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INHIBITION OF HEPATIC MICROSOMAL DRUG METABOLISM BY THE IMMUNOSUPPRESSIVE AGENT CYCLOSPORIN A*

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Abstract—Cyclosporin A (CsA), an orally active immunosuppressive agent, was shown to inhibit cytochrome P-450 dependent biotransformation of drugs in the mouse. It competitively inhibited the hydroxylation of benzo[*a*]pyrene and the N-demethylation of aminopyrine in hepatic microsomes with K_i values of 93 and 1540 μM respectively. This selective inhibition for benzo[*a*]pyrene hydroxylase by CsA was substantiated *in vivo* by selective inhibition of total body clearance of theophylline, but not of antipyrine. CsA was itself N-demethylated by hepatic microsomes with a K_m of 808 μM. CsA interacted directly with cytochrome P-450, causing a reverse type I spectral change in hepatic microsomes. No metabolic intermediate complexes could be demonstrated. These results suggest that CsA has the potential to cause drug interactions involving inhibition of drug biotransformation, particularly of drugs that are metabolised by the same types of cytochrome P-450 which oxidise benzo[*a*]pyrene and theophylline.

Cyclosporin A (CsA) is a powerful immunosuppressive agent which selectively inhibits T-lymphocyte activation [1, 2] and exhibits relatively low myelotoxicity [3]. The advantages of this novel pharmacological agent over conventional steroids or cytotoxic drugs have been demonstrated in a wide variety of clinical situations, particularly in the field of organ transplantation [4, 5].

CsA is a neutral lipophilic compound which contains a number of chemical groups that have the potential to be oxidized by cytochrome P-450 dependent mixed-function oxidases in the liver. Indeed it has been demonstrated that CsA is extensively metabolized in the liver of both animals and man and that most of the metabolism consists of oxidative processes (hydroxylation and N-demethylation) [6]. The many potential sites for oxidation by cytochrome P-450 lead to the speculation that CsA could interfere with the elimination of other drugs which are metabolised by the mixed-function oxidase system in the liver. Such an inhibition could result in a decrease in clearance and an increase in the plasma levels of other drugs used concomitantly and result in toxic drug interactions. To date, several drug interactions have been reported involving other drugs with CsA but none of these involved the inhibition of the metabolism of the other drugs [4].

On the other hand, other drugs such as cytochrome P-450 inducers cause a decrease in CsA toxicity by inducing the metabolism of CsA which results in lowered serum levels of the drug [7, 8]. We now report that CsA is an inhibitor of cytochrome P-450 dependent mixed-function oxidase in the mouse liver and therefore has the potential to cause drug interactions clinically.

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MATERIALS AND METHODS

Materials. Cyclosporin A (CsA) was a gift from Sandoz Ltd., Basel.

Animals. Male Swiss strain mice (30–35 g) obtained from Jackson Laboratories (Bar Harbor, ME) were used throughout these studies. Animals were kept on clay chip bedding and allowed to acclimatize in our facility for at least 1 week before use.

Microsomes. Hepatic microsomes were prepared as described by El Defrawry El Masry *et al.* [9] and were used on the day they were prepared. Microsomal protein levels were determined by the method of Lowry *et al.* [10], using bovine serum albumin as a standard. Cytochrome P-450 and cytochrome b_5 levels in microsomes were determined by the method of Omura and Sato [11].

Enzymatic assays. The N-demethylation of aminopyrine (Aldrich Chemical Co.) was assessed by measuring the amount of $H^{14}CHO$ produced from the incubation of [*methyl*-^{14}C]aminopyrine (sp. act. 118.7 mCi/mmole from NEN Research Products) with microsomes [12]. This technique was utilized in order to measure HCHO produced specifically from aminopyrine without interference from the HCHO produced from CsA. Benzo[*a*]pyrene (Sigma Chemical Co.) hydroxylase activities were measured by the method of Nebert and Gelboin [13]. The N-demethylation of CsA was determined by measuring the amount of formaldehyde produced, over a 10-min incubation period, using the colorimetric procedure based on the Hantzsch reaction [14]. In all enzymatic assays, the amount of CsA which gave the concentrations indicated in the results was added in absolute ethanol and then dried in the test tube prior

to adding liver microsomes (protein concentration of 4.6 mg/ml). We have assumed that the CsA dissolved in the presence of lipid and protein up to a concentration of 5 mM. Concentrations of 5 mM and above produced turbidity and clearly were not taken up by microsomes.

Binding spectra. The binding spectra of CsA with cytochrome P-450 were measured in a suspension of microsomes containing 4.6 mg/ml of microsomal protein in 1.15% KCl [15]. Various amounts of the drug (ranging from 83 to 705 μM) were added in 10 μl dimethyl sulfoxide to 3 ml of microsomal suspension in the test cuvette and compared to 3 ml of microsomal suspension in the reference cuvette containing 10 μl dimethyl sulfoxide. Binding spectra were recorded from 350 to 500 nm in a Pye-Unicam double-beam spectrophotometer.

Metabolic intermediate complexes. The possible formation of metabolic intermediate complexes were examined by incubating CsA (700 μM) with microsomes (4.6 mg protein/mg) and NADPH (0.5 mM) for 20 min at 37° as described by Franklin [16]. The difference spectrum between 400 and 500 nm was obtained by comparing this incubation mixture to identical mixtures incubated for only 15 sec.

Determination of total body clearance of antipyrine and theophylline. The clearance of antipyrine (Sigma Chemical Co.) and theophylline (Squibb Canada) were determined by modification of the methods described by Paxton [17] and Gray *et al.* [18]. Mice were treated with CsA in corn oil (100 mg/kg) in a single dose or three daily doses. Control mice received an equivalent volume of corn oil at the same times. Twenty-four hours after a single dose of CsA or after the third dose of CsA, each animal received a single i.v. dose of antipyrine (5 mg/kg) or theophylline (5 mg/kg). Blood samples were then collected from the orbital sinus at various time intervals and assayed for drug concentrations. Both antipyrine and theophylline followed a one-compartment model of elimination within 10 min of drug administration. Elimination constants and apparent volume of distribution were calculated from the terminal slope of the elimination curve (log concentration vs time) using the method of least squares. Clearance was calculated from the formula

$$\text{Clearance} = V_d \times 0.693/T_{\frac{1}{2}}$$

where V_d is the volume of distribution and $T_{\frac{1}{2}}$ is half-life.

Determination of CsA. Estimations of CsA levels in serum samples obtained 24 hr after the last drug administration were carried out using radioimmunoassay kits supplied by Sandoz Ltd. (Basel) as described by Donatsch *et al.* [19]. This immunoassay does not distinguish between the parent CsA molecule and certain of its metabolites.

Statistics. The mean values for the various parameters were tested for significance using an unpaired Student's *t*-test. Enzyme kinetic data using Michaelis–Menten rate equations were fitted to a hyperbola using the method and computer program described by Barlow [20]. The same method of analysis was used to estimate the binding constants for the interaction of CsA with cytochrome P-450. Similarly, terminal determinants of the elimination curve for theophylline and antipyrine (log concentration vs time) also fitted to a straight line using the method and computer program described by Barlow [20].

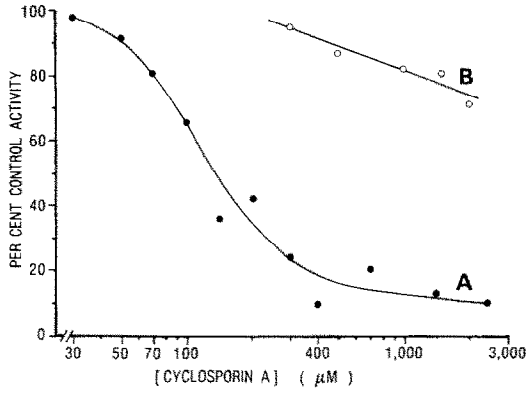

Fig. 1. Inhibition of benzo[*a*]pyrene hydroxylase (A) and aminopyrine *N*-demethylase (B) activity by CsA. Liver microsomes from mice (4.6 mg of microsomal protein/ml) were used to determine the inhibitory effect of CsA on the metabolism of benzo[*a*]pyrene (100 μM) (●) and aminopyrine (50 μM) (○). Control activities equalled 7.70 nmoles 3-hydroxybenzo[*a*]pyrene/mg protein/hr and 12.2 nmoles formaldehyde produced/hr/mg protein respectively.

RESULTS

Effect of CsA on liver microsomal aminopyrine N-*demethylase and benzo*[a]*pyrene hydroxylase activities.* The hydroxylation of benzo[*a*]pyrene and the N-demethylation of aminopyrine were inhibited by various concentration of CsA when added to hepatic microsomes (Fig. 1). The concentration of CsA required for the 50% inhibition (I_{50}) of benzo[*a*]pyrene hydroxylation was approximately 120 μM. The I_{50} for the inhibition of aminopyrine N-demethylation could not be determined as concentrations of CsA greater than 5 mM were not soluble in the incubation mixture. The inhibition of the metabolism of both of these substrates was competitive in nature as indicated by an alteration in the substrate K_m values but no change in the V_{max}, as illustrated in Table 1. The K_i for CsA, calculated by the method of Mazel [21], was 92.9 μM with respect to benzo[*a*]pyrene hydroxylase and 1540 μM with respect to aminopyrine *N*-demethylase.

N-*Demethylation of CsA by hepatic microsomes.* CsA was metabolised *in vitro* by the hepatic microsomal enzymes, resulting in the production of HCHO. A double-reciprocal plot for the *N*-demethylation of various concentrations of CsA is illustrated in Fig. 2. The maximum rate of CsA *N*-demethylation was 60 ± 8 nmoles HCHO/mg protein/hr and the K_m for the reaction was 808 ± 299 μM.

Binding difference spectra of microsomal cytochrome P-450. The addition of CsA to microsomes caused a reversed type I spectral change characterized by an absorbance maximum at 418 nm and an absorbance minimum at 380 nm (Fig. 3). The absorbance changes of CsA followed Michaelis–Menten kinetics and increased with increasing con-

Table 1. Kinetic constants for the inhibition of aminopyrine *N*-demethylase and benzo[*a*]pyrene hydroxylase by CsA

Treatment	Aminopyrine *N*-demethylase K_m (μM)	Aminopyrine *N*-demethylase V_{max} (nmoles HCHO/mg protein/hr)	Aminopyrine *N*-demethylase K_i (μM)	Benzo[*a*]pyrene hydroxylase K_m (μM)	Benzo[*a*]pyrene hydroxylase V_{max} (nmoles 30HBP/hr/mg protein)	Benzo[*a*]pyrene hydroxylase K_i (μM)
Control	154 ± 14	110.9 ± 3.6		86 ± 38	8.4 ± 1.0	
CsA (700 μM)	224 ± 15	108.5 ± 2.6	1540	734 ± 349	8.0 ± 1.9	92.9

The values were obtained using aminopyrine concentrations ranging from 0.05 to 1 mM and benzo[*a*]pyrene concentrations ranging from 0.04 to 1.3 mM. The kinetic constants were calculated from a direct fit of rate of reaction versus substrate concentration values to a hyperbola as described in Methods. In these experiments only a single K_m for aminopyrine was apparent. Each value is expressed as the calculated constant ± error of the estimate. Similar results were obtained from different preparations of microsomes.

centration. The spectral dissociation constant (K_d) and the maximum absorbance change, calculated from a double reciprocal plot, were 407 μM and 0.117 respectively.

Formation of metabolic-intermediate complexes with P-450. This procedure was carried out to show that CsA does not require metabolic activation by the liver microsomes to achieve its inhibitory potency. CsA (1 mM) was incubated with microsomes and 0.5 mM NADPH for periods up to 20 min. When the spectra of these microsomes were compared to similar mixtures that were incubated for 15 sec, no difference spectrum was apparent which indicates that no metabolic intermediate complexes were formed [16].

Effect of CsA on the total body clearance of antipyrine and theophylline. The clearance of a single dose of theophylline was decreased significantly in animals who had received CsA (100 mg/kg) for 1 or 3 days previously (Fig. 4). In contrast, the clearance of antipyrine was unaffected by CsA treatment for 1 or 3 days. The serum levels of CsA in animals killed at the time theophylline or antipyrine was administered were 4.8 ± 0.5 and 5.4 ± 0.5 μg/ml for 1 and 3 day treatments respectively (Table 2). The concentration of cytochrome P-450 in microsomes prepared from these animals was identical to control, and liver weight and microsomal protein concentration were decreased significantly only in animals treated for 3 days.

DISCUSSION

Cyclosporin A (CsA) is a relatively new immunosuppressive agent which is widely used on a chronic basis to prevent rejection following an organ transplant [1, 2]. One of the major disadvantages in the use of this drug has been a high incidence of renal toxicity and, to a much lesser extent, hepatic

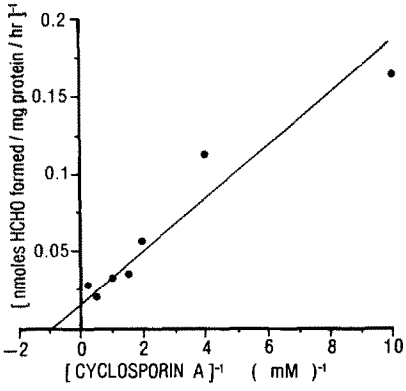

Fig. 2. Double-reciprocal plot of the *N*-demethylation of various concentrations of CsA by hepatic microsomes. Each value is expressed as the mean for two individual incubation mixtures from the same preparation of microsomes. Similar results were obtained with different preparations of microsomes. The kinetic constants reported in Results were calculated from a direct fit of rate of reaction vs substrate concentration values to a hyperbola as described in Methods. The values are expressed as the calculated constant ± error of the estimate. The V_{max} of CsA *N*-demethylation was 60 ± 8 nmoles HCHO/mg protein/hr and the K_m for the reaction was 808 ± 299 μM.

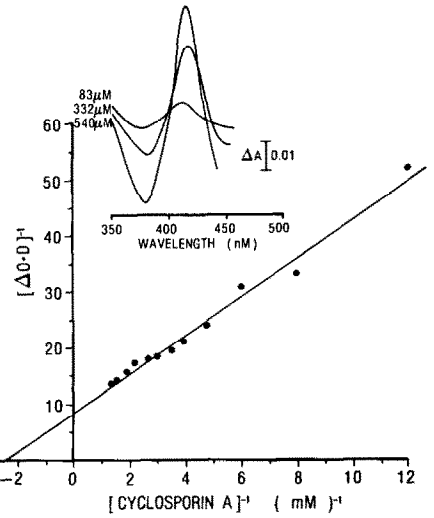

Fig. 3. Double-reciprocal plot of the changes in absorbance produced by the addition of various concentrations of CsA to hepatic microsomes. The changes in absorbance were calculated by the difference in $A_{420-380}$. The lines of best fit were determined by the method of least squares. The spectral dissociation constant derived from a fit of the data to a hyperbola was 407 ± 33 μM. Representative spectra for three concentrations of CsA are shown in the insert of this figure.

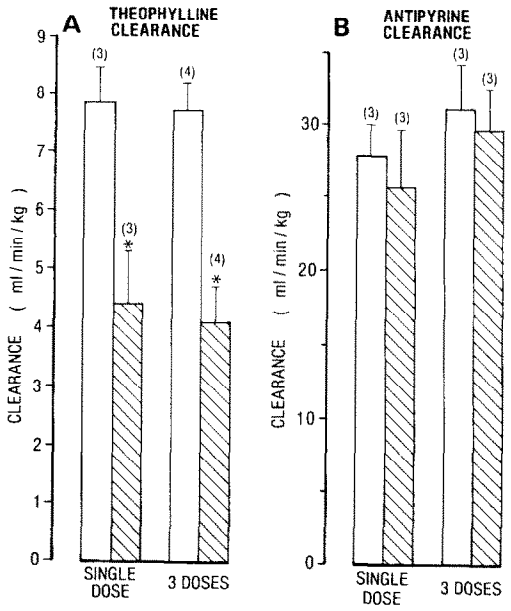

Fig. 4. Theophylline clearance (A) and antipyrine clearance (B) in mice treated with CsA. Mice were treated with a single dose of corn oil (□) or CsA (■) (100 mg/kg, i.p.) or with three doses of corn oil or CsA (100 mg/kg, i.p.), once every 24 hr. Kinetics of theophylline (5 mg/kg, i.v.) and antipyrine (5 mg/kg, i.v.) were determined 24 hr after the last injection. The number of animals are included in the parentheses. (*) Significantly different from control ($P < 0.05$).

toxicity [4, 5]. Several reports have indicated that the extent of CsA toxicity is modified during the concomitant administration of other drugs which appear to alter the metabolism and excretion of CsA. In man, cimetidine [22] and ketoconazole [23] increase the toxicity of CsA by inhibiting its metabolism, whereas rifampicin [24, 25] and phenytoin [26] decrease the effectiveness of CsA by inducing its metabolism. In animals, compounds which induce cytochrome P-450 in the liver are known to decrease the renal toxicity of CsA by causing a decrease in the levels of drug achieved in the serum [7, 8]. We have now shown that CsA can inhibit the metabolism of drugs by cytochrome P-450 in the liver. The hydroxylation of benzo[*a*]pyrene and the *N*-demethylation of aminopyrine in hepatic microsomes were both inhibited by CsA. Although the inhibition was competitive in both cases, benzo[*a*]-pyrene hydroxylation was much more sensitive to the effects of CsA than aminopyrine *N*-demethylation, with over a 16-fold difference in the respective K_i values. This suggests that CsA is much more efficient in inhibiting the enzymatic activity of some isozymes of cytochrome P-450 than others. This idea was substantiated by demonstrating that CsA affected the clearance of drugs *in vivo*. The clearance of theophylline, which is dependent on cytochrome P_1-450 for its metabolism and excretion [27], was impaired significantly in animals treated with CsA. In contrast, the clearance of antipyrine which is dependent on the net activity of several different forms of cytochrome P-450 [28] was unaffected by CsA treatment.

CsA apparently inhibits drug metabolism in the liver by a direct action with cytochrome P-450. The results presented here demonstrate that cyclosporin A bound to cytochrome P-450 and exhibited a reverse type I binding spectrum which is characteristic of interaction at the hydrophilic site of the heme iron [15]. Cyclosporin A apparently does not form metabolic intermediate complexes as no spectral evidence for such complexes could be found when CsA was incubated with microsomes and NADPH. CsA itself is metabolized by cytochrome P-450 to produce HCHO, and simple competition for active sites of the enzyme may well explain the inhibitory properties of this drug. CsA does not appear to decrease or induce the concentration of cytochrome P-450 in microsomes as, after treatment of animals with CsA, normal concentrations of cytochrome P-450 were found. The small (< 9%) decreases in microsomal protein found in CsA-treated animals cannot account for the large decreases in theophylline clearance illustrated in Fig. 4.

The question of real importance raised by this study is the possible inhibition of drug biotransformation in man during the clinical use of CsA. The K_i values for the inhibition of benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase obtained in

Table 2. Hepatic mixed-function oxidase system in mice treated with CsA

Treatment	Microsomal protein (mg/ml)	Cytochrome P-450 (nmoles/mg protein)	Serum CsA levels (μg/ml)
(A)* Corn oil	6.12 ± 0.63	0.619 ± 0.043	ND†
CsA	5.20 ± 0.72	0.596 ± 0.046	4.8 ± 0.5
(B)†‡ Corn oil	7.62 ± 0.36	0.681 ± 0.052	ND
CsA	6.60 ± 0.21§	0.663 ± 0.086	5.4 ± 0.5

* (A) Mice received a single injection of CsA (100 mg/kg) or corn oil, i.p., and were killed 24 hr later, N = 4.

† Below detectable limits (25 ng/ml CsA).

‡ CsA (100 mg/kg) or corn oil was given i.p. every 24 hr for three consecutive days, and the animals were killed 24 hr later.

§ Significantly different from the corn oil treated, $P < 0.05$.

mouse microsomes are well above the serum concentrations which are found during clinical use of CsA in man [23]. However, the tissue concentrations of CsA have been considerably higher than serum levels in animal experiments. For example, the concentrations of CsA in skin, adipose tissue, liver and kidney were approximately three to fourteen times higher than the serum CsA concentration 4–24 hr following the administration of a single dose of CsA [24]. It is therefore very difficult to compare the K_i values determined *in vitro* in microsomes to the tissue concentrations possible in the intact animal and to make predictions on the extent of the interaction in man.

Although it is possible that CsA will inhibit the metabolism of drugs which are metabolized by isoenzymes of cytochrome P-450 that metabolize benzo[*a*]pyrene, it is quite unlikely that CsA will inhibit metabolism of drugs such as aminopyrine which are metabolized by other forms of cytochrome P-450. The selectivity of the inhibition of CsA was also demonstrated *in vivo* where theophylline clearance was impaired but antipyrine clearance was not. The blood levels of CsA achieved in these experiments were greater than those usually found in man but were similar to those reported to cause nephrotoxicity in rodents [8].

In summary, we have demonstrated that CsA is a selective *in vitro* and *in vivo* inhibitor of certain drug oxidations carried out by cytochrome P-450 in the mouse liver. The isozymes which hydroxylate benzo[*a*]pyrene appear to be the most sensitive to this effect. Because the inhibition of certain metabolic pathways may occur at drug levels close to those used in man, the occurrence of drug interaction in humans is a distinct possibility. The interaction will, of course, only occur in a clinical situation with drugs that are metabolised by the specific isozymes of cytochrome P-450 which are affected by CsA (e.g. theophylline).

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