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Pharmacokinetic and pharmacodynamic evaluation of liposomal cyclosporine

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Summary

The pharmacokinetics and renal toxicity of two liposomal intravenous preparations of cyclosporine (CSA) (A, dimyristoylphosphatidylcholine (DMPC) : stearylamine molar ratio 7 : 1; and B, DMPC : dimyristoylphosphatidylglycerol molar ratio 4 : 1) were assessed in the murine model and compared to the commercially available intravenous formulation of CSA (IV) and drug-free controls. No significant differences in steady-state area beneath the whole blood concentration-time curves were found between formulations and IV following 10 mg/kg per day CSA for 10 days. However, the apparent volume of distribution was significantly greater in A compared to B or IV formulations (13.82 ± 2.9 vs. 7.23 ± 3.98 and 7.67 ± 3.01 l/kg, $p < 0.05$), most likely due to the significantly prolonged biologic half-life of this formulation. Although the glomerular filtration rate was not significantly different between A and saline controls (1056 ± 536 and 1130 ± 550 μl/min per g KW, respectively), dosing with B or IV resulted in significant impairment (522 ± 429 and 572 ± 353 μl/min per g kidney weight (KW), respectively; $p < 0.05$). Triglyceride levels remained unchanged in all groups. However, cholesterol concentrations were significantly increased compared to baseline in rats administered formulation A (98 ± 31 vs. 70 ± 19 mg/dl; $p < 0.05$). In summary, dosing of liposomal formulation A resulted in equivalent concentrations of CSA as the IV formulation without the dose-limiting nephrotoxicity. These data offer a nontoxic delivery system for intravenous CSA.

Introduction

Cyclosporine (CSA), a polypeptide derived from the fungus, *Tolypocladium inflatum Gams*, has demonstrated profound immunomodulating activity in cell culture and animal studies (Borel et al., 1977). The predominant immunosuppressive activity of CSA is inhibition of T-lymphocyte proliferation via reversible inhibition of the primary activation pathways (Aszalos, 1988). CSA has been utilized clinically in the prevention of rejection following transplantation of the kidney, liver, bone marrow and pancreas (Matzke and Luke, 1988). Furthermore, CSA has been undergoing trials for the treatment of a variety of autoimmune diseases including diabetes and psoriasis (Talal, 1988).

The usefulness of CSA has been limited due to a broad toxicity profile including nephrotoxicity and hepatotoxicity. Nephrotoxicity may appear acutely, associated with reversible hemodynamic changes (Curtis and Laskow, 1988), or after

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chronic dosing as a result of interstitial fibrosis (Humes et al., 1985). The acute hemodynamic changes, manifested as alterations in renal blood flow and glomerular filtration rate (GFR), may be secondary to the polyoxyethylated castor oil base (Cremophor EL, BASF) present in the commercially available intravenous formulation, and not CSA itself (Luke et al., 1987). Indeed, the finding of an 8-fold increase in toxicity when the intravenous formulation is used compared to oral administration supports these observations (Williams et al., 1986). Due to significant solubility problems with CSA in aqueous solutions, no commercial alternatives to cremophor have been found; hence, the intravenous use of CSA continues to be deleterious. However, an intravenous formulation of CSA is required for immediate immunosuppression in the patient following transplantation.

Multilamellar liposomes are lipid vesicles measuring 1–5 μm that can be used as vehicles for the delivery of lipophilic antimicrobial/antifungal and chemotherapeutic agents (Lopez-Berestein and Juliano, 1987; Emmen and Storm, 1987; Mayhew and Papahadjopolous, 1983; Weinstein and Leserman, 1984; Yatvin and Lelkes, 1982). Properties which make liposomes attractive are their biodegradability, relative lack of immunogenicity and low intrinsic toxicity. Furthermore, liposomes have been used in drug targeting in vivo to prolong the biologic half-life and change the drug distribution (Ellen et al., 1981; Trubetskoy et al., 1987). Due to their particulate nature, liposomes are taken up preferentially by phagocytes of the reticuloendothelial system (RES) in the liver and spleen reducing the delivery of drug to other potential sites of drug-induced toxicity such as the kidney, heart and gastrointestinal tract. Moreover, delivery of drug to the spleen would be advantageous because the immunosuppressant is targeted to one of the major lymphoid organs.

We have previously developed two formulations of intravenous liposomal CSA which retain immunosuppressive activity in vitro (Vadiei et al., 1989). We report here on the disposition and toxicity of these two formulations in the rat compared with the commercially available intravenous formulation.

Materials and Methods

Drug administration

Thirty-four male albino rats (250–350 g CD, Charles River Breeders, Wilmington, MA) were allowed to acclimate to the animal care facility at constant temperature, humidity and 12-h light/dark cycle for a minimum of 2 weeks prior to baseline evaluation. After a 1 ml blood sample was obtained from each rat via tail bleed while lightly etherized, the animal was placed in a metabolism cage (Maryland Plastics) for complete urine collection. After 24 h, the rat was removed, urine quantitated and a second blood sample was obtained following a 12 h fast. Complete urine voiding was prompted by ether inhalation. The procedure was repeated for a further 24 h. The rat was allowed to stabilize for 48 h following the baseline renal function and lipid assessment.

Four groups of rats were randomized (stratified on baseline renal function) to receive intravenous saline (S; $N = 7$), intravenous CSA (IV; $N = 11$), liposomal formulation A (dimyristoylphosphatidylcholine (DMPC) : stearylamine 7 : 1 molar ratio; $N = 8$) or liposomal formulation B (DMPC : dimyristoylphosphatidylglycerol 4 : 1; $N = 8$). These liposomal formulations were characterized as multilamellar vesicles with their size ranging from 1 to 5 μm (Vadiei et al., in press). The dose of drug (2 mg/ml) was 10 mg/kg per day as a single i.v. dose for 10 days. All dosage forms were prepared daily to avoid degradation problems; dosage was determined by daily weights. To avoid changes in renal function due to weight loss, all rats were pair-fed. Rats were housed in metabolism cages throughout the study period to allow complete urine collection.

On the final dosing day, serial blood samples were obtained via tail bleed at just prior to and 0.5, 1, 2, 4, 6, 8, 12 and 24 h following the intravenous bolus dose. Upon completion of the final blood sample, an inulin clearance was performed on each rat. Briefly, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and the right jugular vein and bladder were cannulated with PE-50 tubing (Clay Adams). A priming dose (25 μCi) of [^{3}H]inulin (American Radiochemicals) was administered followed by a constant infusion

(4 ml/h) of [^{3}H]inulin (5 μCi/ml) for 45 min. Urine was collected in a pre-weighed collection vessel for a period of time sufficient to collect 0.20 ml of urine. Urine volume was quantitated by the difference in weight of the centrifuge tube (specific gravity assumed to be 1.0). Anuria was assumed when less than 0.025 ml of urine was collected within 60 min. Immediately prior to and following the urine collection, blood samples were collected via cardiac puncture, allowed to clot at ambient temperatures, and the serum harvested. Radioactivity was counted in the biologic samples (20 μl sample dissolved in 5 ml cocktail (PCS Amersham)) in a scintillation counter (Beckman LSC). Each rat was subsequently killed with a lethal intraperitoneal dose of pentobarbital (300 mg/kg) and the kidney was removed and weighed.

The study protocol was approved by the Animal Care Committee of the University of Houston prior to experimentation. Rats in the study were maintained in accordance with the guidelines established by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Sample analysis

Whole blood samples for pharmacokinetic analysis were collected in polypropylene centrifuge tubes, thoroughly mixed with sodium EDTA, and immediately frozen at $-20\,^{\circ}$C. CSA quantitation was performed by monoclonal radioimmunoassay (Sandoz Research Institute) which is selective for the parent compound (Brunner et al., 1989a). Percent recovery of cyclosporine in whole blood ranged from 97.5 to 101.3%. Within-day and between-day coefficients of variation did not exceed 9% for the concentration range of 10–1600 ng/ml. Samples exceeding the upper limit of the standard concentration range were diluted with drug-free murine whole blood and reassayed. Urine samples were quantitated for sodium concentrations by an ion-selective procedure (Nova 11 + 11 autoanalyzer). Serum cholesterol and triglyceride levels were quantitated using standard enzymatic research kits (Sigma).

Data analysis: pharmacokinetic studies

The whole blood CSA concentration-time profile was plotted on semilogarithmic paper and the elimination rate constant (K) was estimated by log-linear regression of the terminal points (minimum of four). The area under the whole blood CSA concentration-time curve (AUC) was estimated by the trapezoidal rule for each rat. Half-life was determined by the division of ln(2) by K. CSA clearance (Cl) was calculated by the equation:

$$\mathrm{Cl} = D/\mathrm{AUC}$$

Volume of distribution (V_d) was estimated by the equation:

$$V_d = \mathrm{Cl}/K$$

Data analysis: renal function studies

Inulin clearance (CIN) was estimated by the equation:

$$\mathrm{CIN} = (I_u/I_s) \cdot Q_u$$

where I_u and I_s denote urine and serum concentrations of [^{3}H]inulin, respectively, and Q_u represents the urine volume/unit time standardized to kidney weight (μl/min per g KW). Sodium excretion was calculated by multiplication of the urinary concentration of sodium by Q_u.

Statistical analysis

Sodium excretion rates and fasting lipid levels were compared within and between groups by between-within/ split-plot design (PCANOVA, Human Systems Dynamics). Steady-state pharmacokinetics were compared by ANOVA with post-hoc Newman-Keuls test. Differences in GFR from control rats were compared by two-tailed t-test. A difference was considered significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). The data are presented as means $\pm$ standard deviation ($X \pm$ S.D.).

Results

Pharmacokinetic evaluation

There were no significant differences in peak concentrations following each formulation com-

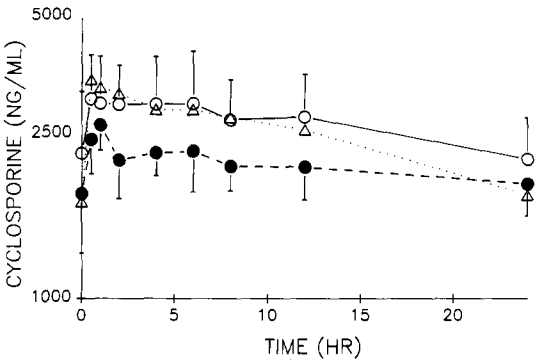

Fig. 1. Mean (±S.D.) whole blood concentration-time profiles of rats administered CSA (solid line), DMPC : SA : CSA (dashed line) or DMPC : DMPG : CSA (dotted line).

pared to CSA alone. Mean whole blood CSA concentration-time profiles are plotted in Fig. 1. Although there were no significant differences in AUC amongst groups, the apparent half-life of CSA incorporated in formulation A was significantly prolonged compared to B and IV products (47.91 ± 13.15 for A vs. 29.82 ± 18.64 for B and 30.95 ± 8.89 h for IV; $p < 0.05$). CSA Cl was not different between groups. Apparent V_d was significantly increased in A compared to B and IV formulations (13.82 ± 2.99 for A vs. 7.23 ± 3.98 for B and 7.67 ± 3.01 l/kg for IV; $p < 0.05$). All pharmacokinetic parameters are listed in Table 1.

Pharmacodynamic assessment

Total body weights were not significantly different amongst groups. Kidney weights did not differ in rats treated with IV (1.25 ± 0.23 g), A (1.46 ± 0.22 g) and B (1.12 ± 0.22 g) compared to controls (1.25 ± 0.29 g; NS). Triglyceride levels were unchanged with 10-day dosing of IV, A or B

TABLE 1

Pharmacokinetic parameters of intravenous CSA preparations (X ± S.D.)

	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	Cl (l h^{-1} kg^{-1})	Vd (l/kg)
CSA	63318 ± 19298	30.95 ± 8.89	0.171 ± 0.048	7.67 ± 3.01
DMPC : SA : CSA	49786 ± 7163	47.91 ± 13.15 [a]	0.204 ± 0.025	13.82 ± 2.99 [a]
DMPC : DMPG : CSA	57998 ± 10769	29.82 ± 18.64	0.177 ± 0.030	7.23 ± 3.98

[a] $p < 0.05$ from CSA.

TABLE 2

Pharmacodynamic parameters of rats treated with saline (S) or CSA as the IV formulation, or incorporated in liposomal formulations A and B (X ± S.D.)

	TG [a]		Chol [b]		E_{Na} [c]		CIN [d]
	Pre	Post	Pre	Post	Pre	Post	
S	59 ± 17	57 ± 14	78 ± 15	76 ± 18	381 ± 256	391 ± 75	1130 ± 550
CSA	61 ± 19	66 ± 21	80 ± 24	89 ± 16	297 ± 123	311 ± 61	572 ± 353 [e]
A [g]	67 ± 13	77 ± 10	70 ± 19	98 ± 31 [f]	262 ± 67	274 ± 68	1056 ± 536
B [h]	48 ± 25	37 ± 20	62 ± 34	65 ± 33	365 ± 143	385 ± 120	522 ± 429 [e]

[a] Triglycerides (mg/dl).
[b] Cholesterol (mg/dl).
[c] Sodium renal excretion (mEquiv./min per 100 g body wt).
[d] CIN (μl/min per g KW).
[e] $p < 0.05$ from saline group.
[f] $p < 0.05$ from pre-therapy.
[g] DMPC : SA : CSA.
[h] DMPC : DMPG : CSA.

formulations compared to baseline or control rats. Although cholesterol levels were unchanged in control, IV or B groups, significant increases over baseline were observed in rats administered formulation A (Table 2).

Renal sodium handling (E_{Na}) was unchanged in all groups. GFR was markedly reduced following 10-day treatment with B or IV (522 ± 429 and 572 ± 353 μl/min per g KW, respectively; $p < 0.05$), whereas GFR remained unchanged in animals treated with A compared to control rats (1056 ± 536 vs. 1130 ± 550 μl/min per g KW; NS).

Discussion

We compared the in vivo pharmacokinetics and renal toxicity of 2 intravenous liposomal formulations of CSA and the commercially available intravenous formulation. A significantly greater apparent distribution of CSA without dose-limiting nephrotoxicity was found with formulation A compared to IV or B formulations, suggesting a potential alternative to the toxic commercial formulation.

Entrapment of CSA in liposomes has been reported by others using different lipid compositions with various entrapment efficiencies (Stuhne-Sekalec et al., 1986a,b). However, efficacy studies of these formulations have not been reported. The nephroprotective effect of liposomal CSA preparations has also been examined in other studies (Smeesters et al., 1988a,b; Hsieh et al., 1985). Kidney function was assessed using microscopy or serum creatinine levels which may not accurately reflect GFR. In the latter study, inulin clearances of rats dosed liposomal CSA were significantly greater than those of rats administered the intravenous product. However, GFR of rats dosed liposomal CSA was 31% lower than the inulin clearance of saline-treated controls.

Liposome encapsulation of drugs has been previously shown to alter the pharmacokinetics including the biological half life and organ distribution (Barza et al., 1987; Lautersztain et al., 1986). In general, drugs entrapped in multilamellar liposomes are rapidly cleared from blood and concentrate preferentially in the RES, namely the liver and spleen. In our study, we found that the area under the drug concentration-time curves and systemic clearances of two liposomal CSA formulations of different lipid compositions yet equivalent size profiles were similar to the IV formulation. However, caution is necessary in the interpretation of the estimates of elimination half-life and volume of distribution in the present study. Since the half-life of CSA is approx. 9–12 h in the murine model (Brunner et al., 1988), blood samples for pharmacokinetic analysis were obtained for a 24-h period. However, in all groups, half-life calculations exceeded the sampling period. The volume of distribution of the IV product was in agreement with previous studies (Brunner et al., 1988) suggesting that the prolongation in apparent half-life may be secondary to changes in hepatic microsomes (Kronback et al., 1988). Optimally, the sampling period should surpass one half-life. In the present study, the apparent half-life of formulation A was 2-fold greater than the sampling period. In so doing, error was introduced in the calculation of the pharmacokinetic parameters. Further studies are in progress using a longer sampling period for accurate characterization of the pharmacokinetics of CSA incorporated in formulation A.

Liposomal-encapsulated drugs have demonstrated reduced toxicity compared to free drug formulations (Lopez-Berestein and Juliano, 1987; Smeesters et al., 1988a,b). CSA is a highly lipophilic compound with a relatively narrow therapeutic index. Due to its lipophilicity, a delivery system for intravenous administration has been developed with the polyoxyethylated castor oil base, cremophor. However, this vehicle has been associated with anaphylactoid reactions when administered in large quantities or too rapidly. Furthermore, acute renal failure secondary to intravenous CSA use may be due to Cremophor EL (Luke et al., 1987). Clearly, a new intravenous formulation is warranted.

Although both IV and B formulations resulted in a 2-fold decrease in GFR after 10 days of dosing in the murine model, no significant differences in GFR were observed in the group administered the liposomal formulation A compared

to saline-treated control rats. The exact mechanism of CSA nephrotoxicity is not presently understood but may be related to shifts in the sympathetic, renin-angiotensin-aldosterone or prostaglandin pathways or vascular congestion (Brunner et al., 1989b; Curtis et al., 1988; Humes et al., 1985). Protection from drug-associated nephrotoxicity with formulation A remains unclear requiring further investigation.

Hyperlipidemia and accelerated atherosclerotic changes have been associated with the clinical use of CSA (Hess et al., 1987; Versluis et al., 1986). In the present study, no changes in cholesterol or triglyceride levels were observed with saline, IV or B formulations. However, baseline cholesterol levels were 40% increased with formulation A. The mechanism of this apparent increase is not understood but may reflect CSA-induced changes in cholesterol absorption from the gut or metabolism within the liver. Preliminary studies, both in vivo and in vitro, have failed to demonstrate decreases in lipoprotein lipase activity associated with CSA (unpublished observations). Since both CSA and cholesterol are dependent on active transport across the gastrointestinal tract, drug-associated changes in chylomicrons may offer an explanation for the relative hypercholesterolemia.

In summary, liposomal formulation A produced a similar blood concentration-time profile as the IV formulation without significant reduction in GFR. Future studies are necessary to compare the immunosuppressive activity of CSA in formulation A versus the commercially available intravenous product in an immunized animal model.

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