Microdialysis Assessment of Microfibrous Collagen Containing a P-Glycoprotein-Mediated Transport Inhibitor, Cyclosporine A, for Local Delivery of Etoposide

Hitoshi Sato,[1] Hidenori Kitazawa,[1] Isao Adachi,[1] and Isamu Horikoshi[1,2]

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Purpose. This study was designed to assess a local drug delivery system of an anticancer agent, etoposide (VP-16), using microfibrous collagen as a drug carrier. For this objective, the microdialysis method was utilized to investigate the local pharmacokinetics of VP-16.

Methods. Microfibrous collagen sheets (CS) containing 20 mg/kg of VP-16 with and without 40 mg/kg of cyclosporine A (CyA) were prepared and applied on the liver surface of rats. VP-16 concentrations in the liver extracellular fluid (ECF) were monitored by a microdialysis method.

Results. The local application of CS containing VP-16 resulted in a relatively long maintenance of drug concentrations in the liver ECF, with very low concentrations in plasma. The inclusion of CyA in the CS resulted in 2-fold and 3-fold increases of the AUC and MRT values of VP-16 in the liver ECF, respectively. The liver ECF-to-plasma AUC ratios of VP-16 were 32–39 and 0.17 with local CS application and iv administration, respectively, indicating a remarkable advantage of the local drug delivery system. A pharmacokinetic interaction experiment suggested that the observed increase of the liver ECF concentrations of VP-16 with CyA resulted from inhibition of the biliary excretion of VP-16 by CyA.

Conclusions. We found that the local delivery of the CS containing CyA on the liver surface is advantageous in terms of the extent and duration of liver ECF drug concentrations, when CyA was included in the CS. The effect of CyA was probably derived from the inhibition of P-glycoprotein-mediated biliary excretion of VP-16 by CyA. The usefulness of the microdialysis technique for the assessment of the local drug delivery system was also demonstrated.

KEY WORDS: etoposide; cyclosporine A; local drug delivery using collagen; microdialysis.

INTRODUCTION

There is considerable current interest in drug delivery improvement for increasing therapeutic efficacy and reducing systemic side effects. Especially, anticancer chemotherapies generally exhibit severe side effects that limit their therapeutic use. One of the major approaches to decrease the systemic side effects of anticancer agents is the local drug application to diseased organs or tissues using an appropriate drug carrier. Several types of local drug carriers have been developed and evaluated including liposomes, microspheres, fibrin, and collagen (1,2). However, the pharmacokinetic behaviors of drugs applied locally with any of these drug carriers have not been well described in a quantitative manner, although such information is required to assess the possibilities and limitations of a drug delivery system on a pharmacokinetic basis. One of the serious problems with cancer chemotherapy is the development of multiple drug resistance (MDR) in malignant tumor cells. MDR has been characterized by diminished drug accumulation in resistant cells, derived from an increased rate of drug efflux by P-glycoprotein. We therefore assessed the microfibrous collagen sheets as a local release carrier of an anticancer agent, etoposide (VP-16), using a tissue microdialysis method to monitor drug concentrations in the rat liver extracellular fluid (ECF). The effect of a P-glycoprotein inhibitor, cyclosporine A (CyA), on the extent and duration of VP-16 exposure to the liver was examined with the goal of blocking the biliary excretion of VP-16 *via* the P-glycoprotein using the collagen preparation containing CyA together with the anticancer agent.

MATERIALS AND METHODS

Materials

Microfibrous bovine dermal collagen sheets (Aviten®) were supplied by Zeria Pharm. (Tokyo, Japan). Injection solutions of etoposide (VP-16) and cyclosporine A (CyA) were obtained from Bristol-Myers Squib Co. Ltd. (Tokyo, Japan) and Sandoz Pharma (Basel, Switzerland), respectively. 5,5-Diphenylhydantoin (phenytoin) sodium salt as an internal standard of VP-16 was purchased from Nacalai Tesque Inc. (Kyoto, Japan). CyA powder was a gift from Sandoz Pharma. Dialysis membrane used for *in vitro* dissolution tests was a reclaimed cellulose membrane (Seamless Cellulose Tubing, Sanko, Tokyo, Japan); its molecular weight cutoff was 12,000–14,000. Microdialysis probes (CMA/20) were obtained from CMA/Microdialysis AB (Stockholm, Sweden); their total length was 24 mm, and the length of the dialysis site was 10 mm; the molecular cutoff of the polycarbonate membrane was 20,000. All other reagents were commercially available and of analytical grade.

Animals

Wistar male rats (250–300g) were obtained from Sankyo Laboratory Co., Ltd. (Toyama, Japan) and allowed free access to standard rodent chow and water.

Preparation of Collagen Sheets Containing Drug(s)

Samples were prepared by adding 20 mg/kg of VP-16 solution (20 mg/ml) to 50 mg of microfibrous collagen sheets (CS) with and without 40 mg/kg of CyA powder dissolved with VP-16 solution. The drug-containing CS were then dried for 3 days at room temperature.

In Vitro Release Profiles of VP-16 from the Collagen Preparations

The *in vitro* release of VP-16 from the CS preparations (1.5 cm × 1.5 cm, 50 mg) was examined by a dialysis method using the reclaimed cellulose membrane tube. A sample (CS or solution as control) was enclosed into the dialysis tube with 1 ml of phosphate buffered saline (PBS), and the tube was

[1] Department of Hospital Pharmacy, Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan.
[2] To whom correspondence should be addressed.

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constantly dialyzed against 100 ml of PBS under constant stirring at room temperature.

Plasma Protein Binding of VP-16

The plasma protein binding of VP-16 (10 or 100 μg/ml) was determined using a micropartition system, Centrifree® (Amicon, MA, USA) in the presence and absence of CyA (10 or 100 μg/ml); the separation of unbound drug was performed by ultrafiltration for 5 min at 4,000 rpm, at room temperature.

Recovery Ratios of VP-16 Across Microdialysis Probes

For *in vitro* recovery of microdialysis, VP-16 was dissolved in Ringer's solution (10 ml) at the final concentration of 100 μg/ml; a microdialysis probe was put into the medium and the microdialysis was started at a constant flow rate (3 μl/min) of Ringer's solution using an automatic infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA). After an adequate lag time (20 min), the dialysate was collected for 1 hr and the concentration of VP-16 was determined by high-performance liquid chromatography (HPLC) as described later. The *in vitro* recovery ratio (R_{vitro}) of VP-16 across the microdialysis probe in Ringer's solution was calculated by the following equation:

$$R_{vitro} = \frac{\text{dialysate concentration}}{\text{surrounding concentration}} \quad (1)$$

For *in vivo* recovery, a microdialysis probe was inserted into the rat liver (center of the right lobe, in parallel with its both surfaces) and fixed by a surgical adhesive, Aron Alpha (Sankyo Co. Ltd., Tokyo, Japan), under anesthesia with sodium pentobarbital. Ringer's solution containing VP-16 (100 μg/ml) was passed through the microdialysis probe at a constant flow rate (3 μl/min) using an infusion pump. One hour after the probe implantation as a stabilization period, the dialysate was collected for 1 hr and the inlet (C_{in}) and outlet (C_{out}) concentrations of VP-16 were determined by HPLC. The *in vivo* recovery ratio (R_{vivo}) of VP-16 across a microdialysis probe in the liver ECF was calculated by the following equation:

$$R_{vivo} = 1 - \frac{\text{effluent dialysate concentration}}{\text{influent dialysate concentration}} \quad (2)$$

The above equation can be derived assuming that the drug recovery from the tissue to the perfusate is the same with the drug loss from the perfusate to the tissue, across the probe membrane. This assumption was utilized for the theoretical justification of the "retrodialysis method" (3) to measure *in vivo* recovery ratio of the microdialysis.

Local Pharmacokinetics of VP-16 Using Microdialysis

Rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the liver was exposed with a midline abdominal incision. The jugular and femoral veins were cannulated with polyethylene tubing (Intramedic PE-50, Clay Adams, Parsippany, NJ, USA). Then, a CS preparation (1.5 cm × 1.5 cm, 50 mg) containing 20 mg/kg of VP-16 with or without 40 mg/kg of CyA was applied on the back surface of the right lobe (n = 4). For liver microdialysis, a microdialysis probe was gently inserted into the center of the right lobe in parallel with the attached CS preparation, and connected to an infusion pump (Model 22, Harvard Apparatus) perfusing Ringer's solution at the flow rate of 3 μl/min. Blood samples were collected from the jugular vein cannula, and effluent dialysate was collected every 1 hr using an automatic fraction collector (DC-1000, Eyela, Tokyo, Japan). As a control experiment, VP-16 and CyA were intravenously injected through the femoral vein cannula (n = 4). Obtained dialysate and plasma samples were stored at −20°C until analysis. Each microdialysis probe was used only once.

Effect of CyA on the Plasma Clearance and Biliary Excretion of VP-16

Rats were cannulated at the femoral artery and vein with PE-50 tubing, the common bile duct with PE-10, and the urine bladder with PE-200 under light ether anesthesia, and placed in a restraining cage. After recovery from anesthesia, the rats received an iv injection of VP-16 (5 mg/kg) with or without CyA (10 mg/kg); the drugs were dissolved in polyethylene glycol 200 containing 10% (v/v) ethanol and mixed immediately before the injection. In the case of VP-16-only injection, the drug solution was mixed with the vehicle. Bile and urine were collected for the indicated time intervals, and blood (approximately 100 μl) was drawn at the middle points of the bile/urine collections after the dosing. Obtained plasma, urine, and bile samples were stored at −20°C until analysis.

Chemical Analysis of VP-16

The concentrations of VP-16 in the dialysate and plasma samples were determined by HPLC (4). Dialysates from the liver microdialysis were injected directly into the HPLC system without any treatment. A portion of plasma samples (100 μl) was added with 5 μl of an internal standard (5,5-diphenylhydantoin), and mixed with chloroform (0.5 ml); the solution was shaken for 10 min, and centrifuged at 3,000 rpm for 10 min. The supernatant was then evaporated under reduced pressure at 50°C. The resultant residue was dissolved in 120 μl of the mobile phase solution, methanol/water (51/49, v/v). An aliquot (100 μl) of the reconstituted samples was loaded into a solvent delivery system equipped with a UV detector (LC module 1, Waters Co. Ltd., Milford, MA, USA) and a data processor (Waters 741). The HPLC conditions were as follows: column; 4.6 × 150 mm stainless-steel column packed with COSMOSIL C_{18} (Chemco, Osaka, Japan), flow rate; 1.0 ml/min; and detection wavelength, 229 nm.

Data Analysis

The concentration of VP-16 in the liver ECF (C_{ECF}) was calculated as:

$$C_{ECF} = \frac{\text{effluent dialysate concentration}}{R_{vivo}} \quad (3)$$

The biliary excretion clearances (CL_{bile}) of VP-16 at designated time intervals were calculated as follows:

$$CL_{bile} = \frac{\text{excretion rate (μg/min)}}{\text{plasma conc. (μg/ml)}} \quad (4)$$

Table I. Plasma Protein Binding and *In Vivo* Recovery Ratio of VP-16 in the Absence or Presence of CyA, and *In Vitro* Recovery Ratio of VP-16 in Ringer's Solution Across the Microdialysis Probes. Mean ± SD (n = 4)

	VP-16 only		VP-16 + CyA	
VP-16 concentration (μg/ml)	10	100	10	100
CyA concentration (μg/ml)	—	—	20	200
Unbound fraction in plasma	16.9 ± 1.3	15.2 ± 1.4	17.1 ± 0.7	14.9 ± 1.9
In vivo recovery ratio in the liver ECF	15.2 ± 1.8	ND	14.7 ± 1.6	14.4 ± 2.0
In vitro recovery ratio in Ringer's solution	32.9 ± 1.5[a]	ND	ND	ND

Note: ND, Not determined ($p < 0.01$).
[a] Significantly different from *in vivo* recovery ratio in the liver ECF, as assessed by Student's t-test.

where plasma concentration of the drug was defined as that observed at the middle time of the bile collection interval; excretion rate was defined as the amount of drug (μg) excreted in bile divided by the time interval (min).

For the kinetic comparison of experimental data, non-compartmental pharmacokinetic parameters (i.e., area under the curve, AUC; mean residence time, MRT) were calculated by moment analysis.

Statistical Analysis

Significant differences between the observed data were assessed by Student's t-test or Welch's t-test.

RESULTS

In Vitro Release Profiles of VP-16 from the Collagen Preparation

The half-lives of VP-16 release from CS and PBS were 224 ± 38 min and 286 ± 56 min (mean ± SD; n = 4), respectively, indicating the lack of sustained release of VP-16 from the CS preparation. Moreover, the addition of CyA in the CS did not influence the releasing profile of VP-16 (i.e., a half-life of 220 ± 28 min).

Plasma Protein Binding of VP-16

The plasma protein binding of VP-16 in the absence and presence of CyA is listed in Table I. The fraction of VP-16 unbound in rat plasma (f_p) was determined as 15–17%, and was not changed with the VP-16 concentration or in the presence of CyA.

Recovery Ratios of VP-16 Across Microdialysis Probes

The *in vitro* and *in vivo* recovery ratios of VP-16 across the microdialysis probes are summarized in Table I. The R_{vivo} in the liver ECF (14–15%) was not changed with the VP-16 concentration or in the presence of CyA. In contrast, the R_{vitro} in Ringer's solution (33%) was significantly higher ($p < 0.001$) than the recovery ratio in the liver ECF.

Local Pharmacokinetics of VP-16 Using Microdialysis

The concentration of VP-16 *vs.* time profiles in the liver ECF and plasma after local drug delivery are presented in Fig. 1. A temporary elevation of the VP-16 concentration in the ECF was observed 1–2 hr after the application of the CS to the liver surface. In contrast, VP-16 concentrations in plasma were significantly lower ($p < 0.01$ with Welch's t-test) than those in the liver ECF after the local CS administration, indicating an advantage of the local drug application with CS to produce higher local concentrations and lower plasma concentrations. The concentration of VP-16 in the ECF was maintained at relatively high levels (>10 μg/ml) for at least 24 hr when CyA was added to the CS preparation together with VP-16, while VP-16 was not detected at 24 hr in the absence of CyA. The plasma and liver ECF concentrations of VP-16 declined more rapidly after iv administration, as shown in Fig. 1C. The non-compartmental pharmacokinetic parameters, as well as the liver-to-plasma AUC ratios, are listed in Table II. All of these parameters were in the rank order of; CS application with CyA > CS application without CyA > iv administration with CyA. The significant differences among these groups are indicated in Table II. There was not a significant difference in the AUCs between the presence and absence of CyA in the CS preparation because of the relatively large inter-individual variation; however, the average AUCs in the plasma and liver ECF were increased by 2–3 fold in the presence of CyA, while the liver-to-plasma AUC ratios were not changed.

Effect of CyA on the Plasma Clearance and Biliary Excretion of VP-16

As shown in Figs. 2A and 2B, the iv co-administration of CyA with VP-16 significantly increased the plasma concentrations (A) and decreased the biliary excretion clearances (B) of

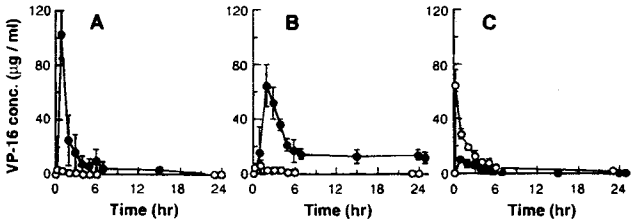

Fig. 1. Plasma (○) and liver ECF (●) levels of VP-16 after local application of collagen sheet (CS) preparations to rat liver surface in the absence (A) or presence (B) of CyA, and after bolus intravenous administration of VP-16 with CyA (C). The doses of VP-16 and CyA are 20 mg/kg and 40 mg/kg, respectively, in all the cases. Each point and vertical bar represent the mean ±SD of 4 rats.

Table II. Pharmacokinetic Parameters of VP-16 in Plasma and Liver ECF After Local Application of Collagen Sheet (CS) Preparations to Rat Liver Surface in the Absence or Presence of CyA, and After Bolus Intravenous Administration of VP-16 with CyA. Mean ± SD (n = 4)

	VP-16 + CS		VP-16 + CyA + CS		VP-16 + CyA (iv)	
Parameter	Plasma	liver ECF	Plasma	liver ECF	Plasma	liver ECF
AUC (hr·μg/ml)	6.70 ± 2.90[a]	207 ± 107[b]	16.1 ± 14.3[c]	451 ± 115[a]	170 ± 24	29.1 ± 7.3
MRT (hr)	1.62 ± 0.25[a]	3.79 ± 1.56	1.98 ± 0.46[d]	10.1 ± 1.8[a,e]	4.66 ± 0.85	2.31 ± 0.44
AUC ratio[f]	32 ± 16[a]		39 ± 18[a]		0.18 ± 0.04	

[a] Significantly different from VP-16 + CyA (iv) ($p < 0.01$) with Welch's t-test.
[b] Significantly different from VP-16 + CyA (iv) ($p < 0.05$) with Welch's t-test.
[c] Significantly different from VP-16 + CyA (iv) ($p < 0.01$) with Student's t-test.
[d] Significantly different from VP-16 + CyA (iv) ($p < 0.05$) with Student's t-test.
[e] Significantly different from VP-16 + CS ($p < 0.01$) with Student's t-test.
[f] Liver ECF-to-plasma AUC ratio.

VP-16 for all the periods of time examined. The total plasma clearances (CL_{tot}) of VP-16 were calculated as 19.2 ± 5.1 and 6.7 ± 1.4 ml/min/kg in the absence and presence of CyA, respectively. Thus, CyA co-administration produced as much as 65% suppression of CL_{tot}. In contrast, the volume of distributions (Vd_{ss}) were 647 ± 90 and 912 ± 148 ml/kg in the absence and presence of CyA, respectively; thus, a modest (40%) increase of Vd_{ss} was caused by CyA. Moreover, as clearly shown in Fig. 2B, CL_{bile} values for all the time periods were significantly decreased by 80–90% when CyA was concomitantly administered. The urinary excretion clearances (CL_{urine}) of VP-16 were approximately 4 ml/min/kg on average and somewhat reduced to 3 ml/min/kg by the CyA co-administration.

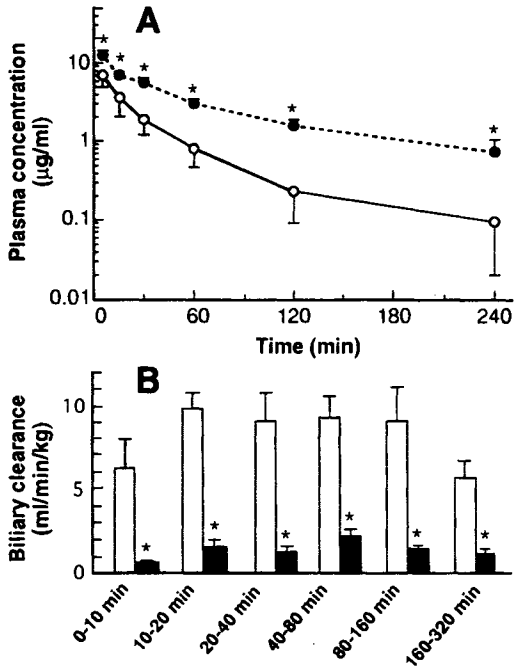

Fig. 2. Plasma concentration profiles (A) and biliary excretion clearances (B) of VP-16 after its intravenous administration (5 mg/kg) without (open symbol or column) or with (closed symbol or column) 10 mg/kg of CyA. Each value and vertical bar represent the mean ±SD of 7 (without CyA) and 4 (with CyA) rats. *Significantly different from VP-16-only administration ($p < 0.05$), as assessed by Student's t-test.

DISCUSSION

This study was designed to assess a local drug delivery system of anticancer drugs using microfibrous, biodegradable collagen. We have reported the releasing profiles of several drugs, including anticancer agents, from CS under an *in vitro* condition (2). In the present study, CS preparations containing VP-16 with or without CyA were applied on the liver surface of rats, and the concentration *vs.* time profiles of VP-16 in the liver ECF and plasma were assessed using a tissue microdialysis method. Microdialysis is a useful tool to determine drug concentrations in the tissue interstitial fluid after local application without any contamination by remaining drug on the liver surface or enzymatic degradation during analysis. For liver microdialysis, several *in vivo* microdialysis studies have been performed in rats (5,6). The R_{vivo} value was not changed with the presence of CyA or with VP-16 concentration, and its variation was relatively small (Table I). Therefore, the constant R_{vivo} value (as an average of the observed values) was employed to estimate true concentrations of VP-16 in the liver ECF according to Eq. (2). There may be a concentration gradient of VP-16 in the liver, depending upon the distance between the liver surface (i.e., the CS preparation) and the site of a microdialysis probe. Therefore, a liver ECF concentration of the drug, estimated at the center of the right lobe by microdialysis, should be thought to be a representative, averaged value.

Multiple drug resistance (MDR) of malignant tumors has been recognized as a critical problem for cancer chemotherapy (7). The mechanism of MDR is associated with the overexpression of a membrane glycoprotein, *P*-glycoprotein, which acts as a drug efflux pump (7). CyA, an immunosuppressive agent, is known as a strong MDR-reversing agent (8). The modulation of multidrug-resistance of anticancer drugs by CyA has recently been investigated in clinical trials (9,10); CyA concentrations more than 2,000 ng/ml produced an 80% increase in VP-16 AUC and a 38% decrease in total clearance (10), and the maximum-tolerated level of CyA was 4,800 ng/ml (9). These observations, as well as the *P*-glycoprotein-mediated efflux system in the liver (11), prompted us to use CyA to enhance the VP-

16 levels in the liver, because the liver is the major site of metabolism and biliary excretion of VP-16. It is not easy to compare the apparent Ki value of CyA to inhibit *P*-glycoprotein between *in vivo* and *in vitro* conditions, because of its strong binding to serum proteins (>99%) for *in vivo* situations and unconfirmed binding in culture medium for *in vitro* assays. However, the present results on the *in vivo* interaction between VP-16 and CyA can be compared with a report of Speeg *et al.* (12) in which the biliary clearance and bile/plasma ratio of colchicine, a *P*-glycoprotein substrate, decreased significantly by 39% and 51%, respectively, after bolus administration of CyA in rats. The dose of CyA used in the present study (40 mg/kg) is higher than that used by Speeg *et al.* (2 or 10 mg/kg iv) and should be effective enough to inhibit the energy-dependent efflux of anticancer agents by *P*-glycoprotein at the liver canalicular membranes (11). Thus, the observed effect of CyA on the maintenance of the liver ECF concentrations of VP-16 (Figs. 1A and 1B) may be explained by the inhibitory effect of CyA on the biliary excretion of VP-16 (Figs. 2A and 2B) *via P*-glycoprotein, leading to an enhanced accumulation of VP-16 in the liver ECF.

However, there is another possibility that the intrahepatic concentrations of VP-16 increased due to the inhibition of the hepatic VP-16 metabolism by CyA, considering that both VP-16 (13) and CyA (14) are metabolized by a *P*450 isozyme, CYP3A4. If this latter possibility applies, the biliary excretion of VP-16 should be higher with the co-administration of CyA due to higher intrahepatic concentrations of VP-16. In the present study, however, we found that the biliary excretion of VP-16 was markedly decreased by co-administration of CyA in rats (Fig. 2B). Moreover, the sum of CL_{bile} and CL_{urine} values (14 ml/min/kg) contributed approximately 75% of the CL_{tot} (19 ml/min/kg); it follows that the contribution of metabolism is only 25% and that the metabolic interaction between VP-16 and CyA may not greatly influence the whole body clearance of VP-16. Thus, it is suggested that the suppression of the biliary excretion of VP-16 by CyA is involved for the most part in the pharmacokinetic change of VP-16 caused by CyA, while the metabolic interaction between these drugs is involved to a lesser extent. The modest increase in Vd_{ss} after the concomitant administration of CyA might be attributed to an enhanced accumulation of the drug in certain tissues due to decreased drug efflux, as also suggested for the marked changes in doxorubicin disposition after CyA co-administration (15). In any case, the inclusion of an inhibitor of the transport and/or metabolism of a drug in a carrier may be appropriate not only for overcoming MDR of cancer tumors but also for increasing local drug concentrations by reducing excretion and/or metabolic conversion of the drug when applied to an eliminating organ such as the liver.

The half-life of VP-16 release from CS *in vitro* (3.7 hr) was close to its MRT value after the local administration of VP-16 containing CS without CyA (3.8 hr), suggesting that the sink condition holds for VP-16 in the liver ECF due to its rapid intrahepatic sequestration. However, the long maintenance of VP-16 in the liver ECF was observed in the presence of CyA (Figs. 1A and 1B), due to the above-mentioned effect of CyA on the *P*-glycoprotein-mediated biliary excretion. Thus, it is most likely that the drug transfer across the liver surface membrane is not the rate-determining step of the overall VP-16 transport into the liver, but the intrahepatic sequestration is. In any case, considering that the systemic exposure of anticancer agents often causes severe dose-limiting toxicities such as bone marrow suppression, their site-specific delivery could be highly beneficial depending on the local-to-systemic drug exposure (AUC) ratio. In this respect, an aim of the local drug delivery was fulfilled using the CS preparations containing CyA, at least for 24 hr, as shown in Figs. 1A–1C (i.e., establishment of high concentrations in the target tissue and low concentrations in the systemic circulation).

In conclusion, the CS preparation appears to be an effective carrier for local drug delivery of an anticancer drug, VP-16, provided that an adequate *P*-glycoprotein inhibitor such as CyA is simultaneously contained in the carrier. The effect of CyA was probably derived from the inhibition of *P*-glycoprotein-mediated biliary excretion of the anticancer agent by CyA. We propose a general notion that the inclusion of an inhibitor of transport and/or metabolism of a drug in a drug carrier may be appropriate for effective local drug delivery, provided that the systemic AUC of the drug is much less than the toxic level. In addition, the usefulness of the microdialysis technique for the assessment of a local drug delivery system was demonstrated.

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