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Diltiazem increases blood concentrations of cyclized cyclosporine metabolites resulting in different cyclosporine metabolite patterns in stable male and female renal allograft recipients

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1 Six male and six female stable renal allograft recipients under cyclosporine immunosuppression and without concomitant therapy with drugs known either to induce or inhibit CYP3A enzymes were included in the study and received 180 mg day^{-1} diltiazem for 1 week in a two-period cross-over fashion. Cyclosporine (352 ± 56 mg day^{-1}) was given in two daily oral doses. The daily doses were not changed during the study. Blood samples were collected for 12 h after receiving cyclosporine alone and after receiving diltiazem in addition for 1 week. Cyclosporine and nine of its metabolites were quantified using h.p.l.c.

2 Co-administration of diltiazem caused a 1.6 fold increase of the AUC(0,12 h) of cyclosporine and a 1.7 fold increase of the AUC(0, 12 h) of its metabolites. Analysis of the metabolite patterns showed an over-proportional increase of the AUC(0, 12 h) of the cyclized metabolites AM1c (2.6 fold) and AM1c9 (2.2 fold). The AUC(0, 12 h) values of cyclosporine and the hydroxylated metabolites increased less than two fold.

3 Differences of the AUC(0, 12 h) values of cyclosporine with and without diltiazem were significantly higher in female than in male patients ($P < 0.02$). The differences in the AUC(0, 12 h) values of the metabolites, especially AM1c, tended to be higher in female patients as well.

4 It is concluded that coadministration of diltiazem not only increases the blood concentration of cyclosporine but also those of its metabolites, leads to a shift of the metabolite pattern towards cyclized metabolites, and that the pharmacokinetic changes under diltiazem administration are more prominent in female than in male patients.

Keywords diltiazem cyclosporine metabolism pharmacokinetics renal allograft recipients drug interactions

Introduction

Today the undecapeptide cyclosporine (Sandimmun®, Sandoz, Basle, Switzerland) is the major immunosuppressant after kidney transplantation. Hypertension is a frequent complication of patients receiving kidney transplants and cyclosporine [1]. Co-administration of the antihypertensive calcium channel blocker diltiazem has several advantages. It reduces the incidence of both graft failure and graft rejection episodes [2–4]; several mechanisms have been described such as protection of the renal arterioles against cyclosporine-induced thicken-

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ing of the vessel walls [5] and reduction of ischaemic cell damage and simultaneously improved blood circulation through the kidney by lowering renal vascular resistance [6]. Furthermore, diltiazem reduces the cyclosporine-induced hypersensitivity to angiotensin II. Benzothiazepines such as diltiazem and clentiazem but not calcium channel blockers of the phenylalkylamine or dihydropyridine type were able to prolong heart graft survival in rats [7]. *In vitro* diltiazem exerts immunosuppressive activity by inhibition of lymphocyte proliferation [8]. Due to its highly variable pharmacokinetics and its blood concentration associated toxic side effects, cyclosporine doses have to be adjusted on the basis of its trough blood concentrations. Coadministration of diltiazem and cyclosporine leads to a rise in cyclosporine blood concentrations which requires dose reduction of up to 48% [9–11]. Like diltiazem [12], cyclosporine is metabolised by enzymes of the cytochrome P450 (CYP) 3A subfamily in the liver [13, 14] resulting in more than 30 metabolites [15]. *In vitro* diltiazem proved to be an effective competitive inhibitor of cyclosporine metabolism [12, 16]. Therefore, the increased bioavailability of cyclosporine in combination with diltiazem could be explained by a competitive interaction of diltiazem with the cyclosporine metabolism in the liver. To date, several studies have investigated the pharmacokinetic interaction of diltiazem and cyclosporine [17–20], all of which had at least one of the following major drawbacks: patients taking other drugs known to be CYP3A inhibitors have not been excluded and/or only cyclosporine was measured. A few studies included measurement of single first generation metabolites, that means metabolites which were changed in one position, but reported only trough blood concentrations. Meanwhile studies have shown that especially second generation metabolites which are generated by further metabolism of first generation metabolites, are particularly sensitive to changes of liver function [15]. In this study only stable renal graft recipients under cyclosporine immunosuppression without concomitant therapy with other CYP3A substrates were included. Cyclosporine metabolite patterns including first and second generation metabolites were analysed using h.p.l.c.

Methods

Patients

Twelve stable renal allograft recipients (six males, six females) were enrolled in this two period cross-over study. The demographic data are shown in Table 1. Inclusion criteria were a stable physical condition and transplant function, a minimum of 3 months after transplantation (mean $\pm$ s.d., 12 ± 2.3 months), an age between 18 and 65 years at the beginning of the study, a bodyweight between 50 and 90 kg, stable medication, no changes of cyclosporine dosage for at least 3 weeks, normal liver function and a written, informed consent of the patients after full explanation according to the guidelines of the Declaration of Helsinki and its revisions. Patients with infectious diseases, organ rejection, known liver diseases or contraindications to calcium channel blocker therapy were excluded as well as patients under therapy with drugs known to interact with cyclosporine metabolism [16] such as high dosage corticosteroids, other calcium channel blockers, macrolide antibiotics, antiepileptics, oral antimycotics, contraceptives, and cimetidine. Patients were treated with 176 ± 28 mg cyclosporine given twice daily. After randomization in a cross-over fashion, six patients received additionally to cyclosporine 90 mg diltiazem twice daily for 1 week while the other six patients continued to receive cyclosporine. After 1 week diltiazem was discontinued and the other six patients received 90 mg diltiazem twice daily during the second week of the study. Cyclosporine pharmacokinetics were assessed at the end of the first and the second week. At these instances, 12 h urine was collected. During the study period, cyclosporine and diltiazem doses remained unchanged. None of the patients reported any unwanted effects, had to be excluded for other reasons or withdrew from the study by own will. The study protocol was approved by the ethics committee of the Medizinische Hochschule Hannover, Hannover, Germany.

Table 1 Demographic data of the patients included in the study (mean $\pm$ s.d.)

	Male	*Female*	$< P$
Age (years)	43 ± 3.8	49 ± 3.9	NS
Weight (kg)	82 ± 4.1	61 ± 3.2	0.01
Body mass index (kg m^{-2})	25.5 ± 1.2	23.3 ± 1.1	NS
Cyclosporine dose (mg day^{-1})	363 ± 16	344 ± 14	NS
Cyclosporine dose (mg kg^{-1} day^{-1})	4.4 ± 0.2	5.4 ± 0.2	NS

Methods

At the end of the first and the second week, blood samples were taken before and 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h after the morning cyclosporine dose. Cyclosporine and its metabolites AM1, AM9, AM1c, AM19, AM1c9, AM14N, AM1A, AM4N, and AM4N9 were measured by h.p.l.c. as previously described by Christians *et al.* [21]. In brief, cyclosporin D was added to the blood samples as internal standard. The samples were extracted by solid-liquid extraction using C_{18} reversed phase glass extraction columns. Cyclosporine and its metabolites were separated on a 250×4 mm analytical h.p.l.c. column filled with C_{18} material of 3 μm particle size. A concave acetonitrile/water pH 3 gradient was run, the column temperature was set to 75° C and the chromatograms were recorded at a u.v.-detector wavelength of 205 nm. For identification and calibration, cyclosporine metabolites isolated from bile and structurally identified by mass spectrometry and one- and two-dimensional n.m.r.-spectroscopy as previously described [22] were used. The Hawk's Cay nomenclature of the cyclosporine metabolites [23] was

used. Quality assessment during the study gave the following specification for the quantification of cyclosporine: The lower limit of quantification was 25 μg l^{-1}, the range of reliable response was 25–5000 μg l^{-1}, the calibration curve was linear with $y = 0.97 + 28$ ($r = 0.99$), the recovery was $86 \pm 20\%$ for 50 ng samples ($n = 10$) and $71 \pm 24\%$ for 500 ng samples ($n = 10$). Intra-assay accuracy was -4.9% for 25 ng samples, -0.5% for 250 ng samples, $+3.2\%$ for 500 ng samples and the interassay variation during the study 14.1% for 25 ng samples ($n = 25$) and 6.8% for 250 ng samples ($n = 25$). Pharmacokinetic parameters were calculated using an open two- compartment model using the algorithm implemented in the Topfit pharmacokinetic program (Version 2.0, Gustav-Fischer Verlag, Stuttgart, Germany). Peak blood drug concentrations (C_{max}), time to peak concentration (t_{max}), AUC(0, 12 h) and terminal half-life ($t_{1/2,z}$) were calculated after curve fitting using a biexponential distribution function with first-order input. During the study, patients were checked the day before the pharmacokinetic profiles were taken including physical examination and electrocardiographic controls. Liver function (serum bilirubin concentration, activities of alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, glutamate dehydrogenase, and cholinesterase), the concentrations of electrolytes, glucose, urea, creatinine, triglycerides and cholesterol in serum, the total serum protein concentration, creatinine clearance as well as the concentrations of protein, electrolytes, glucose, ketones, haemoglobin, bilirubin, urobilinogen, nitrogen concentrations in urine, urine osmolarity, urine sediment, red and white blood cell count, and blood coagulation status were determined in the Institut für Klinische Chemie of the Medizinische Hochschule Hannover using standard techniques.

For distribution statistics and comparison of data, the SAS program (Version 6.04, SAS Institute, Cary, NC, USA) was used. Data were copared using the two-period-crossover analysis as described by Hecker [24]. This test is based on the comparison of differences of the observed values (μ) between the two study periods (A,B) in the two groups (diltiazem in period 1 (μ_{1X}) or 2 (μ_{2X})). The following effects were tested:

$$\text{treatment } (\alpha): \quad \alpha = 1/4\ ((\mu_{1A} - \mu_{1B)} - (\mu_{2A} - \mu_{2b}))$$

$$\text{period } (\beta): \quad \beta = 1/4\ ((\mu_{1A} - \mu_{1B)} + (\mu_{2A} - \mu_{2b}))$$

$$\text{carry-over } (\tau): \quad \tau = 1/4\ ((\mu_{1A} - \mu_{1B)} - (\mu_{2A} - \mu_{2b})).$$

The differences were compared using the two-sided *t*-test. Distribution statistics was calculated using the SAS Univariate procedure.

Results

The two-period crossover analysis failed to detect carry-over or period effects. Metabolites AM14N, and AM4N were not detected in blood. After diltiazem, the concentrations of cyclosporine and its metabolites were higher than without diltiazem (Figure 1). The AUC(0, 12 h) of

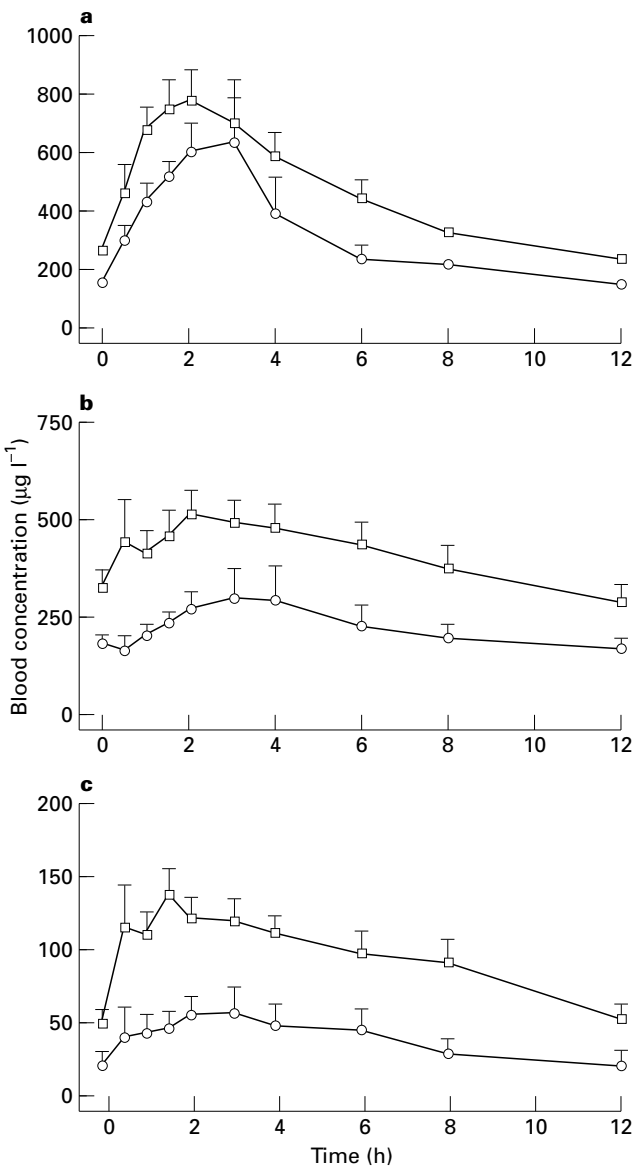

Figure 1 Mean (±s.e. mean, $n = 12$) blood concentrations of cyclosporine (a) and its metabolites AM1 (b) and AM1c (c) with (□) and without (○) coadministration of diltiazem.

cyclosporine was 1.6 fold and that of the sum of all metabolites was 1.7 fold greater with than without diltiazem. Diltiazem modified the cyclosporine metabolite AUC(0, 12 h) values with an over-proportional increase of the metabolites cyclised at amino acid 1 such as AM1c (2.6 fold) and AM1c9 (2.2 fold) as well as the carboxylated cyclosporine metabolite AM1A (2.4 fold). The increase of the AUC(0, 12 h) values of the hydroxylated and demethylated metabolites was in general less than 2-fold (Table 2). The C_{max} of cyclosporine and that of the cyclised metabolite AM1c were significantly higher under diltiazem (Table 2). Diltiazem had no significant effect on the time-to-peak concentration (t_{max}) or on the estimated terminal elimination half-lives ($t_{1/2,z}$) of cyclosporine and its metabolites (Table 2). The changes in cyclosporine metabolite patterns in blood were reflected in 12 h urine. The concentrations of cyclosporine and almost all its metabolites except the demethylated metabolites and the carboxylated metabolite AM1A were increased

Table 2 Pharmacokinetic parameters of cyclosporine and its metabolites with and without co-administration of diltiazem (mean ± s.e. mean, $n = 12$)

	AUC ($\mu g\ l^{-1}\ h$)		C_{max} ($\mu g\ l^{-1}$)		t_{max} (*h*)		*Approximate* $t_{1/2,z}$ (*h*)	
	−diltiazem	*+diltiazem*	*−diltiazem*	*+diltiazem*	*−diltiazem*	*+diltiazem*	*−diltiazem*	*+diltiazem*
Cs	3489 ± 391	5503 ± 560**	737 ± 123	957 ± 104**	1.8 ± 0.2	2.5 ± 0.4	7.3 ± 1.9	9.6 ± 4.4
AM1	2517 ± 384	4956 ± 698**	304 ± 45	604 ± 99	2.0 ± 0.3	2.8 ± 0.4	13.8 ± 2.5	11.3 ± 1.6
AM9	1893 ± 246	2280 ± 306	309 ± 47	306 ± 44	2.5 ± 0.2	3.4 ± 0.6	8.2 ± 1.8	7.3 ± 1.1
AM1c	421 ± 100	1103 ± 141***	88 ± 20	164 ± 20**	1.6 ± 0.5	2.3 ± 0.5	7.4 ± 2.3	9.1 ± 2.5
AM19	712 ± 106	1278 ± 493	100 ± 14	176 ± 40	2.7 ± 0.4	2.8 ± 0.8	13.5 ± 3.1	15.8 ± 4
AM1c9	172 ± 39	379 ± 91	42 ± 8	75 ± 14	1.5 ± 0.4	3.2 ± 1	3.4 ± 0.9	7 ± 4
AM4N9	165 ± 46	229 ± 96	42 ± 11	50 ± 18	4.4 ± 1.2	2.4 ± 0.8	19 ± 16	14 ± 8
AM1A	81 ± 19	192 ± 95	23 ± 5	46 ± 16	2.4 ± 0.9	1.2 ± 0.4	6 ± 2.7	16.4 ± 12.7

Statistics: Data sets were compared using two-period cross over analysis in combination with two-sided *t*-test [24]: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Abbreviation: Cs: cyclosporine.

under concomitant diltiazem administration (Table 3). The increase in metabolite concentrations in urine under diltiazem therapy was most prominent for the metabolites AM1 (2.3 fold), AM1c (3.2 fold) and AM1c9 (4.6 fold).

Although the AUC(0, 12 h) values in male and female patients without diltiazem therapy were not different, the increase in AUC(0, 12) values of cyclosporine and its metabolites was more pronounced in female than in male patients (Table 4). The difference of the AUC(0, 12 h) values of cyclosporine with and without diltiazem was significantly higher in female than in male patients ($P < 0.02$). The differences of the AUC(0, 12 h) values of the metabolites, especially AM1c, tended to be higher in female patients as well (Table 4).

Discussion

Several studies reported an increase in cyclosporine blood concentrations when the patients received diltiazem [17–20]. Detailed analysis of the cyclosporine metabolite pattern in the present study showed that the combination of diltiazem with cyclosporine led not only to an increase of the AUC of cyclosporine but of those of several metabolites as well. The metabolite pattern was shifted with an over-proportional increase in cyclosporine metabolites cyclized at amino acid 1. Since cyclosporine and diltiazem are metabolized by the same CYP enzymes in the liver [12, 13] and diltiazem is a known inhibitor of the cyclosporine *in vitro* metabolism, it can be assumed that diltiazem inhibited cyclosporine metabolism in the liver. This would explain the higher AUC values of cyclosporine in combination with diltiazem but not the parallel increase of the AUC values of its metabolites.

Table 3 Cyclosporine metabolite patterns (measured by h.p.l.c.) in 12 h-urine with and without diltiazem co-administration (mean ± s.e. mean, $n = 12$)

	Without diltiazem ($\mu g\ l^{-1}$)	*With diltiazem* ($\mu g\ l^{-1}$)	P
Cs	280 ± 66	502 ± 207	*
AM1	843 ± 318	1954 ± 662	***
AM9	307 ± 117	559 ± 237	*
AM1c	433 ± 176	1387 ± 368	***
AM19	216 ± 15	917 ± 928	*
AM1c9	322 ± 200	1492 ± 1163	*
AM14N	77 ± 60	98 ± 40	NS
AM4N	98 ± 25	125 ± 176	NS
AM4N9	99 ± 53	88 ± 76	NS
AM1A	63 ± 48	93 ± 58	NS

Statistics: comparison by paired *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Abbreviation: Cs: cyclosporine.

Several studies in which pharmacokinetics after oral and intravenous administration of cyclosporine were compared, have raised evidence for pre-hepatic metabolism of cyclosporine. *In vitro*, small intestinal microsomes metabolised cyclosporine to its major metabolites [25, 26] and after installation of cyclosporine in the small intestine of an anhepatic patient, cyclosporine metabolites were found in the portal vein [27]. Kolars *et al.* [26] showed that the metabolites formed in the small intestinal mucosa also reenter the lumen and this way, metabolism in the small intestine may affect absorption of CYP3A substrates. In a clinical study, Hebert *et al.* [28] showed that CYP3A enzymes in the small intestine might be more important for oral bioavailability than those in the liver. The effects of diltiazem on the pharmacokinetics of cyclosporine found in this study might be explained by inhibition of the small intestinal metabolism of cyclosporine. Less cyclosporine is metabolised in the mucosal cells and excreted back into the intestinal lumen as metabolites. Thus, higher concentration of the unchanged compound reach the liver. Under the assumption that inhibition of CYP3A enzymes in the liver is quantitatively less complete than in the small intestine, due to the higher substrate concentrations, more metabolites are formed resulting in higher AUC values of cyclosporine and its metabolites. An alternative explanation of the increased AUC values of the cyclosporine metabolites is an interference of diltiazem with the elimination of cyclosporine metabolites from the hepatocyte into bile.

There is strong evidence that besides the main

Table 4 AUC(0, 12 h) in male ($n=6$) and female ($n=6$) patients. All data are given as mean ± s.d.

	Difference					
	Female patients		*Male patients*		*(With–without diltiazem)*	
	AUC (0, 12 h) (μg l^{-1} h)		*AUC (0, 12 h) (μg l^{-1} h)*		*AUC (0, 12 h) (μg l^{-1} h)*	
	−diltiazem	*+diltiazem*	*−diltiazem*	*+diltiazem*	*Female*	*Male*
Cs	3402 ± 443	6749 ± 1818**	3576 ± 1954	4256 ± 1116	3347 ± 1741	680 ± 1273*
AM1	2094 ± 333	5360 ± 3280*	3000 ± 1794	4550 ± 1305**	3326 ± 3024	1594 ± 977
AM9	1803 ± 512	2504 ± 1437	1981 ± 1148	2056 ± 539	700 ± 1379	74 ± 813
AM1c	247 ± 162	1190 ± 573**	595 ± 408	1015 ± 419*	943 ± 559	420 ± 313
AM19	629 ± 193	1840 ± 2334	795 ± 494	715 ± 447	1210 ± 2285	−79 ± 276
AM1c9	157 ± 142	541 ± 343	186 ± 137	215 ± 189	384 ± 387	28 ± 111
AM4N9	102 ± 143	298 ± 380	203 ± 135	159 ± 293	195 ± 430	−43 ± 268
AM1A	90 ± 81	293 ± 451	71 ± 53	90 ± 94	202 ± 475	18 ± 86

Statistics: 2-period crossover analysis in combination with one sided *t*-test [24]: *: $P<0.05$, **: $P<0.01$. Abbreviation: Cs: cyclosporine.

metabolic pathway of cyclosporine an alternative metabolic pathway exists. CYP3A enzymes are responsible for hydroxylation and demethylation of cyclosporine [15]. It was assumed that cyclization of amino acid 1 with formation of a furan ring is a non-enzymatic reaction [29, 30]. Meanwhile, several studies showed that cyclization is inducible by several CYP1A inducers such as methylcholanthrene and β-naphthoflavone [31, 32]. Prueksaritanont *et al.* [33] reported that CYP2C6 might be involved in the cyclization of cyclosporine. The existence of an alternative cyclosporine metabolic pathway was used to explain toxicity in patients with low CYP3A concentrations in the liver [34] and *in vitro* the cyclized metabolite AM1c9 increases endothelin production by mesangial cells [35]. The modification of the cyclosporine metabolite pattern by diltiazem can be explained by such an alternative cyclosporine metabolic pathway. While diltiazem partially inhibits the CYP3A-catalysed metabolism in the liver, the CYP enzymes responsible for cyclization are not affected. This results in a relatively higher formation rate of cyclized metabolites compared with hydroxylated and/or demethylated metabolites.

Due to the limited time of 12 h between two cyclosporine doses covering in several cases less than the terminal half-life, the terminal half-lives had to be calculated by extrapolation of the fitted curves and thus represent only approximate values.

This study showed striking differences in the modification of cyclosporine metabolite patterns by co-administration of diltiazem between male and female patients suggesting gender-dependent differences in the metabolism of cyclosporine. That the obvious trend that metabolite concentration differences in female patients are greater than in male patients did not reach statistical significance, might be explained by the small numbers of patients included in the present study. Hunt *et al.* [36] found that the concentration of CYP3A enzymes is 24% higher in female than in male livers and nothing is known about a gender-dependent distribution of CYP3A enzymes in the small intestine. It is important to note that female patients under contraceptive therapy have been excluded from this study. The data available to date are not sufficient to explain these differences.

Analysis of the cyclosporine metabolite pattern showed that diltiazem increases the AUC values of cyclosporine and its metabolites and shifts the metabolite pattern resulting in relatively higher concentrations of cyclosproine metabolites cyclized at amino acid 1. These results can be explained by and support the impact of a significant influence of intestinal metabolism on cyclosporine bioavailability and the existence of an alternative cyclosporine metabolic pathway. The results of this study emphasize the need of a detailed analysis of the metabolite patterns in drug interaction studies with cyclosporine. This aspect is especially important when drugs are combined with cyclosporine only to reduce the cyclosporine doses required to maintain effective blood concentrations [37, 38]. An important result which requires further evaluation with a larger number of patients is the gender-dependent changes of the cyclosporine metabolite patterns in blood when diltiazem is co-administered.

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