Pharmacokinetic Modulation of Irinotecan and Metabolites by Cyclosporin A

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ABSTRACT

The focus of this investigation was to modulate the pharmacokinetics of irinotecan and its metabolites, SN-38 and SN-38G, by possibly reducing biliary excretion, which in turn could lower irinotecan toxicity. We determined the effect of a known cholestatic agent, cyclosporin A (CsA), which is transported across the biliary canalicular membrane by P-glycoprotein, on the biliary excretion of irinotecan and its metabolites. Wistar rats were pretreated with 60 mg/kg CsA 5 min before an i.v. dose of irinotecan at doses levels of 6, 10, and 20 mg/kg. The control groups received irinotecan only. CsA pretreatment resulted in an average increase of 339, 361, and 192% in the area under the plasma concentration-time curve of irinotecan, SN-38, and SN-38G, respectively. Analysis of clearance (CL) of irinotecan indicated a 55 and 81% reduction in the average renal and nonrenal CLs, respectively, in the pretreated groups. The nonrenal CL, which is the primary component of irinotecan CL, includes protein and tissue binding as well as the metabolic and biliary CL of irinotecan. There was no change in the volume of distribution at steady state (indicative of unchanged binding) and in the metabolic conversion of irinotecan to SN-38 due to pretreatment. Therefore, the significant reduction in the systemic CL of irinotecan due to CsA pretreatment was primarily due to lowered biliary excretion. Studies using a photoaffinity analogue of verapamil, 125I[3-azido-3',5'-15I]salicylaminomethyl verapamil (125I[3-azido-3',5'-15I]NAS-VP, AM/7-azido-[3,5-i;15I]salicyl aminomethyl verapamil), and membrane vesicles from the multidrug-resistant cell line, MCF-7/Adr, revealed that irinotecan and metabolites had moderate interaction with P-glycoprotein. Further studies are required to determine the mechanism of the inhibitory effect of CsA on the biliary excretion of irinotecan and its metabolites.

INTRODUCTION

Irinotecan (CPT-11) is a topoisomerase I inhibitor that is currently in Phase II trials in the United States and is approved in Japan and France. After administration, the drug undergoes hydrolysis by the enzyme carboxyl esterase to form a primary metabolite, SN-38, which further undergoes glucuronide acid conjugation (by UDP-GT) to form the corresponding glucuronide, SN-38G (1, 2). The topoisomerase I inhibition and the DNA breaks leading to cell death after irinotecan exposure are due to SN-38 (3), and this primary metabolite determines the activity and toxicity of irinotecan. The major dose-limiting toxicity after irinotecan administration is severe diarrhea that is often unresponsive to common antiarrheal agents. In a previous report, we suggested the possibility that excessive biliary excretion of SN-38, due to decreased glucuronidation in patients, was responsible for the toxicity (4). This observation was supported by the fact that intestinal accumulation of irinotecan and SN-38 caused significant damage to the intestinal mucosa, leading to diarrhea in animal models (5, 6). Moreover, biliary excretion represents the major route of elimination in rats, and irinotecan, SN-38, and SN-38G in the feces accounts for >60% of the dose (2, 7). In humans, biliary concentrations of irinotecan were reported to be 10- to 60-fold higher than concurrent plasma concentrations, whereas those of SN-38 were 2- to 9-fold higher after irinotecan administration (8). In another study, mean bile:plasma ratios for irinotecan were reported to be 70 and 135, and those of SN-38 were 29 and 57 during the first and third weeks of irinotecan administration, respectively (9). High bile:plasma ratios of SN-38G have also been reported (10). The data underscore the significance of biliary excretion of irinotecan and SN-38 and suggest possible intestinal accumulation after repeated dosing. Therefore, an approach to lowering the incidence of dose-limiting diarrhea associated with irinotecan therapy would be to reduce biliary excretion of SN-38.

Possible approaches to decrease biliary excretion would include reduction in bile flow (cholestasis) and reduction in canalicular transport (reviewed in Ref. 11). CsA is a known cholestatic agent and in rats reduces both bile acid-dependent and bile acid-independent flow (12-14). CsA has also been shown to bind to and be transported by P-gp, a M, 170,000–180,000 membrane protein (15-17). P-gp in the bile canalicular membrane is believed to function as an ATP-dependent transporter of cellular xenobiotics (18–20). Induction of the mdr1 gene coding for P-gp was observed during cholestasis (21). CsA in low micromolar-to-nanomolar concentration ranges has also been shown to inhibit effectively the MOAT and the bile salt (taurocholate) transport system (reviewed in Refs. 22 and 23). Thus, CsA could potentially lower biliary excretion of irinotecan and its metabolites by reducing both bile flow and canalicular transport.

The objectives of this investigation were: (a) to determine whether CsA altered the pharmacokinetics of irinotecan, SN-38, and SN-38G; and (b) to investigate whether the binding of CsA to P-gp was a possible mechanism for the alteration in disposition.

MATERIALS AND METHODS

Reagents. Irinotecan solution was a gift from the Yakult Honsha Co. (Tokyo, Japan), CsA (Sandimmune) was purchased from Sandoz Pharmaceutical Co. (East Hanover, NJ), and a total bilirubin assay kit was purchased from Sigma Chemical Co. (St. Louis, MO). 125I[Na was obtained from Amersham (Arlington Heights, IL). The photoaffinity analogue of verapamil, 125I[3-azido-3',5'-15I]NAS-VP (220 Ci/mmol), was synthesized as described previously (24). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Itasca, IL).

Animal Protocol. Experimental procedures were in accordance with the guidelines of The University of Chicago Manual on Laboratory Animals prepared by the Animal Care Committee. Female Wistar rats (~200 gm) having a permanent catheter installed in the right jugular vein were obtained from Charles River Breeding Laboratories (Wilmington, MA). The rats were maintained in metabolism cages for at least 24 h prior to the experiments, and food and water were supplied ad libitum. The animals were divided into three different dose levels of 6, 10, and 20 mg/kg. Within each dose level, the animals were further subdivided into a control group and a pretreated group. The control group received an i.v. bolus (<1 min) administration of irinotecan through the catheter. The pretreated group of rats received an i.v. bolus injection of CsA (60 mg/kg) 5 min before receiving the dose of irinotecan. This would ensure the maximal availability of CsA in the presence of irinotecan. In the 10 mg/kg-dose group, two additional rats received a dose of 30 mg/kg CsA 5 min before irinotecan. The catheter was flushed with 500 μL of physiological saline after each drug administration.

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3 The abbreviations used are: CsA, cyclosporin A; P-gp, P-glycoprotein; CL, clearance: CLr, renal CL; CLm, nonrenal CL; AUC, area under the plasma concentration-time curve; MOAT, multispecific organic anion transport system; UDP-GT, UDP-glucuronyl transferase: [125I][NAS-VP, N-(p-azido-[3,5-125I]salicyl) aminomethyl verapamil].
CsA is dispensed as a solution in 65% Cremophor. The vehicle itself has been reported to reverse the multidrug resistance phenotype caused by P-gp (25, 26). To evaluate the contribution of Cremophor in P-gp-mediated transport of irinotecan and its metabolites, a 65% solution of Cremophor in alcohol was administered to two rats 5 min before an irinotecan dose of 20 mg/kg.

For all rats, serial blood samples (200 µl) were withdrawn through the catheter at predose and at 3, 5, 10, 15, 30, 60, 120, 240, and 360 min after irinotecan administration. After each sample, the catheter was flushed with an equal volume of physiological saline. The samples were immediately centrifuged at 2500 x g for 10 min, and the plasma obtained was stored at −70°C until analysis. Urine samples were collected for a period of 24 h after irinotecan administration. Total irinotecan, SN-38, and SN-38G in 10–20-µl plasma samples and in urine samples diluted 1:5 were quantitated by the reversed-phase high-performance liquid chromatography method as described previously (4). The limit of detection for irinotecan and SN-38 was ~5 ng/ml. SN-38G concentrations were expressed as SN-38 equivalents. Estimation of total (lactone plus hydroxy acid form) versus lactone concentrations was done because: (a) the lactone concentrations reach equilibrium with the total, giving a constant lactone:total ratio; and (b) quantitation of total concentrations is more accurate and convenient. Because the conversion of the lactone form to the hydroxy acid form is primarily pH dependent, the lactone:hydroxy acid ratio would be constant. Sasaki et al. (27) have demonstrated that the AUC of total irinotecan and SN-38 significantly correlated with the AUCs of the corresponding lactone forms. This finding suggests that measurement of total concentrations would have the same clinical significance as those of the lactone forms. Overall, the results would be similar, irrespective of the species (total or lactone form) of the drug and metabolites being quantitated. Total bilirubin content was determined in 40 µl plasma in control rats receiving 6 mg/kg irinotecan and in the corresponding pretreated group, according to the instructions provided in the assay kit.

**Photoaffinity Labeling.** Membrane vesicles from the multidrug resistance-variant human breast cancer cell line MCF-7/Adr, expressing 77-fold resistance to doxorubicin (Adriamycin), were prepared by the nitrogen cavitation and differential centrifugation methods (28). The vesicles (30 µg protein) in Tris-HCl (pH 7.4), 20% PBS, 250 mM sucrose, and 0.02 mM phenylmethylsulfonyl fluoride were photolabeled as described previously (24) using 5 mM [125I]NAS-VP, in the absence or presence of 10 or 100 µM irinotecan, SN-38, SN-38G, or verapamil, in a final volume of 0.05 ml. These drugs were dissolved in DMSO, and the final concentration of DMSO in all photoaffinity labeling experiments was 4%. The reaction mixtures were preincubated for 30 min at 25°C and then irradiated for 20 min with an UV light equipped with two 15-W self-filtering 366-mm lamps. Photolabeled membrane vesicles were analyzed by SDS-PAGE on a 5–15% gradient gel containing 4.5 M urea, followed by autoradiography. The radioactivity associated with P-gp in the absence or presence of the drugs was determined by a gamma counter.

**Data Analysis.** The plasma concentration-time profiles of the control and pretreated groups of rats were analyzed by noncompartmental methods using PCNONLIN, version 4.2 (SCI Software, Lexington, KY). The terminal half-life (t½) was calculated by dividing 0.693 by λ, the slope obtained by log linear regression of the terminal phase of the plasma concentration-time profile. The CL was calculated as the ratio of the dose to AUC. Mean residence time was obtained as the ratio of the area under the first moment curve to AUC. The volume of distribution at steady state (Vdss) was estimated as the product of CL and mean residence time. CLNR was determined as the product of clearance and the fraction of the dose excreted unchanged in the urine. The nonparametric Mann-Whitney test was used to test differences in parameter estimates. A two-sided significance level of <0.05 was considered statistically significant.

**RESULTS**

**Effect of CsA on the Disposition of Irinotecan and Its Metabolites.** Plasma concentrations of irinotecan and SN-38 in control rats after irinotecan administration were comparable with those reported previously (5) (Fig. 1). Plasma concentrations of both SN-38 and SN-38G peaked at ~10 min after irinotecan dosing, with concentrations of SN-38G exceeding that of SN-38 (Figs. 2 and 3). CsA pretreatment was associated with an increase in the irinotecan, SN-38, and SN-38G plasma concentrations (Figs. 1–3). Onset of the progressive increase in the concentrations occurred 15–30 min after irinotecan dose and was sustained during the 6-h sampling period.

There were significant alterations in the pharmacokinetics of irinotecan after pretreatment with 60 mg/kg CsA (Table 1). Across the 3.3-fold dose range, the overall increase in the AUC of irinotecan was 339%. This enhancement was paralleled by a 76% reduction in the CL and a ~3.75-fold increase in the t½. There was no change in the Vdss of irinotecan. Although the CLs of the control groups in the three dose levels were unchanged, the CLs of the three pretreated groups were significantly different (determined by three-way ANOVA). Significantly, the CL at dose 6 mg/kg was 2-fold higher compared with the pretreated group at the 20 mg/kg dose level (1.08 versus 0.53 liter/h/kg; P = 0.02). Both SN-38 and SN-38G also displayed significant enhancement in plasma availability (Table 2). The AUC of SN-38 increased by an average of 361% over the three dosage levels and that of SN-38G increased ~192%. The elimination half-lives of both the metabolites were elevated; the t½ of SN-38 was significantly higher in the pretreated groups as compared with the controls at all three dose levels. Pretreatment with a lower CsA dose (30 mg/kg) resulted in similar effects (Table 3).

Cremophor had a minor contribution in the overall enhancement of
the AUC of irinotecan and its metabolites. Pretreatment with the vehicle resulted in a 13% enhancement for irinotecan, 48% for SN-38, and 25% for SN-38G (data not shown). These increases in plasma concentrations were negligible compared with the effect of CsA.

Urinary irinotecan in control rats accounted on average for 26% of the dose, and SN-38 and SN-38G accounted for 1.6 and 3.8% of the dose, respectively (Table 4). After CsA pretreatment, there were 86, 132, and 87% average increases in the irinotecan, SN-38, and SN-38G urinary excretion, respectively. Irinotecan CL in control rats was composed of 26% CLR and 74% CLNR. There was reduction in both CLR (mean 55%) and CLNR (mean, 81%) in the pretreated groups compared with the controls. Additionally, the CLNR in the 20-mg/kg pretreated group was <50% that of the 6-mg/kg pretreated group.

There was no physical evidence of toxicity associated with CsA pretreatment. There was no evidence of increase in plasma total bilirubin [0.259 ± 0.044 mg/dl (mean ± SD); n = 3] in control rats versus 0.283 ± 0.095 mg/dl (n = 3) in pretreated rats. The urinary output for both groups of rats remained unchanged, which may indicate a lack of CsA-induced nephrotoxicity.

**Interaction of Irinotecan and its Metabolites with P-gp.** To assess the specific interactions of irinotecan, SN-38, and SN-38G with P-gp, their effect on the binding of the photoaffinity analogue of verapamil, [125I]NAS-VP, to the protein was investigated. This photoaffinity analogue has been shown to bind to P-gp specifically and with high affinity (24). As shown in Fig. 4A, 10 and 100 μM irinotecan inhibited the binding of 5 nM of [125I]NAS-VP to P-gp by 64 and 84%, respectively. The inhibitory effect of SN-38 was less than that of irinotecan, and whereas 10 μM SN-38 did not inhibit binding, 100 μM SN-38 inhibited P-gp photolabeling by 57%. Similarly, SN-38G at 10 and 100 μM inhibited the binding of [125I]NAS-VP to P-gp by 53 and 74%, respectively (Fig. 4B). In comparison, verapamil at 10 and 100 μM inhibited P-gp binding by 75 and 90%, respectively.

**DISCUSSION**

Coadministration of CsA with irinotecan resulted in a marked increase in irinotecan, SN-38, and SN-38G plasma concentrations. Pretreatment with 30 or 60 mg/kg CsA produced the same extent of enhancement, which indicates that the maximal inhibitory effect of CsA had been achieved. A more extensive study using lower doses of CsA would indicate the minimum effective dose of CsA. The vehicle Cremophor had insignificant contribution in the enhancement.

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**Fig. 2.** Plasma concentrations of SN-38 after irinotecan administration at a dose of 6 mg/kg (A), 10 mg/kg (B), or 20 mg/kg (C). The control (●) groups were given irinotecan only, and the pretreated (▼) groups were given 60 mg/kg CsA 5 min before the irinotecan dose. Data are the means; bars, SD.

**Fig. 3.** Plasma concentrations of SN-38G after 6 mg/kg (A), 10 mg/kg (B), or 20 mg/kg (C) irinotecan in control (●) groups receiving irinotecan only and in pretreated (▼) groups receiving 60 mg/kg CsA 5 min before the irinotecan dose. Data are the means; bars, SD.
Pharmacokinetic studies of irinotecan in rats have reported nonlinearity over the dose range of 2–40 mg/kg (7). In the present investigation, we observed unchanged CL, possibly because of the narrower dose range used (6–20 mg/kg). However, there was dose dependency in the groups pretreated with CsA; the highest AUC elevation occurred at the irinotecan dose of 20 mg/kg, resulting in a significantly lower CL compared with the pretreated group at 6 mg/kg. A similar trend was observed in the CLNR estimations. The observation suggests saturable elimination kinetics at high doses of irinotecan with concomitant CsA.

To elucidate the mechanism leading to reduced CL in the pretreated rats, we investigated the contribution of the renal and nonrenal components of CL. In control rats, CL is primarily composed of the nonrenal aspect, which is 3-fold that of the renal counterpart. Additionally, given that the reduction in CLNR was 1.5-fold that of CLR in the pretreated rats, the overall decrease in CL could mainly be attributed to reduced CLNR. The CLNR of irinotecan, in turn, would include metabolic conversion to SN-38 and subsequently to SN-38G, metabolism to other as-yet-unreported metabolites, tissue binding, protein binding, and biliary excretion.

There was no change in the fraction of irinotecan hydrolyzed, as indicated by constant mean AUC (ng-h/ml) ratios of SN-38 to irinotecan in the two groups at all dose levels (0.112 and 0.13, 0.072 and 0.08, and 0.072 and 0.067, respectively). However, there was an average decline of ~40% in the terminal CLNR over the dose range of 6–20 mg/kg. However, there was a dose dependency in the pretreated groups. CsA is extensively metabolized primarily by CYP450 enzymes, and the reactions mainly involved are hydroxylation, N-demethylation, cyclization, and oxidation (29). CsA has also been reported to be glucuronylated with the Phase II conjugation reaction, constituting only a minor fraction of CsA dose (30, 31). Thus, there have been no reports of CsA as a substrate of carboxyl esterase. The unchanged SN-38:irinotecan ratio suggests a lack of effect on carboxyl esterase activity. The reduction in the SN-38:SN-38 ratio could possibly be due to inhibition of UDP-GT (assuming CsA and SN-38 are substrates of the same isozyme) or to saturation of the conjugation pathway, a phenomenon that has been observed previously (4). One study isolated an unidentified metabolite from the plasma of a patient receiving irinotecan (32). However, the presence of the metabolite in a larger population of patients, its structure, and the enzyme system involved have yet to be established. Overall, on the basis of available information, CsA pretreatment did not appear to cause significant modifications in the metabolic profile of irinotecan.

The average Vdss in control rats was 3.95 liters/kg, which is >3-fold higher than the body weights of the rats (~200 g), and indicates tissue binding of irinotecan. The drug also exhibits moderate protein binding (33, 34). On the other hand, SN-38 is >95% bound to human serum albumin (33, 34). In plasma, CsA is extensively protein bound; 85–90% is bound to lipoproteins (reviewed in Ref. 35). The unchanged Vdss between the control and pretreated groups leads to the conclusion that CsA did not alter the tissue or protein binding of irinotecan, although protein binding was not directly measured. Because CsA has insignificant binding to albumin, it is unlikely to affect protein binding of SN-38.

There were significant increases in the urinary concentrations of irinotecan and metabolites in pretreated rats that were possibly secondary to elevated plasma concentrations. However, the elevations were not proportional to the concurrent elevations in AUC. For instance, an overall 4.4-fold enhancement of the plasma AUC of irinotecan across the three dose levels in pretreated rats resulted in an average of ~1.9-fold increase in the urinary elimination of unchanged irinotecan. The mean CL of irinotecan in control rats was calculated to be ~0.781 liter/h/kg, which is higher than the glomerular filtration rate of 0.324 liter/h/kg in rats (36). Thus, renal elimination would be represented by glomerular filtration, as well as by active renal secretion. The 55% reduction of CLF in the pretreated rats is suggestive of possible saturation of the active secretory component.

Hence, in summation, the significantly reduced CL of irinotecan by CsA could not be explained by altered metabolism, tissue, or protein binding and thus implicates reduced biliary excretion as the possible mechanism.
cause. Direct estimation of biliary excretion of irinotecan and its metabolites would have provided a more convincing answer. Cannulation of bile duct to measure biliary excretion will be conducted in future experiments. The elevated plasma AUCs of the two metabolites were also primarily due to reduced biliary excretion, although a fraction of the elevated SN-38 AUC could be due to inhibition and/or saturation of UDP-GT activity. The trend toward nonlinearity in CL in the pretreated groups was matched by that of decreasing CLNR. This suggests saturation of the residual biliary transport at high doses of irinotecan and CsA coadministration.

Our second objective was to determine the underlying mechanism contributing to the decreased biliary excretion. Based on the inhibition of [125I]NAS-VP binding to P-gp, irinotecan and metabolites appear to interact with P-gp, with irinotecan and SN-38G being better substrates than SN-38. However, the interaction was modest, and concentrations of the drug and metabolites required to cause significant inhibition of the binding of the verapamil analogue to P-gp were severalfold higher than those obtained in vivo in the rats. Also, the high concentration of SN-38 to inhibit P-gp labeling may not be clinically relevant. However, the low affinity binding may not necessarily reflect the efficacy of transport across the canalicul mem-
decreased biliary excretion. Although the exact mechanism(s) of the decrease is not evident, CsA-related cholestasis could be one of the factors. Clinically, concomitant CsA and irinotecan treatment would allow dose reduction (about 20 to 50% of the maximal tolerated dose) and still achieve comparable systemic exposure of irinotecan and metabolites. More importantly, the risk of intestinal toxicity could be substantially reduced because of: (a) a reduced dose; and (b) lowered biliary accumulation and excretion. It is interesting that concomitant CsA increases “biliary index” by >500%, which conflicts with our previous report (4) of dose-limiting diarrhea as directly related to higher index value. However, the applicability of biliary index is valid only when biliary excretion is unhindered, where an increase in the systemic availability of the compounds would be reflected in the bile. Therefore, CsA coadministration could potentially increase the irinotecan therapeutic index by decreasing the intestinal toxicity associated with prolonged dosing. In addition, to determine possible enhancement of antitumor activity, studies of the effect of concomitant administration of CsA and irinotecan in mice bearing human tumor xenografts are currently under way.

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