

^b means of two separate experiments with the same microsomal preparation

ively, a corresponding concentration-dependent inhibition was observed in the presence of cyclosporin A (10–100 μM; for reasons of solubility no higher concentrations could be tested). Mean *IC*₅₀-values were 300 μM and 65 μM for the formation of α-OH- and 4-OH-midazolam, respectively (see Table 2).

Clinical study

During the pharmacokinetic study with midazolam the steady state blood concentrations of cyclosporin A were largely in the putative therapeutic range of 100–400 ng ml⁻¹ (see Table 1 and Figure 1). Over this time the blood concentrations of cyclosporin A declined with an apparent *t*_{1/2} of 4.4 ± 1.2 h.

The plasma concentration of midazolam following intravenous injection of 0.075 mg kg⁻¹ declined in a biexponential manner (see Figure 2a). Table 3 summarizes relevant pharmacokinetic parameters of midazolam. With the exception of data from one subject the values were

similar to those reported in other healthy subjects.

According to visual analogue scores the midazolam-induced CNS-depression lasted maximally for 4 h. After that time the sedation index had returned to prestudy levels. There was a relationship between the plasma concentrations of midazolam and its pharmacodynamic effect as assessed by the sedation index (Figure 2).

Discussion

It has been proposed that cyclosporin A and midazolam are metabolized by the same cytochrome P-450 isoenzyme (Fabre *et al.*, 1988; Kronbach *et al.*, 1988). Midazolam appears,

Table 3 Pharmacokinetic parameters of midazolam in nine patients treated with cyclosporin A

Subject	<i>t</i> _{1/2} (h)	CL (ml min ⁻¹)	V _{ss} (l kg ⁻¹)
1	5.0	218	0.87
2	1.3	435	0.43
3	1.6	450	0.43
4	1.7	409	0.54
5	1.3	556	0.49
6	2.2	442	0.66
7	2.6	484	1.21
8	3.2	339	0.62
9	2.1	392	0.96
Mean	2.3	414	0.69
± s.d.	1.2	95	0.27
Normal range*	1.5–4.0	350–700	0.6–1.6

* according to Allonen *et al.* (1981); Klotz & Ziegler (1982); Kanto (1985); Reves *et al.* (1985); Dundee *et al.* (1984); Kassai *et al.* (1988); Persson *et al.* (1988).

Figure 1 Steady-state blood concentrations of cyclosporin A during a dosing interval (mean ± s.d.) as measured by r.i.a. using a specific monoclonal antibody in nine patients on maintenance therapy.

Figure 2 Plasma concentration-time profile (mean $\pm$ s.d.) of midazolam in nine patients following a single intravenous dose of 0.075 mg kg^{-1} (a) and sedation index (mean $\pm$ s.d.) assessed using five visual analogue scales (b). The bottom plot (c) shows the relationship between the plasma concentration of midazolam and its sedative effects.

therefore, to be an ideal drug for the investigation of the potential for cyclosporin A to act as an inhibitor of drug metabolism. Results of previous *in vitro* experiments with liver microsomes prepared from macrolide-treated rabbits have indicated that the immunosuppressive agent attenuates midazolam metabolism in a concentration-dependent manner (Fabre *et al.*, 1988). From the data presented by these authors an IC_{50} -value of about $5 \mu\text{M}$ can be estimated.

To establish whether this interaction also occurs in man the pharmacokinetics of midazolam were investigated in kidney-transplanted patients receiving cyclosporin A. In addition, further *in vitro* experiments were carried out to assess the inhibitory potential of cyclosporin A.

The spectral changes induced by cyclosporin A indicate that it binds avidly ($K_s = 0.4 \mu\text{M}$) to rat liver microsomes. Similar type I spectral changes have been observed (no K_s -value pro-

vided) upon addition of cyclosporin A to liver microsomes obtained from phenobarbitone-induced male rats (Augustine & Zemaitis, 1986). Recent binding studies with a purified human cytochrome P-450 fraction (cyclosporin A oxidase) gave a K_s -value of $25 \mu\text{M}$ for cyclosporin A (Combalbert *et al.*, 1989). It is possible, therefore, that cyclosporin A and other drugs given concomitantly might compete for the same cytochrome P-450 binding sites resulting in inhibition of the metabolism of co-administered drugs.

In order to explore this possibility we investigated the *in vitro* metabolism of midazolam with microsomes isolated from rat and human liver samples. In both species the formation of α -OH- and 4-OH-midazolam was found to be inhibited by cyclosporin A. It became obvious that the more active rat liver microsomes demonstrated a much higher susceptibility (see Table 2) to inhibition by cyclosporin A and the IC_{50} -values were in good agreement with those described for induced rabbit liver microsomes (Fabre *et al.*, 1988). When rats were pretreated with cyclosporin A (25 or 50 mg kg^{-1} daily) *in vivo* the oxidative metabolism of various model drugs (e.g. ethylmorphine, aniline) was impaired *in vitro* (Augustine & Zemaitis, 1986); the conjugation of paracetamol, however, appears to be unaffected (Galinsky *et al.*, 1988).

Mean IC_{50} -values for human liver microsomes were $65 \mu\text{M}$ (4-OH-midazolam) and $300 \mu\text{M}$ (α -OH-midazolam; the major metabolite in man). These *in vitro* concentrations, however, are much higher than therapeutic blood concentration in patients, which vary between 83 nM (100 ng ml^{-1}) and 332 nM (400 ng ml^{-1}). Similarly, the cyclosporin A doses used in the above investigations with rats were higher by a factor of 4 to 9 than those used in humans. Therefore, *in vitro* to *in vivo* extrapolation of these findings is difficult.

For this reason we studied the pharmacokinetics of midazolam in allograft recipients who were on long-term prophylactic treatment with cyclosporin A. Because cyclosporin A is relatively toxic we performed this study in patients needing this immunosuppressant for prevention of organ rejection rather than in healthy volunteers. All subjects receiving the intravenous test dose of midazolam had therapeutic blood concentrations of cyclosporin A (see Figure 1 and Table 1) and the highest concentration was 1100 ng ml^{-1} ($0.9 \mu\text{M}$). Apparently, these concentrations were not sufficient to affect the hepatic elimination of midazolam. In our patients the plasma clearance and elimination half-life of midazolam were in the normal range (see

Table 2). Thus, with therapeutic doses and under clinically relevant conditions no interaction between the two drugs could be observed.

According to the results summarized in Figure 2, there was an almost parallel and rapid decline of the plasma concentrations of midazolam and its sedative effects. Applying the pharmacodynamic $E_{\max}$ -model an EC_{50} -value of 156 ng ml⁻¹ was calculated and an apparent $t_{1/2}$ of 1.3 h was estimated for the duration of action. Such plasma drug concentration-effect relationships have been described previously in healthy volunteers (Allonen *et al.*, 1981) as well as in surgical patients (Persson *et al.*, 1988). Likewise, the sedative effects were of short duration confirming the findings of others (Allonen *et al.*, 1981; Dundee *et al.*, 1984; Kanto, 1985; Kassai *et al.*, 1988; Klotz & Ziegler, 1982; Persson *et al.*, 1988; Reves *et al.*, 1985). Hence the pharmacokinetic and pharmacodynamic measurements both suggest that the dosage of midazolam needs no adjustment when it is administered to patients receiving treatment with cyclosporin A.

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Furthermore, a single intravenous dose of midazolam appeared to be without effect on the disposition of cyclosporin A; the concentration of the immunodepressant declined during a dosing interval with a mean apparent $t_{1/2}$ of 4.4 h, a value which is at the lower end of the normal range (Ptachcinski *et al.*, 1986; Rodighiero, 1989).

In conclusion, our combined *in vitro/in vivo* approach to the evaluation of the potential of cyclosporin A to interfere with the metabolism of co-administered drugs emphasizes the caution required when extrapolating experimental data to the clinical setting. Although *in vitro* experiments did indicate that cyclosporin A is a relatively potent inhibitor of drug metabolism, such effects could not be substantiated in cyclosporin treated patients when midazolam was used as a substrate.

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