COMPARISON OF BIOAVAILABILITY AND METABOLISM WITH TWO COMMERCIAL FORMULATIONS OF CYCLOSPORINE A IN RATS

JENS KOEHLER, THOMAS KUEHNEL, FRIEDER KEES, KLAUS HOECHERL, AND HORST F. GROEBECKER

Department of Pharmacology and Clinical Pharmacology, University of Regensburg (J.K., F.K., K.H., H.F.G.); and Clinic of Head and Neck Surgery, University Hospital Regensburg (T.K.), Regensburg, Germany

(Received November 7, 2001; accepted February 26, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The bioavailability and metabolism of cyclosporine A (CsA) capsules were compared with two bioequivalent (Food and Drug Administration approved) preparations in rats. Two groups of Wistar-Kyoto rats were given 10 mg/kg q.d. of Sandimmun Neoral (NEO), Novartis Pharma, and CsA (United States Pharmacopeia modified), Eon Labs (EON), as capsules dissolved in water by oral gavage. After reaching steady-state (SS), rats were euthanized 2, 4, 8, 12, and 24 h after dosing. Parallel to this investigation, a single dose (SD) study was also performed. CsA and CsA metabolite concentrations of AM1, AM4N, and AM9 were determined by high-performance liquid chromatography in kidney, whole blood, and urine. The bioavailability of EON was 15% lower [area under the curve (AUC)SS blood CsA, 27.9 ± 3.69 mg·h/l] in the blood and was 40% lower (AUCSS kidney CsA, 136.2 ± 21.2 mg·h/l) in the kidney in contrast to NEO (AUCSS blood CsA, 32.1 ± 4.32 mg·h/l and AUCSS kidney CsA, 220.8 ± 29.5 mg·h/l). In contrast, the plasma AM4N level was significantly elevated in group receiving EON (AUCSS blood AM4N, 4.1 ± 0.42 mg·h/l) compared with the other group treated with NEO (AUCSS blood AM4N, 2.9 ± 0.39 mg·h/l). In the kidneys, no significant differences were observed concerning the AM4N concentrations of NEO (AUCSS kidney AM4N, 11.8 ± 1.87 mg·h/l) versus EON (AUCSS kidney AM4N, 12.1 ± 2.14 mg·h/l, but AM1 was increased (AUCSS kidney AM1, 54.3 ± 11.2 mg·h/l) in comparison to NEO (AUCSS kidney AM1, 20.5 ± 3.56 mg·h/l). Furthermore, EON produced a larger amount of AM4N in the urine (5.8 ± 0.85 μg/24 h versus 2.2 ± 0.95 μg/24 h). Similar results were obtained with the SD study. Although the clinical consequences of our results remain at present unknown, the data suggest differences in CsA disposition that may affect drug efficacy and safety and merit further investigation in humans.

CsA is still the center of immunosuppressive regimen after solid organ transplantation and is further employed in the treatment of autoimmune disorders. It is known that CsA1 possesses very high inter- and intra-individual pharmacokinetic variability (Kahan et al., 1996) if orally applied in a lipophilic solvent (e.g., olive oil). Absorption in the small intestine, mainly by passive diffusion (LeGrue et al., 1983), is highly bile-dependent when using such an oil-based formulation. Necessary emulsification of the crude oil-in-water droplet mixture formed by intestinal digestion (Drewe et al., 1992) varies according to the presence of food, composition of the gastric fluids (e.g., bile salts), and bowel motility (Grevel, 1986). As a consequence, erratic absorption has been cited as the main reason for the variable bioavailability of CsA after orthotopic liver transplantation. Of further clinical interest are a variety of drug interactions that increase CsA bioavailability by inhibiting CsA metabolism (e.g., ketoconazole; First et al., 1989) or diminish it by inducing the degrading enzymes (e.g., phenytoin; Freeman et al., 1984). In addition, known cases of diseases such as cholestasis and hyperactive bowel motility are also able to reduce CsA absorption.

Moreover, CsA bioavailability can be enhanced by drugs increasing gastric emptying (e.g., metoclopramide; Wadhwa et al., 1987). With the intention to reduce the digestive influence on CsA absorption, pharmaceutical research was focused on a microemulsion preconcentration formulation with self-emulsifying properties, which immediately forms a microemulsion upon contact with the aqueous gastrointestinal fluids and thus enhances oral bioavailability. The first microemulsion product, Sandimmun Neoral, has improved immunosuppressive efficacy due to a better oral bioavailability, lower pharmacokinetic variability, and better dose-linearity compared with the former Sandimmun (Dunn et al., 2001). But the need to reduce costs in the health system in nearly every country leads to generic substitutions of the original products. Different generic manufacturers now produce CsA formulations with modified bioavailability (CsA United States Pharmacopeia modified; approved as AB-rated by the Food and Drug Administration) for generic substitution of the original product Sandimmun Neoral. In this study, we concentrated on evaluating the bioequivalence of the generic EON compared with NEO in rats primarily by comparing the occurrence of CM in whole blood, kidneys, and urine and the bioavailability of CsA in the kidneys. It is known that small changes in the pharmacological inactive excipients of a galenic formulation can profoundly alter drug absorption...
Moreover, does generic substitution lead to an altered risk of graft loss? (Opelz, 2001). Reduced immunosuppression caused by low CsA exposure can have a negative clinical outcome (e.g., graft loss) and consequently increase the costs for the health system (Kahan, 1999).

CsA undergoes extensive first pass metabolism (e.g., by the CYP3A in the rat, located in the gastrointestinal mucosa and in the liver). Primary CM are mono- and dihydroxylated (e.g., AM1, AM9) or demethylated (AM4N) derivatives of CsA (Wenger, 1990). The possible activity or renal side effects of these CM are still controversial topics. Experiments to determine immunosuppressive or toxic activity of CM have been restricted to in vitro and animal tests (Fahr et al., 1990). However, it is nearly impossible to extrapolate these findings to those transplanted patients showing elevated CM levels, since their appearance is time and tissue dependent. Furthermore, possible additional or synergetic effects have to be considered as well. AM4N is mainly generated by the gastrointestinal CYP3A in humans and rats (Schwinghammer et al., 1991), therefore we focused on the detection of this CM for the comparison of the two CsA preparations and discussed its possible role as a marker for the extent of CsA absorption. We compared CsA and CM (AM1, AM4N, and AM9) pharmacokinetics in whole blood and in the kidneys after single dose and under steady-state conditions between NEO and EON. Additionally, CsA and CM recovery in the urine were obtained from a 24-h postdose collection. For the detection of possible renal damage, the serum creatinine levels were determined as well.

Materials and Methods

Experimental Design. Seventy adult normotensive Wistar-Kyoto rats (Charles River, Sulzfeld Germany) weighing 250 to 280 g were randomized into two groups and each of these groups subdivided into five groups containing seven animals each. The capsules of NEO (Novartis Pharma Ltd., Basel, Switzerland) and EON (Eon Labs Manufacturing, Inc., Laurelton, NY) were dissolved for peroral application by a gavage. The emulsions (containing 2 mg/ml) were freshly prepared each day; one capsule of the investigated products (containing 100 mg of CsA each) was put into a graduated glass flask, dissolved by adding sterile isotonic sodium chloride solution up to 50 ml, and mixed on a magnetic stirrer for 30 min at room temperature. Group 1 was given NEO and group 2 received EON; a rat weighing 250 g received 1.25 ml of the prepared emulsion, corresponding to 10 mg/kg · day. On day seven, six rats of each main group were held in metabolic cages to obtain urine over 24 h. On day eight, one rat of each subgroup was killed before the final dosing (t = 0) to obtain the trough levels (C_{0h}). All remaining rats in the five subgroups were euthanized after inhalation of sevoflurane 2, 4, 8, 12, and 24 h after the final dosing for tissue collection.

The SD experiment was performed in the same manner but without predose determination of trough levels. Wistar-Kyoto rats (n = 60) were randomly divided into five groups containing six animals each. After single dosing of 10 mg/kg, the animals were killed at the same timepoints as previously described. One group was held in metabolic cages for 24 h to obtain the urine. CsA concentration and the concentration of the CM were determined in whole blood, kidney, and also in the urine by high-performance liquid chromatography (HPLC) in both study designs.

Sample Preparation. Tissue samples were finely cut with a razor blade on ice, weighed to obtain 100-ng samples, and homogenized with an ultra-turrax (IKA Labortechnik, Staufen, Germany) after adding 1 ml of HPLC-grade water and 100 μl of internal standard solution (5 μg/ml cyclosporine D in methanol; Recipe Chemicals and Instruments GmbH, Munich, Germany). Afterward, proteins were precipitated by adding 2 ml of acetonitrile-methanol solution containing 10% zinc sulfate (Recipe Chemicals and Instruments GmbH). One milliliter of whole blood and urine samples was spiked directly with the internal standard and precipitation reagent. After centrifugation, the supernatants were transferred into disposable extraction columns (Recipe C_{18} encapsulated, adsorbed at the solid phase by passing the samples slowly through the columns using a vacuum box, and then washed with acetonitrile-water solution and later with heptane (Recipe Chemicals and Instruments GmbH). HPLC samples were obtained by eluting CsA and AM4N with 300 μl of ethanol-ethyl acetate solution (Recipe Chemicals and Instruments GmbH). The eluated samples were diluted with 100 μl of HPLC-grade water (Recipe Chemicals and Instruments GmbH) and purified by vortexing with 1 ml of heptane. After phase separation, the lower layer was used for HPLC analysis. The determination of serum creatinine levels was performed with a conventional assay (Roche Molecular Biochemicals, Mannheim, Germany) using the Jaffé-method with protein precipitation (Koch and Heimens, 1979). One milliliter of rat serum was used for analysis.

HPLC Conditions. The method was adapted from Brozmanova et al. (2000). The chromatographic equipment consisted of pump LC 10AT, autosampler SIL 10A, UV detector SPD 10AV set at 205 nm, and Class 10 integration software (Shimadzu Europe, Duisburg, Germany). Separation was performed using a Luna phenylhexyl column (i.d. 150 × 4.6 mm; Phenomenex, Aschaffenburg, Germany) with acetonitrile/methanol/water (200:80:140, v/v/v) as eluent. At a flow rate of 1 ml/min (column temperature 75°C), AM9
eluted at 6.1 min, AM1 at 6.8 min, AM4N at 8.4 min, CsA at 13.3 min, and finally cyclosporine D (internal standard) at 16.7 min.

**Statistical Evaluation and Pharmacokinetic Analysis.** Pharmacokinetic parameters were determined by standard noncompartmental analysis. Maximum plasma concentration ($C_{\text{max}}$) and time of $C_{\text{max}}$ ($t_{\text{max}}$) were noted directly. The area under the plasma concentration-time curves (AUC) from 0 to 24 h of CsA and AM1, AM4N, and AM9 were calculated by the linear trapezoidal rule. $C_{\text{max}}$ and AUC obtained from EON were compared with NEO by analysis of variance with multiple comparison followed by the Student’s $t$ test. Blood SS (a), blood SD (b), kidney SS (c) and kidney SD (d) were compared separately. $P < 0.05$ were considered significant and indicated in the tables as superscript letters (a, b, c, d, respectively).

**Results**

Rats treated with EON showed a nonsignificant lower AUC (about 15%) and a different absorption profile of CsA in whole blood compared with NEO in the rat, both under SS and SD conditions (Fig. 1A; Table 1). In contrast to the CsA levels, the AM4N concentration was significantly elevated in the blood (Fig. 1B) in the EON group compared with NEO, again under both conditions. The AM4N whole blood levels following NEO administration were in the range described previously by Kovarik et al. (1994). Furthermore, under SS conditions, the profile of AM1 was altered but not significantly increased (Fig. 1B; Table 1), whereas the AM9 levels did not differ at all between both preparations. There were no observable changes in the CM values under SD conditions, except AM4N (Fig. 1C; Table 1).

The described differences in whole blood were even more pronounced in the kidneys. The AUC (CsA) of EON was about 40% lower (Fig. 2A; Table 2) than the AUC of NEO, whereas the AM4N levels in the kidneys interestingly were not affected by this circumstance (Fig. 2B; Table 2). In addition to our findings in the blood, the AM1 concentration was significantly higher in the EON SS group, whereas the other metabolites did not differ between NEO and EON under SS conditions. No differences were observed concerning the metabolites under SD conditions (Fig. 2C; Table 2) between NEO and EON. The only metabolite recovery that was increased in the urine was that of AM4N in the SS as well as in the SD experiment (Fig. 3, A and B), whereas the CsA excretion over 24 h did not show any observable alteration. No significant changes were observed in the serum creatinine levels of all groups (NEO SD, 0.41 ± 0.08 mg/dl; EON SD, 0.45 ± 0.07 mg/dl; NEO SS, 0.50 ± 0.13 mg/dl; EON SS, 0.51 ± 0.11 mg/dl).

**Discussion**

Our experimental findings in rats show significant differences in the CsA absorption rate, bioavailability, and CM profiles between two approved bioequivalent preparations, NEO and EON, both under SD conditions.

---

**Table 1**

Pharmacokinetic parameters of CsA and CM in rats after 10 mg/kg p.o. under SS and SD conditions obtained in the blood

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$</th>
<th>$C_{\text{max}}$</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEO (h)</td>
<td>EON (h)</td>
<td>NEO (mg·h/l)</td>
</tr>
<tr>
<td>CsA</td>
<td>2</td>
<td>8</td>
<td>2.7 (0.48)</td>
</tr>
<tr>
<td>AM1</td>
<td>4</td>
<td>8</td>
<td>0.3 (0.05)</td>
</tr>
<tr>
<td>AM4N</td>
<td>4</td>
<td>8</td>
<td>0.12 (0.04)</td>
</tr>
<tr>
<td>AM9</td>
<td>2</td>
<td>4</td>
<td>1.37 (0.25)</td>
</tr>
</tbody>
</table>

Panel A: CsA: NEO SS: □, EON SS: ■. Panel B: AM1 NEO: □, AM1 EON: ■. Panel C: AM4N NEO: ◊, AM4N EON: □. AM9 NEO: ●, AM9 EON: ◆. Results are expressed as means ± S.D. *<P < 0.05; **<P < 0.01; ***<P < 0.001.

Panel A. CsA: NEO SS: □, EON SS: ■. Panel B. AM1 NEO: □, AM1 EON: ■. Panel C. AM4N NEO: ◊, AM4N EON: □. AM9 NEO: ●, AM9 EON: ◆. Results are expressed as means ± S.D. *<P < 0.05; **<P < 0.01; ***<P < 0.001.
tested under identical experimental conditions. Furthermore, our results could be masked by the tremendous amount of variability in CsA bioavailability in transplanted patients in the clinical setting. The possible clinical consequences of the use of generic CsA will become evident after several years and after multiple switching between different generic formulations. Therapeutic drug monitoring (TDM) for CsA after solid organ transplant mostly uses the CsA \( C_0 \) levels as an input for further dose adjustment. Our data show, that in spite of the inequivalence of metabolism, absorption rate, and differences in CsA kidney levels, the \( C_0 \) levels in the blood were not affected by this circumstance at all, and therefore no striking differences would have been observed in the traditional TDM between NEO and EON. It is known that there exists no correlation between the amount of the oral dose and the steady-state trough concentration (Grevel, 1986). The only way to avoid early rejection, because of low exposure to CsA, is to use the \( C_2 \) or \( C_4 \) methods, AUC monitoring, or limited sampling strategies (e.g., absorption profiling) (Dunn et al., 2001) as input for the TDM, but these are more difficult to implement in the daily clinical routine as compared with measuring the \( C_0 \) levels, since the time window at e.g., 2 h, where the blood sample would have to be drawn, is much smaller (approximately 10 min) than by using \( C_0 \) levels. Another problem of CsA therapy is the malabsorbers. It has been suggested that specific CM measurements in the urine can be used to identify these patients as early as possible to avoid rejection episodes (Christians et al., 1991). We found elevated AM4N levels in the blood, kidney, and urine combined with an incomplete and delayed CsA absorption in the EON group. But we neither know if CsA malabsorption (e.g., due to genetic disposition) always leads to high AM4N levels, nor how much the CM profile is generally influenced in the clinical situation, where drug interactions (e.g., statins, antibiotics, diuretics) must be considered as well. Further studies with human volunteers compared with renal-transplanted patients are necessary to elucidate these connections. As mentioned in the introduction, it remains unclear if elevated levels of AM4N contribute to nephrotoxicity or if they have useful immunosuppressive activity, because these findings are mainly based on in vitro experiments (Radeke et al., 1992). In cell culture, AM4N showed changes consistent with vacuolization seen in tubular cells exposed to CsA in vivo, whereas other CM that circulate in higher concentrations (e.g., AM1) did not cause such damages (Copeland et al., 1990). Although extrapolation to humans is not possible at this time, our data in rats suggest the need of further investigation.
References


