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Metabolism of the macrolide immunosuppressant, tacrolimus, by the pig gut mucosa in the Ussing chamber

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1 The macrolide tacrolimus (FK506), used as an immunosuppressant, is a cytochrome P450 (CYP) 3A substrate in the liver. The metabolism of tacrolimus and the transport of its metabolites in the pig gut was studied in the Ussing chamber. Tacrolimus and its metabolites were quantified by h.p.l.c./mass spectrometry.

2 In the Ussing chamber, demethyl, didemethyl, hydroxy and hydroxy-demethyl tacrolimus were generated. Their formation was concentration- and time-dependent. The metabolite pattern was not different from that after incubation of tacrolimus with human small intestinal microsomes.

3 The metabolite formation was highest in the duodenum and declined in the order duodenum > jejunum > ileum > colon > stomach.

4 Since tacrolimus metabolism was inhibited by the specific CYP3A inhibitors, troleandomycin and ketoconazole, we concluded that these enzymes are involved in intestinal metabolism of tacrolimus.

5 Tacrolimus metabolites re-entered the mucosa chamber (>90%) and passed through the small intestinal preparation into the serosa chamber.

6 It is concluded that tacrolimus is metabolized in the intestine, that the metabolites are able to re-enter the gut lumen and also enter into the portal vein and that small intestinal metabolism and transport is at least in part responsible for the low oral bioavailability of tacrolimus

Keywords: Tacrolimus; drug metabolism; gut mucosa; pre-hepatic metabolism; intestinal mucosa; Ussing chamber

Introduction

Tacrolimus (FK506, Fujisawa, Osaka, Japan) is used as an immunosuppressant in transplantation medicine (European FK506 Multicentre Liver Study Group, 1994; The U.S. Multicenter FK 506 Study Group, 1994) and is under investigation in the therapy of immune diseases (Peters *et al.*, 1993). It has a 23-member macrolide lactone structure ($C_{44}H_{69}NO_{12}$, molecular weight: 803.5 Da) and is isolated from *Streptomyces tsukubaensis*. Tacrolimus has a low and highly variable oral bioavailability of approximately 20% ranging between 5-67% (Wallemacq & Reding, 1993). More than 10 phase I metabolites have been structurally identified (Iwasaki *et al.*, 1993, 1995; Schüler *et al.*, 1993; Lhoëst *et al.*, 1994). In the rat and human liver, tacrolimus undergoes *O*-demethylation at C(13), C(15) and C(31) and/or hydroxylation at C(12) and C(19). CYP3A enzymes are responsible for demethylation in the human and rat liver (Sattler *et al.*, 1992; Vincent *et al.*, 1992; Shiraga *et al.*, 1993). Demethylation of tacrolimus is specific for CYP3A enzymes (Shiraga *et al.*, 1993; Karanam *et al.*, 1994). Mainly *O*-demethylated metabolites are present in blood of liver and kidney graft recipients (Christians *et al.*, 1992a; Gonschior *et al.*, 1994).

Cytochrome P450 (CYP) 3A enzymes account for 70% of the total concentration of CYP enzymes in the small intestine and about 20% in the liver (Watkins *et al.*, 1987). It has been hypothesized that metabolism by CYP3A enzymes in the small intestine is, at least in part, responsible for the low and variable oral bioavailabilities of most drugs that are substrates of these enzymes (Watkins, 1990; Kolars *et al.*, 1992). Furthermore, there is strong evidence that, in the case of cyclosporin, a significant fraction of the metabolites formed in the gut mucosa re-enters the gut lumen (Kolars *et al.*, 1992). Tacrolimus is a CYP 3A substrate with a low and variable oral bioavailability which might be caused in addition to first pass metabolism in the liver, as in the case of cyclosporin, by significant metabolism in the small intestine. The models used to study drug metabolism in the gut comprise isolated mucosal microsomes and a rat isolated intestinal loop. Both methods have their limitations. Isolated microsomes give no information as to whether the metabolites enter the gut lumen or the portal vein and the isolated gut loop is an unstable, quickly fading system. In the present study an Ussing chamber (Ussing, 1949; Ussing & Zerahn, 1951) was used to study small intestinal metabolism and transport of tacrolimus.

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Methods

Preparation of Ussing chamber

Samples of different parts of the pig gastrointestinal tract were obtained from the local slaughterhouse. The samples were rinsed with 1.32% NaCl and immediately stored in ice-cold Krebs-Henseleit buffer (pH 7.4) saturated with carbogen (95% O_2, 5% CO_2). The lamina mucosa was stripped from the lamina muscularis and as far as possible from the lamina serosa. The mucosal patches were mounted in the Ussing chamber. The area on each side of the chamber was 1.13 cm^2. Both chambers were filled with 10 ml Krebs-Henseleit buffer (pH 7.4) the osmolality of which was adjusted to 300 mosm l^{-1} with mannitol. The buffer was circulated in the closed system at 20 ml min^{-1}. Carbogen bubbles and warming of the ascending loop were used as the driving force. The chambers were kept at 37°C by water jackets. The potential difference across the mucosal preparation was recorded to monitor tissue viability (AC Mikroclamp, Aachen, Germany). Under the conditions described, the mucosa stabilized within 30 min. The conductivity was constantly about 20 mS cm^{-2}. The system was stable for at least 8 h. Tacrolimus was added 45 min after the setting up of the Ussing chamber.

Metabolism of tacrolimus in the Ussing chamber

If not stated otherwise, tacrolimus, dissolved in acetonitrile/water (pH 3) 70/30 v/v, was added to the mucosal chamber, resulting in a final concentration of 10 μmol l^{-1} and was incubated in the Ussing chamber for 4 h. All experiments were performed in triplicate. To study the time-dependent formation of tacrolimus metabolites, metabolism was stopped and samples taken after 0 h (controls), 0.5, 1, 2, 3 and 4 h of incubation. The specific CYP3A inhibitors, troleandomycin and ketoconazole (Chang *et al.*, 1994; Yun *et al.*, 1995) as well as the CYP3A substrate verapamil (Kroemer *et al.*, 1993) were added to the mucosal side to evaluate potential drug interactions with the small intestinal metabolism of tacrolimus. The resulting final concentrations were 0, 5, 10, 25, 50 and 100 μmol l^{-1}. These drugs were pre-incubated in the Ussing chamber for 1 h. Tacrolimus and its metabolites were quantified after 4 h incubation.

Metabolism of tacrolimus by small intestinal microsomes

To confirm that pig gut mucosa constitutes a valid model, microsomes from the proximal small intestine of different species (rat, $n=5$; dog, $n=3$; pig, $n=10$; human, $n=14$) were isolated as previously described (Porteous *et al.*, 1979; Pinkus, 1981). Tacrolimus (final concentration 7.5 μmol l^{-1}) was incubated with 1 ml microsomes in phosphate buffer pH 7.4 (1.5 g protein l^{-1}) and 0.5 ml of an NADPH generating system. The NADPH generating system consisted of 2 mmol l^{-1} EDTA, 10 mmol l^{-1} $MgCl_2$, 0.84 mmol l^{-1} NADP, 18 mmol l^{-1} isocitric acid and 667 u l^{-1} isocitrate dehydrogenase (all from Sigma, Deisenhofen, Germany). After 20 min incubation the reaction was stopped by addition of 0.5 ml acetonitrile. The samples were extracted and the metabolites formed quantified by h.p.l.c. as described by Sattler *et al.* (1992).

Quantification of tacrolimus and its metabolites by h.p.l.c./m.s.

Tacrolimus and its metabolites were quantified in the Ussing chamber by a modification of a previously published h.p.l.c./m.s. method (Christians *et al.*, 1992a). In brief, to 1 ml buffer solution from the mucosa of serosa compartment of the Ussing chamber, the internal standard 32-O-acetyl tacrolimus (10 μl of 1 mmol l^{-1}) and 2 ml methanol/saturated zinc sulphate in water 50/50 v/v for protein precipitation were added. After centrifugation at 2500 *g*, the supernatants were loaded on glass extraction columns which have previously been washed with 3 ml acetonitrile and sulphuric acid (pH 3). Samples were washed on the columns with 3 ml sulphuric acid (pH 3) and 0.5 ml hexane. Tacrolimus and its metabolites were eluted by 1.5 ml dichloromethane. Dichloromethane was evaporated and the sample reconstituted in 300 μl mobile phase (methanol/water 90/10 v/v). One hundred and fifty μl was injected into the h.p.l.c. system. Tacrolimus and its metabolites were isocratically eluted from the analytical column. The flow was set to 0.4 ml min^{-1} and the column temperature to 40°C. Chemical ionization with methane was used and negatively charged ions were recorded. The mass spectrometer was run in the single ion monitoring mode and was focused on the following ions: tacrolimus (803.5 amu), 32-O-acetyl tacrolimus (internal standard, 845.5 amu), demethyl tacrolimus (789.5 amu), didemethyl tacrolimus (775.5 amu), demethylhydroxy tacrolimus (805.5 amu), hydroxy tacrolimus (819.5 amu), and dihydroxy tacrolimus (835.5 amu). The metabolites were identified by their retention time and the m/z of their molecular ion. The metabolites had a retention time 0.7 min shorter and the internal standard 0.5 min longer than that of tacrolimus. Since the concentration was far beyond the linear range (0.25 pmol l^{-1}–0.25 nmol l^{-1}), tacrolimus could not be quantified in the mucosal chamber and a signal was detected in the chromatograms of the other ions. This did not influence quantification of the metabolites or the internal standard since those were separated from tacrolimus on the analytical column. H.p.l.c./m.s. allowed quantification of several metabolites with different molecular weights. It was possible to determine in how many positions a metabolite was demethylated and/or hydroxylated but not in which site(s) of the molecule (Christians *et al.*, 1992a). Thus, individual metabolites such as the O-demethylated metabolites could not be distinguished and only the concentration of demethyl tacrolimus (i.e. the sum of all demethylated metabolites) could be given when the tacrolimus metabolite concentrations were quantified by h.p.l.c./m.s.

Materials

For quantification of tacrolimus and its metabolites, an h.p.l.c. system 1090A connected by a particle beam interface to a 5989A single stage quadrupole mass spectrometer was used. Data were processed using MS-ChemStation Software, Version C.01.05 (all Hewlett Packard, Waldbronn, Germany). Tacrolimus and its metabolites were extracted in glass extraction columns (Krannich, Göttingen, Germany) filled with 25–40 μm C_{18} material (LiChroprep, Merck, Darmstadt, Germany) and were analyzed on a Spherical C_{18} analytical column (3.9 x 150 mm, 5 μm particle size, Waters Millipore, Milford, U.S.A.). All solvents for extraction and h.p.l.c./mass spectrometry (h.p.l.c./m.s.) analysis were of h.p.l.c. grade and purchased from Merck (Darmstadt, Germany). Tacrolimus was a gift from Fujisawa (Osaka, Japan). The tacrolimus metabolites 13-O-demethyl, 15-O-demethyl, 31-O-demethyl, 12-hydroxy and 13,15-O-didemethyl tacrolimus were isolated after incubation of tacrolimus with human liver microsomes and their structures were verified by mass spectrometry and n.m.r. spectroscopy as previously described (Christians *et al.*, 1991; Schüler *et al.*, 1993). They were used for identification and calibration of the metabolites. The internal standard 32-O-acetyl tacrolimus was synthesized, isolated and structurally identified by n.m.r. spectroscopy according to Christians *et al.* (1992a). Ketoconazole was obtained from Janssen (Neuss, Germany). Troleandomycin and verapamil were purchased from Sigma (Deisenhofen, Germany).

Results

Comparison of tacrolimus metabolism with proximal small intestinal microsomes of different species showed that pig was not significantly different from man in respect to metabolite formation rate and the final concentration of 13-O-demethyl tacrolimus (Table 1). Comparison of the metabolite patterns by mass spectrometry confirmed that those generated by pig and human small intestinal microsomes were similar. After

Table 1 Metabolism of tacrolimus by proximal small intestinal microsomes of different species

Species	*Formation rate* (pmol $min^{-1}mg^{-1}$ protein)	*Concentration* (μmol l^{-1})
	of 13-O-demethyl tacrolimus	
Rat ($n=5$)	14.3±4.8	0.3±0.7
Dog ($n=3$)	40.1±24.1	0.9±0.2
Pig ($n=10$)	60.3±26.4	1.5±0.3
Man ($n=14$)	54.2±29.2	1.8±0.4

Tacrolimus in a final concentration of 7.5 μmol l^{-1} was incubated with isolated microsomes of the small intestine (1.5 g protein l^{-1}) for 20 min. The concentrations of 13-O-demethyl tacrolimus were determined with h.p.l.c. by u.v. detection according to Sattler *et al.* (1992). An analysis of variance (GLM procedure, SAS, version 6.05, SAS Institute, Cary, NC, U.S.A.) showed significant differences ($P<0.03$). Data are given as mean±standard deviation.

incubation of tacrolimus with pig duodenal mucosa in the Ussing chamber, demethyl, didemethyl, hydroxy, and hydroxy-demethyl tacrolimus were formed. Dihydroxy tacrolimus was not found. The concentration of hydroxy-demethyl tacrolimus was only above the detection limit of the h.p.l.c./m.s. assay when high tacrolimus concentrations and long incubation times were used. Therefore, this metabolite was not included in the figures. The formation rates were 276.5 ± 23.2 pmol l^{-1} demethyl tacrolimus min^{-1} cm^2, 98.5 ± 9.6 pmol l^{-1} hydroxy tacrolimus min^{-1} cm^2, and 159.3 ± 12.5 pmol l^{-1} didemethyl tacrolimus min^{-1} cm^2. After 4 h incubation, 2% of the tacrolimus concentration added to the mucosa-compartment was detected in the serosa-compartment of the Ussing chamber (Figure 1). Metabolites were mainly found in the mucosal-compartment, but small quantities close to the detection limit of the h.p.l.c./m.s. method were present in the serosal-compartment (Figure 1). No metabolites were detected on either side when tacrolimus was added to the serosal- instead of the mucosal-compartment. Tacrolimus metabolism in the Ussing chamber was concentration- and time-dependent (Figures 2 and 3). Metabolite formation was different in mucosa from various sections of the gastrointestinal tract (Figure 4). The highest concentration of tacrolimus metabolites was detected in the duodenum. It decreased in the following order: duodenum > ileum > jejunum > colon. No metabolites were found in the stomach mucosa (Figure 4). Addition of the specific CYP3A inhibitors troleandomycin (Figure 5) and ketoconazole as well as the CYP3A substrate, verapamil, significantly inhibited demethyl tacrolimus formation. Troleandomycin, 100 μmol l^{-1} inhibited demethyl tacrolimus formation by 65% (half maximal inhibition concentration (IC_{50}): 82 μmol l^{-1}) and 100 μmol l^{-1} verapamil by 69% (IC_{50}: 91 μmol l^{-1}). The strongest inhibitor was ketoconazole with 80% inhibition of demethyl tacrolimus formation at 25 μmol l^{-1} (IC_{50}: 9 μmol l^{-1}).

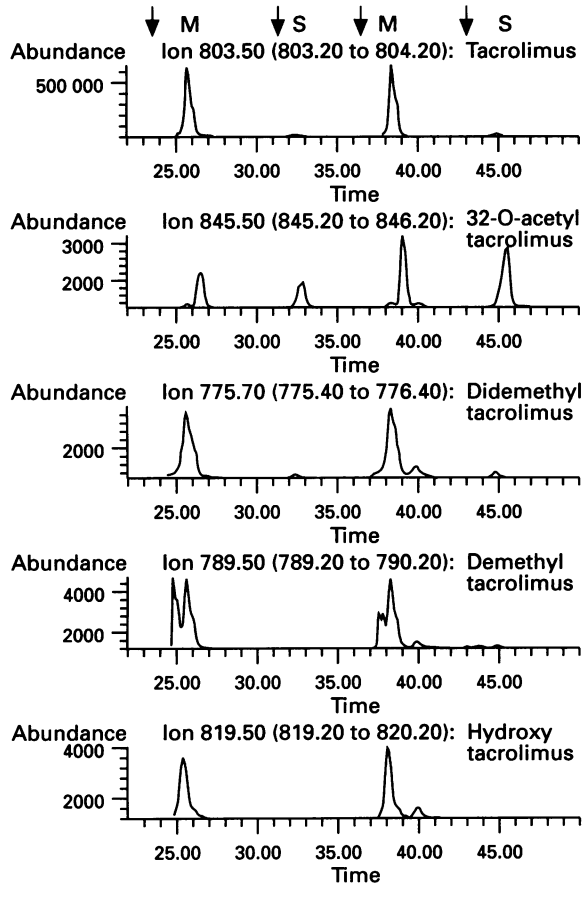

Figure 1 Metabolism of tacrolimus and distribution of the metabolites in the Ussing chamber. The tacrolimus metabolites were quantified by h.p.l.c./mass spectrometry (m.s.) after incubation of 10 μmol l^{-1} tacrolimus with a pig mucosa preparation in the Ussing chamber for 4 h. One ml of the perfusion buffer of the mucosa (M) or serosa (S) compartment was extracted by solid-liquid extraction. 32-O-acetyl tacrolimus was added as internal standard. H.p.l.c./m.s. conditions: eluent: methanol/water 90/10 v/v, C_{18}, 5 μm 3.9.150 mm analytical column, column temperature 40°C, flow: 0.4 ml min^{-1}, chemical ionization with methane, detection of negatively charged ions, single ion monitoring. Four representative ion chromatograms are shown. Arrows: top: injection into the h.p.l.c./m.s. system.

Discussion

Ussing chambers have been used to study intestinal transport of ions, monosaccharides, amino acids, fatty acids and drugs (Back & Rogers, 1987; Rogers *et al.*, 1987; Schröder *et al.*, 1991; v. Engelhardt *et al.*, 1993) as well as intestinal metabolism of xenobiotics (Rogers *et al.*, 1987; Tija *et al.*, 1991). Our

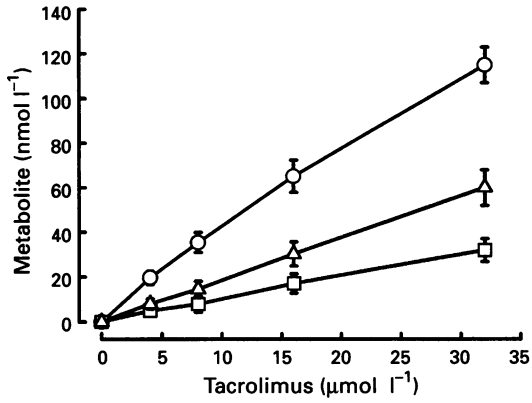

Figure 2 Concentration-dependent formation of demethyl (○), didemethyl (△) and hydroxy tacrolimus (□) by pig duodenal mucosa in the Ussing chamber. Tacrolimus was incubated for 4 h. The metabolites in the mucosal chamber were analysed by h.p.l.c./m.s. Each data point is the mean ± standard error ($n = 4$).

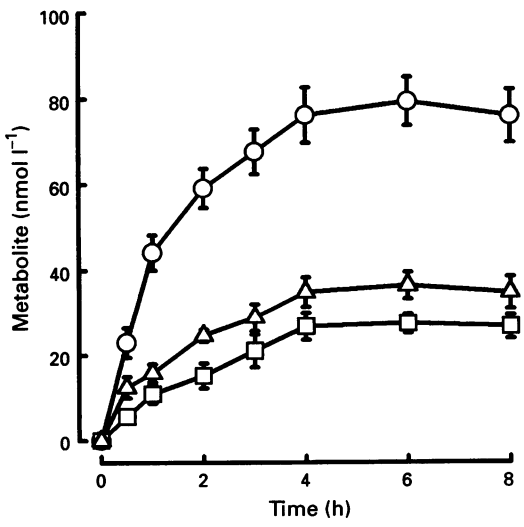

Figure 3 Time-dependent formation of demethyl (○), didemethyl (△) and hydroxy tacrolimus (□) by pig duodenal pig mucosa in the Ussing chamber: 20 μmol l^{-1} tacrolimus was incubated for 0–8 h. The metabolites in the mucosal-chamber were analysed by h.p.l.c./m.s. Each data point is the mean ± standard error ($n = 3$).

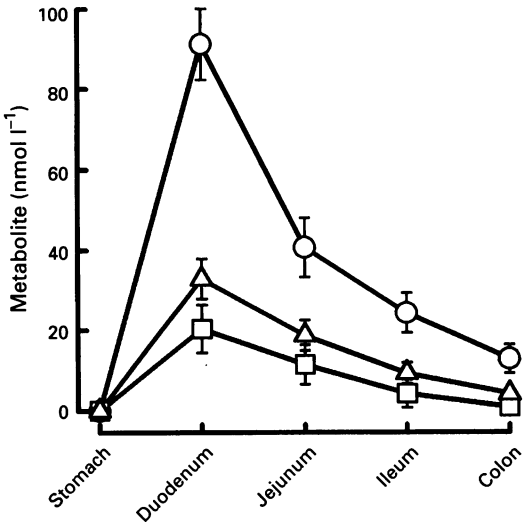

Figure 4 Formation of demethyl (○), didemethyl (△) and hydroxy tacrolimus (□) by mucosa preparations from different parts of the pig gut in the Ussing chamber: 10 μmol l^{-1} tacrolimus was incubated in the Ussing chamber for 4 h. The metabolites formed were quantified in the mucosa chamber by h.p.l.c./m.s. The metabolite concentrations were measured in the mucosa compartment of an Ussing chamber after 4 h incubation of tacrolimus (10 μmol l^{-1} final concentration added to the mucosal compartment) with mucosal preparations from different parts of the pig gut. Each data point is the mean ± standard error (n = 3).

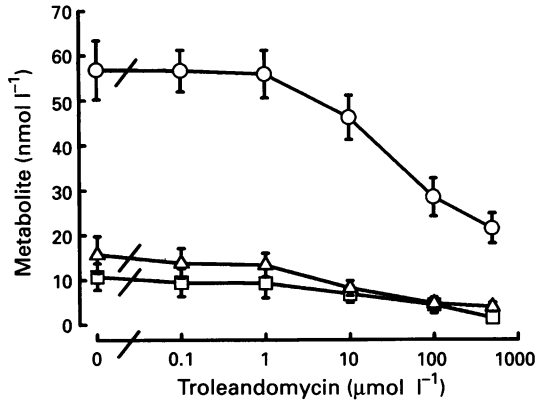

Figure 5 Inhibition of tacrolimus metabolism by the pig duodenal mucosa by the specific CYP3A inhibitor troleandomycin. Troleandomycin was added to the mucosal chamber and pre-incubated for 60 min. Then tacrolimus was added (final concentration 10 μmol l^{-1}) and incubated for 4 h. The concentrations of demethyl (○), didemethyl (△) and hydroxy tacrolimus (□) were quantified by h.p.l.c./m.s. Each data point is the mean ± standard error (n = 4).

study demonstrates that tacrolimus undergoes significant metabolism in the gut.

The rate of metabolism of tacrolimus is highest in the duodenum and jejunum, the part of the gastrointestinal tract where tacrolimus is absorbed. The site-dependent metabolism in the pig gastrointestinal tract is similar to the distribution of CYP3A described by Kolars *et al.* (1994) in man using immunoblot, Northern blot and polymerase chain reaction. The rather low transport of unchanged tacrolimus through the mucosal preparation from the mucosal- to the serosal-compartment of about 2% is in accordance with earlier observations with other drugs (Rogers *et al.*, 1987; Tija *et al.*, 1991). Restrictions apply especially when the quantitative results of this *in vitro* model are transferred to tacrolimus-treated patients. However, since tacrolimus metabolites were found on both sides of the mucosal preparation, it can be assumed that *in vivo* tacrolimus metabolites formed in the mucosal cells are able to either enter the portal vein or re-enter into the gut lumen. It remains to be evaluated whether the mechanism responsible for re-entrance of the tacrolimus metabolites into the gut lumen is passive diffusion or active transport. Inhibition of tacrolimus metabolism by the specific CYP3A inhibitors troleandomycin and ketoconazole proved that, as in the liver, these enzymes in the gut are also responsible for tacrolimus metabolism. Therefore, it is likely that all known CYP3A inhibitors and inducers will be able to modify the intestinal metabolism of tacrolimus. In a pharmacokinetic study of cyclosporin, Hebert *et al.* (1992) showed that induction of intestinal CYP3A by rifampicin reduced bioavailability more markedly than the induction of hepatic metabolism. Our study suggests that, in addition to the first pass metabolism in the liver, intestinal metabolism of tacrolimus in combination with re-entrance of the metabolites into the gut lumen is at least in part responsible for its low and perhaps its variable bioavailability. CYP3A inhibitors may increase and CYP3A inducers may reduce absorption of tacrolimus.

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