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SHORT COMMUNICATION

Modulation Effects of Cyclosporine on Etoposide Pharmacokinetics and CNS Distribution in the Rat Utilizing Microdialysis

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ABSTRACT. In the present study, we evaluated the pharmacokinetics of the chemotherapeutic agent etoposide (ET) under steady-state conditions and examined its extent of distribution into the CNS of conscious animals. An i.v. infusion of 15 mg/kg/hr was administered to nine rats. Each of the nine rats also received the potent multidrug resistance (MDR) modulator cyclosporine (CSA). Upon the addition of CSA, the i.v. treated animals demonstrated a 53% decrease in ET clearance. This decrease resulted in a greater than 2-fold increase in the steady-state concentrations of ET. The corrected brain-blood ratio (BBR_{corr}) was 0.36 ± 0.18 prior to CSA treatment, and although CNS concentrations increased upon the addition of CSA, there was no increase in the BBR_{corr} (0.24 ± 0.10). The present study demonstrates that the increase of ET in the CNS following CSA is a result of a decrease in ET systemic clearance and not an inhibition of ET efflux from the CNS. BIOCHEM PHARMACOL 51;7: 987–992, 1996.

KEY WORDS. etoposide; pharmacokinetics; microdialysis; central nervous system (CNS); P-glycoprotein; multidrug resistance

The MDR[‖] phenomenon includes cross-resistance among the naturally derived cancer agents, e.g. anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxins (ET, teniposide), Vinca alkaloids (vinblastine, vincristine), taxanes (taxol, taxotere), and various other lipophilic compounds [1]. This drug resistance is associated with the *MDR1* gene, which encodes for a protein known as P-gp (160–180 kDa). This protein has been shown to act as an energy-dependent efflux pump that transports substrates out of the cell before cytotoxic effects can occur. Recently, P-gp has been shown to be expressed in several normal tissues including the CNS, kidney, liver, and colon [1–7]. More specifically, expression has been detected at the renal proximal tubule in the kidney, the biliary canalicular membrane of the liver and the endothelial cells at the BBB and blood–testes barrier. Expression of this membrane-bound protein at these localized regions in the kidney and liver suggests a secretory role of P-gp. Alternatively, expression at the BBB suggests that P-gp serves as a pump to eliminate toxic or potentially harmful compounds out of the CNS.

In rodents, there are two different drug-transporting P-gps that seem to function in a manner similar to that of human MDR1 P-gp. These proteins are designated as mdr1a (or mdr3) and mdr1b (or mdr1). In rodents, the *mdr1a* gene is highly expressed at the BBB [8]. Mice homozygous for disruption of the *mdr1a* gene have been shown to be sensitive to the toxic effects of P-gp substrates. Studies with vinblastine demonstrated an increased accumulation of vinblastine in brain tissues of *mdr1a* gene-deficient animals. These animals also showed profound alterations in pharmacokinetics and tissue distribution when compared with controls, although detailed CNS distribution studies were not performed [9].

Clinical studies in humans, combining MDR modulators with the chemotherapeutic agents, have shown toxicities consistent with enhanced uptake of these drugs in the CNS. These data are complicated by the fact that the majority of the MDR modulators also alter the pharmacokinetic profile of the chemotherapeutic agent. The pharmacokinetic effects that MDR modulators have on these chemotherapeutic agents have been best characterized with CSA and its analogs (i.e. PSC-833), which are among the most potent inhibitors of the MDR transporter [10]. Evidence suggests that these toxicities are due to an increase in the CNS drug concentrations of the neoplastic agent(s), although the mechanism of this increased concentration has yet to be delineated. This evidence has led to the current study in which the objective was to determine, utilizing the microdialysis technique, if CSA causes an increase in CNS concentrations of ET by inhibiting its active trans-

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‖ *Abbreviations:* ET, etoposide; CSA, cyclosporine; MDR, multidrug resistance; BBB, blood–brain barrier; BBR_{corr}, brain–blood ratio corrected; P-gp, P-glycoprotein; and $f_{u,s}$, fraction of drug unbound in serum.

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port out of the CNS, and/or if CSA causes an alteration (decrease) in the systemic clearance of ET resulting in increased CNS concentrations.

MATERIALS AND METHODS

Chemicals

ET (Vepesid) and teniposide (VM-26) were obtained from the Bristol Myers Co., Wallingford, CT. CSA (Sandimmune) was purchased from the Sandoz Pharmaceutical Corp., East Hanover, NJ; and antipyrine from the Ransdell Co., Louisville, KY.

Animals

Adult male Sprague–Dawley rats, weighing between 340 and 500 g, were obtained from Harlan Laboratories (Indianapolis, IN) and utilized in all *in vivo* experiments. Animals were maintained under a 12:12 hr light:dark cycle and had access to food and water *ad lib.* prior to the studies.

Drug Administration and Sampling Protocol

ET drug solution was prepared by diluting commercial Vepesid (20 mg/mL ET) 1:1 with 0.9% NaCl. Cannulas were implanted into the femoral vein (serial blood sampling) and femoral artery (ET administration) prior to the microdialysis probes. ET was administered as an i.v. infusion (15 mg/kg/hr) for 9 hr to nine adult male Sprague–Dawley rats. Serial blood samples were collected hourly beginning at the third hour after initiating the ET infusion. Rats also received either 5 mg/kg CSA i.v. (N = 7) 4.5 hr after initiating the ET infusion or 5 mg/kg CSA i.p. (N = 2) b.i.d. for 3.5 days prior to initiating the infusion of ET. This CSA dose has been shown previously to allow for MDR reversing levels of CSA to be achieved in the rat [11].

In Vivo *Microdialysis*

An artificial cerebral spinal fluid (pH 7.35) was prepared and contained (in mM): Na, 155; K, 2.9; Ca, 2; Mg, 0.7; Cl, 138; HCO_3, 25; and glucose, 6.0. In all experiments, delivery of the microdialysate was accomplished by a microinjection pump (CMA/100; Carnegie Medicin) at 2 μL/min. Dialysate samples were taken very 30 min. Commercial probes (4 mm in length) were utilized in the CNS (CMA/12; Carnegie Medicin) and jugular vein (CMA/20 soft probe; Carnegie Medicin). For brain microdialysis probe implantation, the rat was mounted in a Kopf stereotaxic frame. Supported by a intracerebral probe guide, the CMA/12 microdialysis probe was implanted into the frontal cortex with the coordinates of 1.0 mm lateral and 3.0 mm anterior to bregma. All surgeries were performed under anesthesia with ketamine (85 mg/kg, i.p.) and acepromazine (1.6 mg/kg, i.p.). Anesthesia was maintained with periodic intramuscular injections of this combination as needed. Body temperature was maintained at 37° with an electric heating pad checked via a rectal thermometer. Animals were allowed to recover for approximately 24 hr prior to initiating the experiment.

Retrodialysis

Relative ET microdialysis probe *in vivo* recovery was estimated utilizing antipyrine as a retrodialysis marker. Corrected ET concentrations were obtained by dividing the ET concentrations from the dialysate by the percent loss of antipyrine as it flowed through the respective probe.

Data and Statistical Analysis

Serum concentrations were averaged at 3 and 4 hr and 8 and 9 hr to obtain pre- and post-treatment steady-state levels of ET, respectively. Similarly, dialysate concentrations were averaged at various times to obtain the unbound concentration at steady-state, 3.5–4.5 hr pre-treatment cyclosporine, and 7.5–9 hr post-treatment cyclosporine. The CL_s was calculated from $CL_s = Ro/C_{ss}$, where Ro is the rate of infusion and C_{ss} is the concentration at steady-state, respectively. The $CL_{u,corr}$ (corrected unbound clearance) was calculated from the $CL_{u,corr} = Ro/C_{ss,unbound}$ from the jugular dialysate at the times noted above. The BBR_{corr} (brain–blood ratio corrected) was calculated as follows: $BBR_{corr} = brain[ET]_{corr}/jugular[ET]_{corr}$, as determined from the microdialysis probes. Data are displayed as means ± SD. Statistics were performed utilizing the Wilcoxon Signed Rank test with the *a priori* level of significance set at $P < 0.05$.

ET Protein Binding Determinations

Protein binding *ex vivo* for serum was determined for ET by equilibrium dialysis from samples obtained from individual rats (N = 4) used in this study. Protein binding was performed in both the absence and presence of CSA. Briefly, serum samples were dialyzed in Plexiglass cells against 0.133 M, pH 7.2 phosphate buffer at 37° for 5 hr. The fluids in the dialysis cell were separated by a Spectra por 2 dialysis membrane (Spectrum Medical Industries, Inc., Terminal Annex, LA) (mol. wt cutoff = 12,000–14,000). At the end of dialysis, serum and buffer samples were collected and analyzed for ET. The $f_{u,s}$ was calculated as the ratio of buffer to serum drug concentrations.

Assay Methodology

ET was analyzed by a modification of the HPLC method described by Stiff *et al.* [12]. Dialysate samples were analyzed by a specialized microbore HPLC system that allows determination of small sample volumes, i.e. 2–5 μL. Chromatography was performed on an HPLC component system consisting of a CMA/200 microsampler, an FL-45 detector, and a PM-80 pump [Bioanalytical Systems (BAS), Inc., West Lafayette, IN]. The mobile phase consisted of 10 μM

ammonium acetate buffer, pH 5.0–5.5:methanol (54:46), and the effluent was monitored by fluorescence detection at Ex 230 nm and Em 325 nm, respectively. Separations were carried out on a C_{18} (5 μm particle size) 100 × 1.0 mm column (BAS) with a flow rate of 0.1 mL/min. Assay sensitivity was 15 ng/mL with a 5-μL injection (70 pg on column). Rat serum samples were extracted with methylene chloride utilizing Teniposide as the internal standard. Antipyrine was also quantified by HPLC via modification of Brouwer *et al.* [13].

RESULTS

Infusion regimens in the rats resulted in the achievement of steady-state concentrations for ET by 3 hr (Fig. 1A). Similarly, after the addition of CSA most of the animals obtained steady state by approximately 7.5 hr. Figure 1A also demonstrates that ET dialysate concentrations for both CNS and jugular paralleled each other, with CNS concentrations being much lower than jugular. In the i.v. studies, CSA (5 mg/kg) was given as a bolus dose over a 3 to 5-min period at the 4.5-hr time point. Upon the addition of CSA i.v., both the jugular and CNS dialysate concentrations increased dramatically (Fig. 1A). This pattern was similar in all of the animals studied. In four of the seven animals, blood was also taken for ET analysis and protein binding determinations. Figure 1B demonstrates that serum ET concentrations also paralleled the jugular dialysate concentrations.

The mean pre $CL_{u,corr}$ of 290 mL/min/kg (±404) was reduced significantly upon the addition of CSA when compared with the post $CL_{u,corr}$ of 83 mL/min/kg (±90) (Table 1). Although there was a large variation between the animals, all the animals showed a decrease in the $CL_{u,corr}$ upon the addition of CSA. On the average, there was a 53% decrease in the $CL_{u,corr}$. This decrease resulted in a greater than 2-fold increase in the jugular dialysate concentrations. This increase in jugular dialysate concentrations resulted in a substantial increase in the CNS dialysate concentrations (94% mean increase). Although concentrations in the CNS increased, there was no increase in the BBR_{corr} for ET (Table 1). The mean BBR_{corr} for ET prior to the CSA treatment was 0.36 (±0.18), as compared with 0.24 (±0.10) for the treated group.

Comparisons of the systemic clearance (CL_s) showed a trend similar to that of the microdialysis data (Table 2), with a substantial decrease in the elimination of ET observed. Values of $f_{u,s}$ in the rats demonstrated that CSA had no effect on ET binding to serum proteins (Table 2), with a mean pre $f_{u,s}$ of 0.34 (±0.07) and a post $f_{u,s}$ of 0.33 (±0.04), respectively.

DISCUSSION

Microdialysis is a technique that samples unbound substances in extracellular fluid, and has been utilized in numerous pharmacokinetic studies including those on the effects of cocaine, acetaminophen, and ethanol [14–16]. Microdialysis has also been used to compare the kinetics of substances in the brain with those in the blood. In previous studies, we demonstrated that comparisons of Adinazolam and its N-demethylated metabolite in dialysates from brain and blood revealed a BBR of 0.63 and 0.37, respectively [17]. Other authors [18], have shown that after intravenous aluminum citrate injection, aluminum has a BBR of 0.12 for the frontal cortex. Similarly, other studies have shown that compounds like diazepam have a BBR equal to 1 [19]. These studies demonstrate the usefulness of the microdialysis technique in evaluating the distribution and pharma-

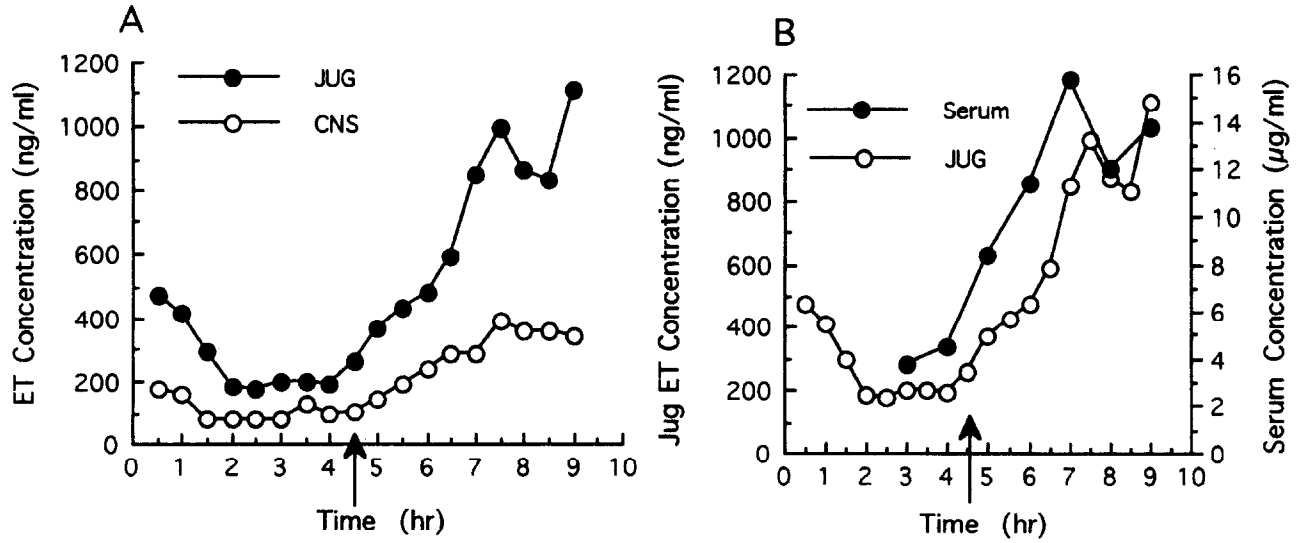

FIG. 1. (A) Corrected ET concentrations versus time in dialysates from probes implanted in the jugular vein (●) and frontal cortex (○) of a representative animal (rat No. 15). The arrow denotes when 5 mg/kg of CSA was given. (B) Plot of serum (●) and jugular dialysate (○) ET concentrations versus time in a representative animal (rat No. 15). The arrow denotes when 5 mg/kg of CSA was given.

TABLE 1. Corrected unbound systemic clearance ($CL_{u,corr}$) and brain–blood ratio corrected (BBR_{corr}) parameters for ET as determined by microdialysis, both pre and post CSA treatment i.v., for each individual rat (N = 7)

Rat. No.	Pre $CL_{u,corr}$ (mL/min/kg)	Post $CL_{u,corr}$ (mL/min/kg)	$CL_{u,corr}$ (% decrease)	Pre BBR_{corr}	Post BBR_{corr}
13	141	48	66	0.58	0.21
14	211	76	64	0.33	0.22
15	1168	282	76	0.49	0.39
16	38	24	27	0.18	0.14
17	67	41	39	0.16	0.13
18	360	76	79	0.54	0.36
19	42	34	19	0.21	0.22
Mean (±SD)	290 (404)	83* (90)	53 (24)	0.36 (0.18)	0.24* (0.10)

* $P < 0.05$ (Wilcoxon Signed Rank).

cokinetics of various xenobiotics. In this study, we have utilized the microdialysis technique for the first time to evaluate the pharmacokinetics of ET and its CNS distribution, in the presence and absence of a P-gp modulator (CSA).

To date, several known modulators of P-gp have been identified, including verapamil, CSA, PSC-833, and tamoxifen. In this report, we demonstrated that CSA does modulate the pharmacokinetics of ET in the rat. *In vitro* studies have shown that CSA is a potent modulator of P-gp at concentrations of 2–3 μM [1]. In addition, clinical studies have been undertaken to evaluate the modulation effects of CSA on various neoplastic agents including ET [20]. Recent clinical data suggest that moderate to severe CNS toxicities have been associated with the addition of various P-gp modulators [1,21]. Authors have attributed these toxicities to an inhibition of P-gp at the BBB, although human studies have significant limitations and cannot confirm this hypothesis. The establishment of an animal model to evaluate various modulators and their effects on various neoplastic agents is critical to a better understanding of the role that P-gp plays in the transport of neoplastic agents at the BBB, with regard to therapeutics and potential side-effects that might occur during the inhibition or alteration of P-gp.

In the present study, we demonstrated that CSA is a potent modulator of ET *in vivo*, decreasing the CL_u on the average by 53%. There was considerable interanimal variation in the $CL_{u,corr}$ for ET (Table 1) with the change in pre-treatment $CL_{u,corr}$ highly correlated with the pre-treatment $CL_{u,corr}$ value ($r^2 = 0.999$). The most dramatic changes were associated with the animals that had the highest clearances pre-treatment. Current data in our laboratory suggest that CSA inhibits the variable portion of ET clearance in the rat. This could possibly be due to the various expression of P-gp (i.e. at the canalicular membrane in the liver and/or renal proximal tubule) within this animal population and/or cytochrome P450 levels. Protein binding data suggest that CSA had no effect on ET binding to serum proteins. This observation is important since clinical trials combining CSA with ET have shown significant increases in the volume of distribution of ET when compared with ET alone. The current data suggest that the increased ET volume of distribution observed with the addition of CSA is possibly the result of MDR modulation and enhanced uptake of the drug into tissues and not an alteration in the binding of ET to serum proteins.

The significant decrease observed in the clearance of ET with CSA gives evidence that CSA must be inhibiting the elimination of ET, possibly through the inhibition of P-gp transport at the hepatic canalicular membrane and/or renal tubule. Alternatively, CSA could be inhibiting the metabolism of ET via cytochrome P450 [22]. In our studies, CSA doses above 5 mg/kg in combination with ET resulted in significant toxicity, complicating pharmacokinetic analysis; thus, increased CSA doses could not be evaluated with this ET dosing regimen. Although CSA levels were not quantified in that study, previous authors have shown that CSA

TABLE 2. CL_s parameters for ET, both pre and post CSA treatment i.v., for each individual rat (N = 4), and protein binding determinations

	Pre CL_s (mL/min/kg)	Post CL_s (mL/min/kg)	Pre $f_{u,s}$	Post $f_{u,s}$
13	30	11	0.41	0.34
14	22	11	0.29	0.31
15	61	19	0.27	0.30
18	63	15	0.40	0.38
Mean (±SD)	44 (21)	14* (4)	0.34 (0.07)	0.33 (0.04)

*$P < 0.05$ (Wilcoxon Signed Rank).

at 2 mg/kg (i.v. bolus) decreases the renal clearance of colchicine by approximately 50% without significantly affecting the glomerular filtration rate [11]. They also showed that CSA at 10 mg/kg had no effect on *p*-aminohippurate or ranitidine renal clearance, suggesting that CSA must be inhibiting another transporter other than the organic anion or cation transporter (i.e. P-gp). Indirect evidence for the role of P-gp in biliary drug excretion comes from *in vitro* experiments using canalicular membranes of hepatocytes in which daunorubicin transport across these membranes is a unidirectional process that can be inhibited by classical MDR modulators (i.e. verapamil) [23]. Another study evaluated tissue distribution changes with doxorubicin when combined with CSA in a similar rat model. In this study, higher doses of CSA (12.5 and 25 mg/kg) were given via an i.p. injection. The doxorubicin was then administered i.v. 30 min after the CSA administration. These authors also observed marked increases in doxorubicin concentrations in the liver, adrenals, and kidney. The study failed to show doxorubicin distribution changes in the brain after CSA. This study also did not measure CSA levels in these rats [24].

The BBR_{corr} for ET prior to CSA treatment was 0.36. A BBR of less than unity at steady state for ET suggests that ET is actively transported out of the CNS, possibly through P-gp, which has been identified on endothelial cells associated with the BBB in normal rat brain [5]. After i.v. treatment with CSA, there was no increase in BBR_{corr} for ET (0.24). In this study, CSA had no effect on the efflux of ET out of the CNS. This was in spite of the fact that CSA decreased the elimination of ET. These results also suggest that the increase in levels in the CNS in the rat are associated with an increase in the steady-state serum concentrations and not an inhibition of P-gp at the BBB. In addition, two of the nine animals were dosed with CSA at 5 mg/kg i.p. b.i.d. for 3.5 days prior to the infusion of ET, and these animals showed a BBR similar to that of animals in the i.v. experiments (0.33 and 0.34), showing that multiple dosing of CSA also had no effect on the BBR of ET in the rat.

In the current study, the interaction of a P-gp modulator (CSA) and its effects on the CNS distribution and elimination of ET are described. We conclude that CSA has no effect on the BBR of ET, but is a potent inhibitor regarding the elimination of ET. We also describe for the first time an animal model utilizing microdialysis to evaluate P-gp modulators and their effects on chemotherapeutic agents. Currently other P-gp modulators in combination with ET and other MDR-related chemotherapeutic agents are being evaluated in this system. These studies may lead to a better understanding with respect to the physiological role P-gp plays in the CNS as well as help to predict and explain toxicities seen with other MDR modulators.

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