

## Crephor EL-mediated Alteration of Paclitaxel Distribution in Human Blood: Clinical Pharmacokinetic Implications

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### Abstract

We have determined the *in vitro* and *in vivo* cellular distribution of the antineoplastic agent paclitaxel (Taxol) in human blood and the influence of Crephor EL (CrEL), the vehicle used for i.v. drug administration. In the absence of CrEL, the blood:plasma concentration ratio was  $1.07 \pm 0.004$  (mean  $\pm$  SD). The addition of CrEL at concentrations corresponding to peak plasma levels achieved after the administration of paclitaxel (175 mg/m<sup>2</sup> i.v. over a 3-h period; i.e., 0.50%) resulted in a significant decrease in the concentration ratio ( $0.690 \pm 0.005$ ;  $P < 0.05$ ). Kinetic experiments revealed that this effect was caused by reduced erythrocyte uptake of paclitaxel by polyoxyethyleneglycerol triricinoleate, the major compound present in CrEL. Using equilibrium dialysis, it was shown that the affinity of paclitaxel for tested matrices was (in decreasing order) CrEL > plasma > human serum albumin, with CrEL present at or above the critical micellar concentration ( $\sim 0.01\%$ ). Our findings in the present study demonstrate a profound alteration of paclitaxel accumulation in erythrocytes caused by a trapping of the compound in CrEL micelles, thereby reducing the free drug fraction available for cellular partitioning. It is proposed that the nonlinearity of paclitaxel plasma disposition in patients reported previously should be reevaluated prospectively by measuring the free drug fractions and whole blood:plasma concentration ratios.

### Introduction

A substantial number of clinical studies with the antineoplastic agent paclitaxel (Taxol) have been performed to date and have revealed a nonlinear disposition of the drug, which may have significant implications in that greater-than-expected increases in systemic exposure may result from a given increase in dose (1). For example, 3-h infusions of paclitaxel at 135 mg/m<sup>2</sup> resulted in a mean  $C_{\max}^2$  of 3.3  $\mu\text{M}$  and a mean AUC of 10.4  $\mu\text{M}\cdot\text{h}$ , whereas at 175 mg/m<sup>2</sup>, the mean  $C_{\max}$  and AUC values were 5.9 and 18.0  $\mu\text{M}\cdot\text{h}$ , respectively (2). Thus, a 30% increase in dose results in an 80% increase in the  $C_{\max}$  and a 75% increase in the AUC. In line with results from dose escalation studies in humans, paclitaxel disposition in mice also increased disproportionately with doses increasing from 2 to 10 or 20 mg/kg (3). Subsequent studies have demonstrated that both distribution and elimination appeared to be linear processes in the absence of CrEL, the formulation vehicle used for i.v. drug administration (4). Because plasma concentrations of CrEL in mice and humans are within the same range, it is very likely that CrEL also plays an important role in nonlinear paclitaxel disposition in humans (5, 6). At present, the

biochemical mechanisms responsible for the dose-dependent interaction of CrEL with the pharmacokinetics of paclitaxel remain unclear. It has been suggested that CrEL might interfere with P-glycoprotein-mediated biliary drug secretion, thereby influencing paclitaxel elimination (7). However, recent studies indicate that drug-transporting P-glycoproteins are not essential *per se* for normal hepatobiliary secretion of paclitaxel (8, 9) and that the disposition of CrEL itself limits the potential to modulate P-glycoprotein activity *in vivo* (10).<sup>3</sup> In the present study, we have determined the *in vitro* and *in vivo* cellular distribution of paclitaxel in human blood in an effort to extend our insight into the role of CrEL in the pharmacokinetics of paclitaxel.

### Materials and Methods

**Chemicals and Reagents.** Paclitaxel powder and a sterile solution of paclitaxel formulated in a mixture of CrEL and dehydrated ethanol USP (1:1, v/v) at 6 mg/ml were obtained from the Bristol-Myers Squibb Co. (Wallingford, CT). Stock solutions of paclitaxel at 1 mg/ml in DMSO were stored at  $-80^\circ\text{C}$  and diluted further in methanol-DMSO (1:1, v/v) before use. CrEL, pure castor oil, and human serum albumin (fraction V) standards were obtained from Sigma Chemical Co. (St. Louis, MO), and Coomassie Brilliant Blue G-250 was obtained from Bio-Rad Laboratories (Munich, Germany) as a concentrated solution in 85% (w/v) phosphoric acid-ethanol (2:1, v/v). Five reversed-phase HPLC fractions of crude CrEL, each with progressively increased hydrophobicity, were kindly donated by Dr. David Kessel (Wayne State University, Detroit, MI; Ref. 11). Miscellaneous chemicals and reagents were of the highest grade available and originated from Rathburn (Walkerburn, United Kingdom). Purified water was obtained by filtration and deionization using a Milli-Q-UF system (Millipore, Bedford, MA) and was used throughout.

**Experimental Techniques.** Samples of human blood were obtained from four healthy volunteers in glass vials containing lyophilized sodium heparin as an anticoagulant and were used within 1 h after collection. Aliquots of the blood were centrifuged for 5 min at  $3000 \times g$  ( $4^\circ\text{C}$ ) to separate plasma, which was transferred to a clean polypropylene tube and then stored frozen at  $-20^\circ\text{C}$  until used. Erythrocyte suspensions were prepared with freshly donated heparinized blood from which the plasma and buffy coat were removed by aspiration. The cells were washed twice, each time with 3 ml of ice-cold phosphate buffer containing 0.01 M potassium phosphate, 0.137 M sodium chloride, and 2.7 mM potassium chloride, in the presence of 0.05% (w/v) glucose at pH 7.4 and resuspended in the same buffer to give the desired hematocrit. For preparation of platelet-rich plasma, heparinized blood was centrifuged at  $200 \times g$  for 20 min at room temperature to pack down the erythrocytes and leukocytes. The resulting supernatant, with very low contamination from RBCs and leukocytes, was used as platelet-rich plasma.

Blood:plasma ratio experiments were conducted using aliquots (2 ml) of the various matrices, which were placed in a  $37^\circ\text{C}$  shaking water bath for 5 min before the addition of paclitaxel in the presence or absence of CrEL. At the time required for maximal cellular paclitaxel uptake at equilibrium, duplicate aliquots of 250  $\mu\text{l}$  were withdrawn from the incubation tubes (Eppendorf,

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<sup>2</sup> The abbreviations used are:  $C_{\max}$ , peak drug levels; AUC, area under the concentration-time curve; CrEL, Crephor EL; HPLC, high-performance liquid chromatography.

<sup>3</sup> J. van Asperen, A. Sparreboom, O. van Tellingen, and J. H. Beijnen. Crephor EL masks the effect of mdr1a P-glycoprotein on the plasma pharmacokinetics of paclitaxel in mice, submitted for publication.

Table 1 Blood:plasma concentration ratio of paclitaxel in the absence and presence of various concentrations of CrEL, castor oil, and HPLC fractions of CrEL<sup>a</sup>

Compound added (%)	Blood:plasma ratio	Percentage change vs. control (%)	<i>P</i> <sup>b</sup>
None	1.07 ± 0.004		
CrEL (0.01)	1.09 ± 0.009	+1.83	0.387
CrEL (0.05)	0.990 ± 0.015	-9.35	0.012
CrEL (0.10)	0.901 ± 0.017	-15.8	0.003
CrEL (0.50)	0.690 ± 0.005	-35.5	<0.0001
CrEL (1.0)	0.625 ± 0.008	-41.6	<0.0001
Castor oil (0.50)	1.23 ± 0.171	+13.0	0.061
CrEL fraction 1 (0.50)	1.06 ± 0.008	-0.94	0.520
CrEL fraction 2 (0.50)	0.926 ± 0.018	-13.5	0.043
CrEL fraction 3 (0.50)	0.763 ± 0.055	-28.7	0.010
CrEL fraction 4 (0.50)	0.645 ± 0.051	-39.7	0.003
CrEL fraction 5 (0.50)	0.943 ± 0.039	-11.9	0.103

<sup>a</sup> Paclitaxel was used at an initial concentration of 1 µg/ml and incubated in whole blood for 15 min at 37°C before fractionation and HPLC analysis. Ratio data are presented as mean values ± SD of (at least) triplicate measurements.

<sup>b</sup> Probability value *versus* control (unpaired two-sided Student's *t* test).

Hamburg, Germany) and kept at -80°C for 5 min to achieve complete hemolysis. The remaining blood fraction was centrifuged, and the supernatant was diluted 4-fold with drug-free human plasma to determine plasma concentrations as described above.

Equilibrium dialysis was accomplished at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> using test cells made from 1.5-ml polypropylene microtubes (Eppendorf) carrying a 250-µl inside recess in the lids. The experiments were carried out with 250-µl aliquots of paclitaxel-containing (1 µg/ml) plasma or a 40 mg/ml solution of human serum albumin in 0.01 M phosphate buffer (pH 7.4) against an equal volume of the same buffer. Spectra/Por 3 dialysis tubing with a *M<sub>r</sub>* 12,500 cutoff (Spectrum Medical, Kitchener, Canada) was soaked in 0.9% (w/v) sodium chloride solution before use. The time to reach equilibrium was determined in preliminary experiments and ranged between 5 and 24 h, depending on the composition of the fluid in the receptor compartment. The ratio of drug concentrations measured by HPLC in the buffer and plasma or serum albumin solution after dialysis was taken as an estimate of the unbound (free) fraction of paclitaxel. Because the volume shift during dialysis was negligible (<10%), the results were used directly without applying a correction factor. In both the blood:plasma ratio and equilibrium dialysis experiments, it was confirmed that the total drug recovery from all of the fractions was equal to the amount of paclitaxel added to blood, plasma, or buffer mixtures.

**Clinical Pharmacokinetics.** The patient studied was a 65-year-old female suffering from recurrent ovarian cancer after earlier cisplatin-containing chemotherapy. She received paclitaxel formulated in CrEL-ethanol at an absolute dose of 256 mg supplemented with 100 µCi of [*G*-<sup>3</sup>H]paclitaxel (specific activity, 2.4 Ci/mmol; radiochemical purity, 99.7% by HPLC; Moravik Biochemicals, Inc., Brea, CA). The majority of the tritium is in the *m*- and *p*-positions of the aromatic rings, with minor amounts in the 10-, 3'-, and 2-position of the taxane ring system. The dose was administered as a 3-h i.v. infusion in 500 ml of sterile and isotonic sodium chloride, after a standard premedication comprising dexamethasone (10 mg, i.v.), clemastine (2 mg, i.v.) and ranitidine (50 mg, i.v.). Blood samples (~5 ml) were obtained in glass tubes with lyophilized sodium heparin at the following time points: immediately before dosing; at 0.5, 1, 1.5, 2, 2.5, and 3 h after start of infusion; and at 5, 15, 30, and 45 min and 1, 2, 4, 6, 8, 12, and 24 h after end of infusion. Aliquots (1 ml) of whole blood were immediately stored frozen at -20°C, and the remaining blood was centrifuged at 4000 × *g* for 5 min to separate plasma. The clinical protocol was approved by the Rotterdam Cancer Institute Ethics Board, and the patient gave informed consent before study entry. Concentrations of paclitaxel were measured using an isocratic reversed-phase HPLC method with UV detection at 230 nm, as described previously (12). The analytical procedure for CrEL was based on a colorimetric dye-binding assay using Coomassie Brilliant Blue G-250 (13), with modifications as described (6).

**Results**

**In Vitro Partition in Blood Fractions.** The time to equilibrium of paclitaxel between whole blood and plasma, as determined by measuring the concentration on blood cells at several time points at 37°C

and calculating the rate of migration, was reached within 15 min of pooling cells and plasma. At this time point, a blood:plasma ratio of 1.07 ± 0.004 (mean ± SD) was observed, which was independent of the paclitaxel concentration, indicating that the cellular concentration is proportional to the concentration in plasma water. This shows that the rate of exchange between blood cells and plasma water is effectively instantaneous on the time scale of paclitaxel disposition and that the steady-state load on cells represents approximately half of the total blood concentration. The addition of CrEL at concentrations corresponding to peak plasma levels achieved after the administration of paclitaxel (175 mg/m<sup>2</sup> i.v. over a 3-h period; *i.e.*, 0.50%; Ref. 10) resulted in a significant decrease in the blood:plasma concentration ratio (0.690 ± 0.005; *P* < 0.05). Additional experiments revealed a clear concentration-dependent effect of CrEL on this ratio, with maximal inhibition of cellular paclitaxel uptake at a CrEL total blood concentration of 1.0% (Table 1). At this concentration, the blood:plasma ratio averaged 0.625 ± 0.008, suggesting a distribution of paclitaxel merely outside blood cells, with ~88% of total paclitaxel associated with the plasma fraction, assuming a mean hematocrit of 0.45. Incubation of paclitaxel in whole blood with castor oil, the major component in the CrEL vehicle before polyoxyethylation, showed no evidence of significant alterations in cellular uptake with a mean blood:plasma ratio of 1.23 ± 0.17 (*P* > 0.05). Experiments with reversed-phase HPLC fractions of CrEL indicated that the effect of the unfractionated vehicle was not observed with the hydrophilic components present in the first fractions, which mainly contained polyethylene glycol and oxyethylated glycerol (Table 1), but is primarily caused by compounds from fraction 4, *e.g.*, polyoxyethylene-glycerol triricinoleate along with fatty acid esters of polyethylene glycol.

The distribution of paclitaxel within an artificial mixture of washed erythrocytes occupying 45% of the total volume and buffer indicated RBC:buffer concentration ratios of 3.70 ± 0.061 and 0.649 ± 0.014 in the absence and presence of CrEL, respectively, further indicating that in the latter case, paclitaxel is only distributed in the water phase (Table 2). The apparent contradiction between the low blood:plasma ratio and high accumulation into erythrocytes was shown to be due to the compensating effect of plasma protein binding, which has been observed previously for a variety of other drugs (for an overview, see Ref. 14), including paclitaxel (15). This is borne out by the finding that in the presence of plasma proteins, cellular partitioning of paclitaxel in erythrocytes was markedly impaired (Table 2). Surprisingly, paclitaxel accumulation in platelets was less affected by CrEL than erythrocyte uptake (Table 2), with a platelet:plasma ratio in the order of ~300, taking into account the low total platelet volume in blood (~4 µl/ml blood). As suggested recently by Wild *et al.* (16), this high degree of platelet uptake likely reflects a tight binding of paclitaxel to the intracellular tubulin/microtubule system and has previously been

Table 2 Distribution of paclitaxel in artificial binary mixture systems of the main blood fractions in the absence and presence of CrEL<sup>a</sup>

Binary system	CrEL (%)	Total:extracellular ratio	Percentage change vs. control (%)	<i>P</i> <sup>b</sup>
Erythrocytes/buffer <sup>c</sup>	None	3.70 ± 0.061		
	0.50	0.649 ± 0.014	-82.5	<0.001
Erythrocytes/plasma <sup>c</sup>	None	1.03 ± 0.021		
	0.50	0.664 ± 0.007	-35.5	0.002
Platelets/plasma <sup>d</sup>	None	1.33 ± 0.037		
	0.50	1.26 ± 0.024	-5.26	0.018

<sup>a</sup> Paclitaxel was used at an initial concentration of 1 µg/ml and incubated in whole blood for 15 min at 37°C before fractionation and HPLC analysis. Ratio data are presented as mean values ± SD of (at least) triplicate measurements.

<sup>b</sup> Probability value *versus* control (unpaired two-sided Student's *t* test).

<sup>c</sup> Hematocrit, ~0.45.

<sup>d</sup> Platelet count, ~750 × 10<sup>9</sup>/L.

shown to occur with other tubulin-interactive drugs, such as the *Vinca* alkaloids (16).

**Binding Interactions.** To gain insight into the mechanism underlying this phenomenon, equilibrium dialysis was used to determine the effects of CrEL on the fraction of unbound paclitaxel. Preliminary experiments verified that CrEL could not cross the semipermeable dialysis membrane, as indicated by the absence of detectable levels in the buffer compartment after dialysis. As predicted by earlier experiments (5), paclitaxel was found to bind extensively to human plasma ( $89.1 \pm 0.595\%$ , mean  $\pm$  SD;  $n = 3$ ) in the absence of CrEL, with human serum albumin accounting for approximately 50% of the total binding. In the presence of CrEL (0.50%), however, a clear and statistically significant decrease in the free (unbound) drug fraction of up to 66% ( $P = 0.013$ ) and 92% ( $P = 0.002$ ) was seen for plasma and human serum albumin, respectively. Interestingly, equilibrium dialysis experiments performed with CrEL in the absence of any plasma proteins revealed that at a spiked concentration of 0.50%, a free drug fraction of only 0.044 was observed, suggesting that paclitaxel affinity for CrEL is substantially greater than that for either plasma or human serum albumin. This binding affinity for CrEL was distinctly concentration dependent, with no change in the free fraction of paclitaxel at CrEL concentrations below 0.01% [*i.e.*, the amount of surfactant corresponding to the critical micellar concentration in aqueous solutions (17)] and maximal binding (at a free drug fraction of  $\sim 0.017$ ) in the presence of CrEL at a concentration of 1.0%. Equilibrium dialysis of paclitaxel-containing plasma against a buffer with CrEL resulted in a virtually quantitative shift of drug ( $90.1 \pm 1.09\%$ ) to the receptor fluid, further pointing to a preferential binding to the surfactant.

**In Vivo Pharmacokinetics.** Fig. 1 shows the logarithmic concentration-time curves of paclitaxel in whole blood and plasma (*i.e.*, the unbound ultrafiltrate fraction plus the protein/CrEL-bound blood fraction; Fig. 1A) and of CrEL in plasma (Fig. 1B) after an i.v. dose of 256 mg was administered to a female patient with advanced ovarian cancer. Similar to our *in vitro* partition experiments, a distinct CrEL concentration dependency was noted for the whole blood:plasma concentration ratio and the unbound drug fraction (Table 3). This suggests that erythrocytes form a secondary transport system in whole blood, which becomes less significant as the CrEL concentration increases; hence, the unbound drug concentration decreases. In all, these data appear to indicate that CrEL micelles act as the principal carrier of paclitaxel in the systemic circulation.

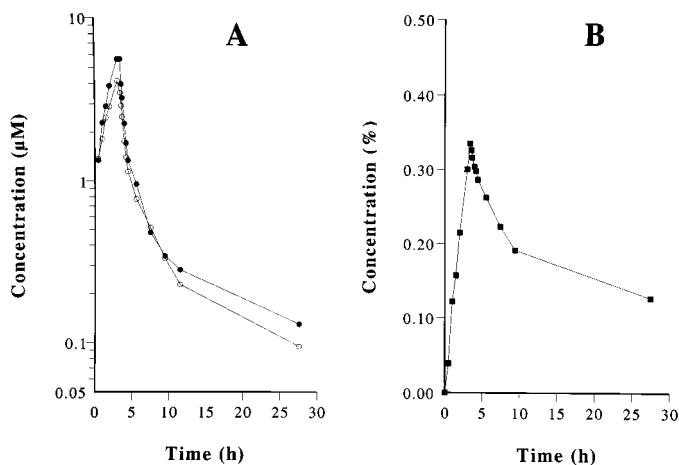


Fig. 1. Concentration versus time curves of paclitaxel in whole blood (○) and plasma (●; A) and of CrEL (■) in plasma (B). Pharmacokinetic data were obtained from a female patient receiving the drug formulated at 6 mg/ml in a mixture of CrEL-dehydrated ethanol USP (1:1, v/v) at an absolute dose of 256 mg.

Table 3 CrEL concentration-dependent blood:plasma ratio and unbound paclitaxel fraction in plasma of a patient<sup>a</sup>

Time (h) <sup>b</sup>	CrEL (%)	Blood:plasma ratio	Unbound fraction
0.50	0.039	1.02	0.076
1.0	0.122	0.795	0.057
1.5	0.157	0.851	0.038
2.0	0.215	0.749	0.028
2.5	0.300	0.737	0.022
3.0	0.335	0.623	0.016

<sup>a</sup> Paclitaxel was administered as an i.v. infusion over a 3-h period at an absolute dose of 256 mg to a single female with advanced ovarian cancer.

<sup>b</sup> Sample collection time point after the start of a 3-h i.v. infusion of paclitaxel.

## Discussion

In the present study, we have shown that CrEL, the formulation vehicle used for i.v. drug administration, causes a profound alteration of paclitaxel accumulation in erythrocytes by reducing the free drug fraction available for cellular partitioning. This effect was also observed in the absence of plasma proteins, indicating that it was not caused by altered protein binding or an increased affinity of paclitaxel for protein dissociation products that are produced by the action of CrEL on native lipoproteins (11, 18). The data indicate that erythrocytes form a secondary transport system in whole blood, which becomes less significant as the CrEL concentration increases, and hence, the free drug fraction decreases. Current data suggest that drug trapping occurs in micelles composed primarily of polyoxyethylene-glycerol tricinoleate and that these micelles act as the principal carrier of paclitaxel in the systemic circulation.

The findings of extensive RBC uptake of paclitaxel at low CrEL levels and the high binding to plasma proteins and CrEL micelles may have a substantial impact on the interpretation of the drug's pharmacokinetic behavior that has not been fully appreciated. Previously, paclitaxel disposition in animals and humans has been reported to be nonlinear, with disproportional relationships between changes in the dose and the resulting plasma AUCs and peak plasma concentrations. This nonlinearity has been speculated to result from two separate saturable processes, one in distribution and one in elimination (both described by Michaelis-Menten kinetics), based on the usefulness of complex mathematical pharmacokinetic models to accurately describe plasma profiles of paclitaxel over a wide dosage range (1, 2). The authors have generated estimates of the maximal process rate (*i.e.*,  $V_{max}$ ) and the paclitaxel concentration associated with  $0.5 \times V_{max}$  (*i.e.*,  $K_m$ ) and observed that the  $K_m$  estimate for the distribution process is much smaller than that for elimination. This suggested that distribution should be the first process to exhibit saturation. Our present data indicate that the resulting disproportional accumulation of paclitaxel in plasma, which is most pronounced with the drug administered by the 3-h infusion schedule (1), is related to a CrEL-mediated alteration of drug distribution within the circulation during paclitaxel infusion. This is exactly what is observed clinically in our patient and is supported by our previous finding that, in spite of nonlinear paclitaxel kinetics in plasma, tissue levels of the parent drug and its known mono- and dihydroxylated metabolites in mice have a linear relationship with the dose administered (3). The paradox of disproportional increases in plasma concentrations and linear distribution processes in tissues is likely caused by the fact that the plasma comprises a relatively small fraction of the total volume available for paclitaxel distribution and that, simultaneously, the CrEL-drug complex is not stable enough to substantially reduce the amount of drug that exists in the body in the active, diffusible, unbound form. In addition, it is also possible that the equilibrium between blood and tissues is not based solely on paclitaxel dissolved in the plasma water phase but also on direct drug transport from loaded erythrocytes to

tissues (14). This hypothetical transport may be of great importance for paclitaxel in cases of low concomitant CrEL levels that are associated with low dose schedules. Regardless of the uptake processes of the tissues, our current findings indicate that the operation of Michaelis-Menten kinetics, the postulated cause of nonlinear paclitaxel disposition in plasma, is not related to saturable tissue binding or disproportional elimination kinetics but appears to be an artifact caused by paclitaxel dose-related levels of CrEL in the blood. We are currently prospectively reevaluating the linearity of paclitaxel pharmacokinetics in humans using different infusion schedules by measuring free drug fractions and blood:plasma ratios, based on the expectation that if the fraction of drug that is bound changes appreciably with concentration in the concentration range of interest, then the AUC of free (unbound) paclitaxel should be a linear function of the dose administered.

The existence of CrEL in blood as large polar micelles with a highly hydrophobic interior also raises the possibility of additional complexities in case of combination chemotherapy regimens with paclitaxel. For example, fluorescence studies on the interaction between anthracycline drugs and different surfactants indicated that daunorubicin, although relatively hydrophilic with an octanol:water partition ratio of  $\sim 3.5$ , is readily incorporated into CrEL micelles (17). Thus, in the systemic circulation, micellar incorporation of anthracyclines may result in altered cellular distribution and a concomitantly increased plasma concentration. In this regard, it is interesting to note that both paclitaxel (in the clinical formulation) and CrEL alone strongly affect the plasma pharmacokinetics of another anthracycline antineoplastic agent, doxorubicin, in both rodents and humans (7, 19). Similarly, CrEL is known to decrease etoposide clearance in rats (20), and preliminary findings in humans treated with a combination of paclitaxel ( $\geq 175$  mg/m<sup>2</sup> over a 3-h period) and i.v. etoposide confirmed these observations.<sup>4</sup> At present, we are investigating the effects of micellar incorporation on the biodistribution and pharmacokinetics of doxorubicin and etoposide and the role of CrEL as a determinant of the increased incidence and severity of hematological toxicity in clinical trials (7) with a combination of paclitaxel and doxorubicin.<sup>5</sup>

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