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ORIGINAL ARTICLE

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Pharmacokinetics of N-2-chloroethylaziridine, a volatile cytotoxic metabolite of cyclophosphamide, in the rat

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Abstract *Objectives*: The objectives of this study were to characterize pharmacokinetics of N-2-chloroethylaziridine (CEA) in the rat model and assess the in vivo fraction of total clearance of phosphoramide mustard (PM) that furnished CEA to circulation. *Methods*: The disposition of CEA was investigated following separate intravenous (iv) administrations of PM, synthetic CEA, and their combination to the Sprague-Dawley rats. In addition, in rats receiving prodrug cyclophosphamide (CP), plasma concentrations of CP and its metabolites, 4-hydroxycyclophosphamide (HOCP), PM, and CEA, were simultaneously quantified using GC/MS and stable isotope dilution techniques. *Results*: Following iv administration of synthetic CEA, concentrations of CEA declined biexponentially with the mean terminal half-life and total body clearance of 47.5 min and 167 ml/min/kg, respectively. Urinary excretion of unchanged CEA was 0.164% of the administered dose. CEA was found to be the major circulating metabolite after iv administration of precursor PM to rats. The fraction of total clearance of PM that furnished CEA to circulation was estimated to be 100%, indicating virtually complete availability of the metabolite to circulation once formed. In rats administered with CP, PM exhibited the highest plasma and urinary concentrations compared to HOCP and CEA. *Conclusions*: For the first time, CEA was demonstrated to be an important in vivo metabolite of CP in the present study. In light of the poor permeability and in vivo stability of PM, the ultimate DNA alkylator, the findings obtained in this study suggested that CEA may contribute significantly to the overall antitumor activity of prodrug CP.

Keywords Cyclophophamide · Phosphoramide mustard · N-2-Chloroethylaziridine · Pharmacokinetics · GC/MS assay

Abbreviations CP: Cyclophosphamide · HOCP: 4-hydroxycyclophosphamide · PM: Phosphoramide mustard · CEA: N-2-chloroethylaziridine · NNM: Nornitrogen mustard

Introduction

Enzymatic activation by CYP2B and CYP3A [1, 2] of cyclophosphamide (CP), a widely used antineoplastic prodrug, generates the primary metabolite, 4-hydroxycyclophosphamide (HOCP) [3–6]. HOCP rapidly undergoes a reversible ring-opening reaction to give aldophosphamide, which is further decomposed into phosphoramide mustard (PM) and acrolein. PM is one of the major circulating metabolites after oral administration of CP to animals and humans. Also, PM itself is a potent cytotoxic agent and is generally believed to be ultimate alkylating metabolite of CP in vivo [7, 8]. PM is a polar unstable molecule which undergoes facile degradation in vitro. It was expected that as PM degraded in solution, its cytotoxicity would diminish. However, Dr. Chan's laboratory found that completely degraded PM solution still retained about 85% of the cytotoxicity against L1210 and human CCRF-CEM cells [9]. N-2-chlororthylaziridine (CEA) was subsequently found to be the major degradation product of PM, and the entire activity of the degraded PM solution can be accountable by the breakdown product, CEA [9].

CEA has long been known as a volatile metabolite of CP. In 1968, about 2% of the administered dose was reported to be eliminated as CEA in the exhaled air of rats receiving CP [10]. Since then, little information has

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been obtained regarding this metabolite. The cytotoxicity of CEA and its contribution to the overall antitumor activity of CP have been unknown until recently. Chan's laboratory reported that CEA was found to be the major in vitro degradation product of PM [11]. In CYP2B1 and CYP3A4- expressing V79 Cells treated with CP, cytotoxicity as determined by MTT assay increased with time and with an increase in CP concentration whereas no cytotoxicity was observed for parental nontransfected V79 cells [2]. In addition, the generation of metabolites HOCP, PM, and CEA by CP-treated cells transfected with CYP3A4 or CYP2B1 cDNA was demonstrated in the same study. Flowers et al reported that CEA rather than acrolein was responsible for the airborne cytotoxicity as the volatile metabolite of 4-hydroperoxycyclophosphamide, and amount of generated CEA was sufficient to volatilize, diffuse across wells and cause significant neighboring well cytotoxicity [12]. Shulman-Roskes et al. using NMR and HPLC techniques found that P-N bond scission observed at neutral pH did not occur in the parent PM to produce nornitrogen mustard, rather it was an aziridinium ion derived from PM which underwent P-N bond hydrolysis to give CEA [13]. These in vitro data strongly suggest that CEA may be an important metabolite of prodrug CP. The disposition of CEA in animals and humans has not been investigated and little pharmacokinetic information is available for this metabolite due to the lack of a suitable analytical method. A quantitative analytical method of CEA in biological fluids has been established by Lu and Chan using GC/MS and stable isotope dilution techniques [11]. In the present study using the assay methods developed for CP and metabolites (HOCP, PM, and CEA) by this laboratory [4, 5, 11], we investigated the pharmacokinetics of derived and synthetic CEA in detail, including an assessment of its total clearance, volume distribution, and the fractional clearance of the precursor PM which furnished CEA to circulation following intravenous administration to rats. Also, the disposition of CP and its major metabolites, HOCP, PM and CEA were assessed in rats administered with CP.

Materials and methods

Chemicals and apparatus

CP and PM were supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). CEA, 4-HydroperoxyCP (HOOCP), [β-$^2H_4$]HOOCP, [β-$^2H_4$]CP, [β-$^2H_4$]CEA, [α,β-$^2H_8$]CEA, [α,β-$^2H_8$]PM and [β-$^2H_4$]PM were synthesized in this laboratory [11, 14]. 4-HydroxyCP (HOCP) and [β-$^2H_4$]HOCP were prepared by reduction of HOOCP and [β-$^2H_4$]HOOCP with sodium thiosulfate immediately before use. HPLC-grade methanol and methylene chloride were obtained from Curtin Matheson Scientific (Brea, CA). N, O-bis(trimethyl-silyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL). C-18 reversed-phase resin (Bondesil, 40 μm) was purchased from Analytichem International (Harbor City, CA). Kuderna-Danish (K-D) concentrator tube (5 ml capacity) and micro Snyder column (three bulbs) were purchased from ACE Glass (Louisville, KY).

Animal dosing and sampling

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 280–340 g were used in the present study. The research protocol was approved by the Institutional Animal Care and Use Committee at the Ohio State University. The left jugular and femoral veins of each rat were cannulated under ether anesthesia one day prior to the dosing [15]. Rat chow (Tekland, Indianapolis, IN) and water were given ad libitum. Approximately 24 h after the surgery, each rat was administered the appropriate amount of test articles in 0.3 ml of saline solution via the femoral vein cannula, followed by flushing the catheter with 0.2 ml saline. Total sixteen rats were divided into three dose groups (5, 6, and 5 animals each group, respectively), and then were administered intravenously with synthetic CEA at doses of 5, 10, and 20 mg/kg, respectively. Six other rats were given CP at 20 mg/kg, and nine other rats were dosed PM at 20 mg/kg. One rat was given [α,β-$^2H_8$]PM at 30 mg/kg and CEA at 10 mg/kg simultaneously by iv bolus administration. For pharmacokinetic study of CEA, blood samples (0.1–0.3 ml each) for plasma were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min post-dose via the jugular vein cannula and then placed into heparinized sample tubes in an ice bath. The assay detection limit of CEA only allowed us to adequately assess the pharmacokinetic profile of CEA at the low dose of 5 mg/kg. The blood sampling time was shortened slightly to 180 min for the pharmacokinetic study of PM, and extended to 360 min for the pharmacokinetic study of CP to accommodate the assay sensitivity and half-lives of the test articles. Urine samples within 24 h post-dose were also collected on dry-ice by using metabolic cages for all pharmacokinetic studies. Blood samples for plasma were immediately centrifuged at 1,500×g for 10 min at 4°C prior to the collection of plasma. All the plasma and urine samples were frozen at −80°C until analysis.

Sample preparation

Plasma and urine samples for the analysis of CP and HOCP [4, 5, 16, 17] were thawed in a water bath, followed by adding 100 μl of saline containing 1 μg [β-$^2H_4$]CP and 1 μg [β-$^2H_4$]HOCP, and 200 μl of 1M KCN-$NaHSO_3$ (pH 8) and then extracting with 5 ml methylene chloride. After centrifugation at 1,500×g for 10 min, the organic phase was separated and evaporated

to dryness under a stream of nitrogen. The residue was derivatized with 35 μl BSTFA for 40 min at 120°C, and an aliquot (1 μl) of the derivatized samples was injected onto GC/MS. Plasma samples for CEA analyses [11] were extracted with 3 ml methylene chloride after adding 200 ng CEA-d_4. The organic phase was placed into a K-D tube concentrator (ACE Glass, INC. Louisville, KY) and the volume was reduced to approximate 10 μl in a 90°C water bath. Then, approximately 2 μl of aliquot was injected onto GC/MS.

Plasma samples for total PM determination [16] were each loaded onto a disposable Poly-prep minicolumn (Bio-Rad, Richmond, CA) containing 40 mg C-18 reversed-phase resin, and the resin was washed with 1 ml of cold saline, followed by centrifugation at 1,500×*g* for 10 min to remove as much water as possible. The minicolumn was then eluted with 1 ml of methanol, and the methanol extract was evaporated to dryness under a stream of nitrogen. The final residue was derivatized by 35 μl of BSTFA at 120°C for 40 min, and 1 μl of aliquot was analyzed by GC/MS.

Gas chromatographic-mass spectrometry analysis

GC/MS analyses of CP, HOCP, PM, and CEA were carried out on an ITS40 ion trap mass spectrometer directly coupled to a 3300/3400 Varian gas chromatography (Walnut Creek, CA) and a CTC A200S autosampler with a capillary splitless injector (Finnigan MAT, San Jose, CA). A DB-5 fused-silica capillary column (30 m×0.25 mm I.D.) (J & W Scientific, Folsom, CA) was used. Chemical ionization mode was used with ammonia as the reagent gas. Helium was used as the carrier gas with a head pressure set at 15 psi. For the analysis of CEA and its deuterium-labeled analogs, the temperature of the injection port, transfer line and source were maintained at 200, 220, and 230°C, respectively. The temperature of the oven was programmed at 60°C for 4 min and increased to 150°C at a rate of 5°C/min. The retention times of the CEA, CEA-d_4, and CEA-d_8, were 3.6, 3.6, and 3.5 min, respectively. Ions at m/z 106, 110 and 114 for corresponding CEA, CEA-d_4 and CEA-d_8 were quantified by selected ion monitor mode.

For the analysis of CP and HOCP, the injector, transfer line, and source temperatures were maintained at 240, 260, and 230°C, respectively. The oven temperature was programmed at 150°C for 2 min and increased to 190°C at 5°C/min, and then to 250°C at 15°C/min. The final temperature was held for 3 min. The retention times for trimethylsilyl derivatives of CP, CP-d_4, HOCP, and HOCP-d_4 were 12.3, 12.2, 17.5, and 17.4 min, respectively. Ions at m/z 333, 339, 412, and 416 were monitored. For the analysis of PM, PM-d_4 and PM-d_8, the same GC temperature program was used. The trimethylsilyl derivatives of dehydrogen chlorinated PM and its d_4 and d_8 analogs were eluted at retention times of 10.2, 10.1, and 10.1 min, and ions at m/z ratios of 329, 333, and 337 were monitored.

Stability evaluation of PM and CEA in rat plasma

Precursor PM at 0.076 mM was added into 5 ml of fresh rat plasma and then incubated at 37°C in a water bath. Two aliquots (50 μl each) were sampled at 0, 5, 10, 20, 30, 45, 60, 90, 120, 190, 315, 422, 645, 1,330, 1,500, 1,680, 2,790, 3,105, and 3,315 min following initiation of the incubation. The plasma samples was then mixed with appropriate amounts of the internal standards PM-d_4 and CEA-d_4 and quantified for concentrations of precursor PM and degradation product CEA by GC/MS [11]. The change in concentrations of PM and CEA was therefore followed to assess the stability.

Plasma protein binding of CEA in rat and human plasma

Five replicate CEA samples spiked in 2 ml of fresh rat plasma at 6.8 and 13.8 μM were each incubated at 37°C for 30 min. A 0.2 ml of aliquot was removed for the analysis of total CEA, and the remaining plasma was placed into an ultrafiltration unit (Amicon Centrifree, Danver, MA) and centrifuged at 3,500×*g* for 15 min to obtain the ultrafiltrate. 200 μl of the ultrafiltrate was removed for the analysis of unbound CEA. The protein binding in human plasma was also performed at 10 μM level by using the same method ($n = 6$ replicates). Plasma protein binding was estimated using the following equation:

Plasma protein binding % = (total CEA−free CEA)/total CEA

Partition of CEA between plasma and red blood cells

The ex vivo partition experiment of CEA was performed as follow. After CEA in 0.3 ml of saline at 10 mg/kg was administered into rats via the femoral vein, 0.2 ml of blood was each collected via jugular vein at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min following intravenous administration. Each blood sample was divided into two portions: one for analysis of plasma concentration, the other for analysis of blood concentration. For the later, four-fold volume of distilled water was added to disrupt red blood cells [18]. The CEA concentration in erythrocyte was estimated using the following equation:

$$C_{rbc} = \{C_b - [C_p(1 - H_t)]\}/H_t$$

where H_t was the hematocrit (0.46), and C_b and C_p were the CEA concentrations in blood and plasma, respectively.

The in vitro partition of CEA was also evaluated. 1 ml of fresh heparinized rat whole blood was incubated with CEA at 10 μM at 37°C for 30 min. Then, half of the blood sample was centrifuged at 1,500×*g* for 10 min to obtain plasma for the analysis of CEA (C_p). The other

half was analyzed for CEA blood concentration (C_b), and C_{rbc} was estimated by using the same equation above.

Data analysis

The pharmacokinetic analyses were performed by compartmental and noncompartmental analysis using WinNonlin (Pharsight Corporation, NC). A weighting factor of $1/y^2$ was used in most of the fitting. An appropriate compartment model was selected based on the smallest values of standard error (SE), the weighted sum of square (WSS) and Akaike's Information Criteria (AIC). Pharmacokinetic parameters such as total plasma clearance (CL_T), mean resident time (MRT) and steady-state volume of distribution (V_{ss}) were estimated by noncompartmental analysis. The area under the concentration-time curve (AUC) and area under the first moment curve (AUMC) were calculated using trapezoidal method and extrapolated into time infinity. Statistical analyses were performed by 1-tailed Student's *t* test or by ANOVA.

The fraction of total clearance of PM that furnishes the primary metabolite CEA in the circulation, f_m, was estimated from the following equation:

$$f_m = [AUC_{\text{derived CEA}}/D_{PM}]/[AUC_{CEA}/D_{CEA}]$$

where $AUC_{\text{derived CEA}}$ and AUC_{CEA} are the areas under the concentration-time curves of the derived CEA and preformed CEA, respectively. D_{PM} and D_{CEA} are the doses of PM and preformed CEA.

The fraction of PM that was transformed into CEA in rat plasma, f_d, was calculated using the following equation:

$$f_d = AUC_{CEA}/(Co/K_{CEA})$$

where AUC_{CEA} is area under the concentration-time curve of degradation product CEA. Co is the initial concentration of PM in rat plasma and K_{CEA} is the apparent first-order degradation rate constant of CEA in plasma.

Results

In vitro conversion of PM into CEA in rat plasma

The concentration-time profiles of PM and generated CEA following the incubation of 0.076 mM PM in fresh rat plasma at 37°C are presented in Fig. 1. As shown, PM declined monoexponentially with a half-life of 55.4 min. The concentration of generated CEA peaked at 361 min and then declined slowly with a half-life of 488 min. The fraction of PM that is converted into CEA in rat plasma, f_d, was estimated to be 41%. Approximately 32% of PM was converted to CEA in pH 7.4 sodium phosphate buffer and the apparent degradation

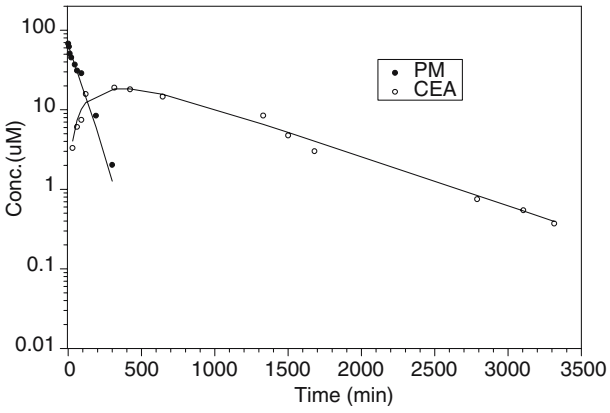

Fig. 1 The concentration-time profiles of phosphoramide mustard (PM) and N-2-chloroethylaziridine (CEA) following incubation of PM at 0.076 mM in fresh rat plasma at 37°C. The fraction of PM that was transformed into CEA, f_d, was estimated using the equation: $f_d = AUC_{CEA}/(Co/K_{CEA})$; where AUC_{CEA} is area under concentration-time curve of degradation product CEA. Co is the initial concentration of PM and K_{CEA} is the apparent first-order degradation rate constant of CEA in plasma

half-life of CEA in the buffer was 1,210 min observed in the previous study [11]. CEA appeared to be more stable in the buffer than in rat plasma, indicating that the plasma protein may destabilize the CEA. This is consistent with the observation that GSH concentrations affect the stability of CEA in the buffer [13].

Pharmacokinetics of CEA in rats

Following a single intravenous bolus administration of synthetic CEA to male Sprague-Dawley rats, mean concentration-time profiles of CEA at doses of 5, 10, and 20 mg/kg are presented in Fig. 2. The derived mean pharmacokinetic parameters of CEA are summarized in Table 1. The plasma concentration of CEA declined biexponentially with a mean terminal half-life of 44.5 min. The apparent volume of distribution in central compartment (Vc) was 3.68 L/kg, indicating extensive tissue distribution of CEA in rats. The urinary excretion of unchanged CEA within 24 h at 10 mg/kg was 0.16% of the administered dose, suggesting that the major elimination route of CEA is via metabolism or/and expired air. Total body plasma clearance of CEA at 10 mg/kg was 165 mL/min/kg (greater than liver blood flow), further suggesting that the elimination via extrahepatic metabolism or/and expired air may be important for CEA [19]. No dose dependent kinetics was observed for all pharmacokinetic parameters except for C_o and AUC at three different doses ($P > 0.1$). Mean AUC values were proportional to the dose administered; indicating that pharmacokinetics of CEA in the rat was linear in the dose ranged from 5 to 20 mg/kg. The plasma protein binding of CEA in rat plasma was estimated to be 49.6 ± 5.1 ($n = 5$) and $48.0 \pm 4.8\%$ ($n = 5$) at 6.8 and 13.8 μM, respectively. The plasma protein binding of CEA in human plasma was estimated to be

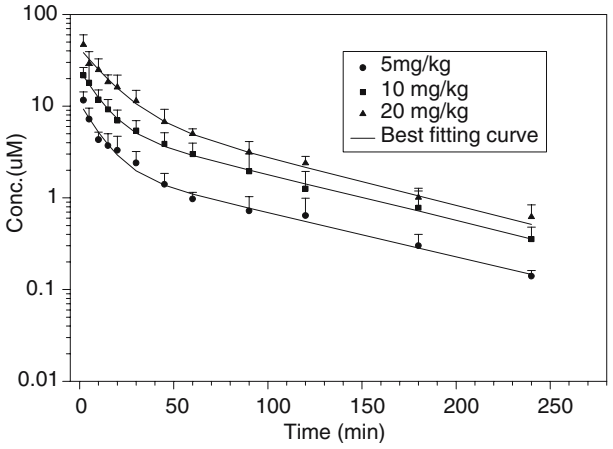

Fig. 2 Mean plasma concentration-time profiles of N-2-chloroethylaziridine following a single intravenous administration of synthetic N-2-chloroethylaziridine to the Sprague-Dawley rat at doses of 5, 10, and 20 mg/kg

54.2 ± 4.9% ($n = 6$) at 10 μM, similar to the observation in rat plasma. Both in vitro and ex vivo partition studies indicated that the partition ratio (K_b) of CEA between erythrocyte and plasma was approximately 1.2:1, slightly in favor of red blood cells.

Disposition of derived CEA following intravenous administration of its precursors, PM and CP, to rats

The representative plasma concentration-time profiles of PM and derived CEA following a single intravenous bolus dose of PM at a dose of 20 mg/kg are shown in Fig. 3. The derived mean pharmacokinetic parameters of PM and CEA are summarized in Table 1. The plasma concentrations of PM declined monoexponentially with a short half-life of 15.7 min. The volume of distribution and total body clearance of PM was 0.419 L/kg (less than total body water) and 18.4 mL/min/kg (33.3% of liver blood flow), respectively. CEA was a major circulating metabolite and achieved the peak concentration at the first sampling timepoint (5 min postdose) following the intravenous administration of PM. The derived CEA decayed biexponentially with a terminal phase half-life of 47.1 min, similar to the observation after the direct intravenous administration of synthetic CEA ($P > 0.1$). The 24 h urinary excretion of unchanged PM and generated CEA following the administration of PM was 7.0 and 3.9% of the administered dose, respectively.

Combined the results obtained in the pharmacokinetic analysis of precursor PM and generated CEA with the data obtained following intravenous administration of synthetic CEA, the fraction of total clearance of PM that furnished CEA to circulation (f_m) was estimated to be 1.03. The f_m value indicated that CEA was almost completely available to circulation once it was formed from PM. A similar f_m value was also observed in the animal following simultaneous intravenous administration of the deuterium labeled precursor, PM-d_8, and unlabeled preformed CEA. The synthetic CEA and derived CEA from PM were simultaneously quantified in the same animal and the effect of individual variation on the estimation was therefore eliminated. The f_m value was observed to be 1.06, consistent with the estimation using group mean values from separate administrations.

In rats administered with CP, the plasma concentrations of CP and its metabolites, HOCP, PM and CEA were measured using the GC/MS and isotope dilution techniques. The presence of CEA in plasma and urine was demonstrated for the first time in this study. The representative plasma concentration-time profiles of CP, HOCP, PM, and CEA are presented in Fig. 4. Plasma concentrations of CP declined monoexponentially in two of six rats with a mean terminal half-life of 51.6 min. Similar to the result obtained in the previous study [4], the concentrations of HOCP declined in parallel with those of parent CP, indicating that the formation of HOCP from CP was rate-determining for the disposition of the derived HOCP. Also, PM was observed to be the major metabolite of CP in urine and plasma and no appreciable amount of nor nitrogen

Table 1 Mean(±SD values) pharmacokinetic parameters of derived and synthetic N-2-chloroethylaziridine following intravenous administrations of precursor phosphoramide mustard or N-2-chloroethylaziridine to Sprague-Dawley rats

Pharmacokinetic parameter	CEA				PM 20 mg/kg ($n = 9$)
	Synthetic			Derived[a] ($n = 9$)	
	5 mg/kg ($n = 5$)	10 mg/kg ($n = 6$)	20 mg/kg ($n = 5$)		
Co (μM)	13.4 ± 6.3	27.6 ± 5.8	61.7 ± 19.0	20.1 ± 5.8	169 ± 52
$t_{½}\alpha$ (min)	6.60 ± 3.85	5.43 ± 3.88	5.40 ± 3.75	4.24 ± 2.77	–
$t_{½}\beta$ (min)	51.8 ± 11.4	48.4 ± 16.3	42.4 ± 7.4	47.1 ± 17.5	15.7 ± 3.6
V_C (L/kg)	4.08 ± 1.54	3.58 ± 0.74	3.37 ± 1.19	–	–
V_{SS} (L/kg)	9.21 ± 1.88	8.19 ± 2.15	6.89 ± 2.49	–	0.419 ± 0.190
MRT (min)	51.3 ± 11.4	57.3 ± 17.1	45.8 ± 7.6	74.8 ± 25.3	27.7 ± 5.2
CL_T (L/min/kg)	0.184 ± 0.043	0.165 ± 0.072	0.152 ± 0.049	–	0.0184 ± 0.0062
AUC (μM min)	267 ± 50	688 ± 215	1,341 ± 397	631 ± 115	3,729 ± 1,146
% Dose in 24 h urine	–	0.164 ± 0.088	–	3.91 ± 1.90	7.02 ± 4.25

[a] derived CEA was generated from its precursor, PM

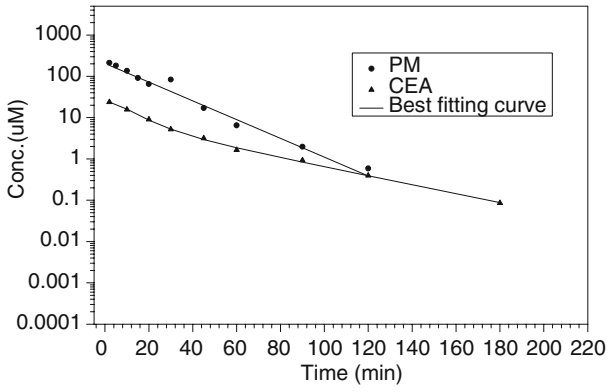

Fig. 3 The representative concentration-time profiles of phosphoramide mustard (PM) and derived N-2-chloroethylaziridine (CEA) in the Sprague-Dawley rat following intravenous administration of phosphoramide mustard at a dose of 20 mg/kg. The fraction of PM that furnished CEA in the circulation, f_m, was estimated using the equation: $f_m = [AUC_{derived\ CEA}/ D_{PM}]/[AUC_{CEA}/D_{CEA}]$; where $AUC_{derived\ CEA}$ and AUC_{CEA} are group mean AUC of the derived CEA and preformed CEA, respectively. D_{PM} and D_{CEA} are the doses of PM and preformed CEA

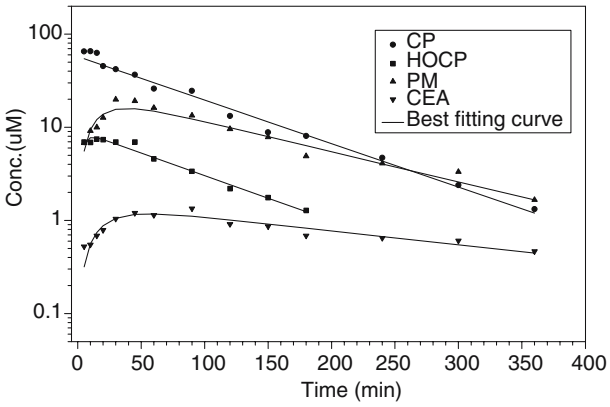

Fig. 4 The representative concentration-time profiles of cyclophosphamide, and its metabolites, 4-hydroxycyclophosphamide, phosphoramide mustard, and N-2-chloroethylaziridine, in the Sprague-Dawley rat following intravenous administration of cyclophosphamide at a dose of 20 mg/kg

mustard was observed in plasma. Mean derived pharmacokinetic parameters of CP, HOCP, PM, and CEA in rats administered with CP are presented in Table 2. The volume of distribution and total body clearance of CP was 0.671 L/kg (equal to total body water) and 11.1 mL/min/kg (20.1% of liver blood flow), respectively. The metabolite-to-parent AUC ratios of HOCP, PM, and CEA in rats were estimated to be 18.2, 74.7 and 4.27%, respectively. Mean urinary excretion of unchanged CP, and metabolites HOCP, PM, and CEA as the percentage of administered dose in 24 h urine were 0.532, 6.2, 63.6, and 1.3%, respectively. Overall, the pharmacokinetic parameters for CP, HOCP, and PM are similar to the observations in the previous study from this laboratory [4] and the pharmacokinetics of CEA was also investigated for the first time in this study. As shown in Table 2, the half-life values of CP, HOCP, PM, and CEA following the intravenous administration of CP were 52, 51, 92, and 100 min, respectively. CEA exhibited the longest half-life and mean residence time compared to CP, HOCP and PM. Of note, in cancer patients treated with CP ($n = 3$), similar concentration-time profiles of CP, HOCP, PM, and CEA were also observed with PM exhibiting the highest in concentration compared to HOCP and CEA (data not shown).

Discussion and conclusion

Although CP has been widely used in cancer chemotherapy for many years and its pharmacokinetics has been investigated in numerous studies [5, 6], the metabolism and disposition of this drug is still not fully understood. PM has generally been recognized as the ultimate intracellular cytotoxic metabolite of CP [6, 7]. The previous studies by this laboratory demonstrated that the P-N bond cleavage of PM to form NNM only occurred readily at a low pH in vitro [11, 16]. No appreciable NNM was detected in the incubation of PM in a pH 7.4 sodium phosphate buffer and CEA instead of NNM was found to be a major in vitro degradation product of PM at a physiological pH [11, 16]. At a pH above the pK_a of PM, the hydrolytic pathway of PM was via the formation of aziridinium ion, followed by a nucleophilic attack [11, 13, 16, 20, 21]. These in vitro data suggested that the cleavage of P-N bond of PM to form CEA could also be readily in vivo. The in vivo studies by this laboratory [4, 5] showed that no appreciable amount of circulating NNM was detected in both rodents and humans treated with the precursors, CP or HOCP. Although the rapid removal of generated NNM by bicarbonate in the blood stream could be the possible reason for that observation, the major part of PM was metabolized through the enzymatic cleavage of P-N bond of aziridinium ion to form CEA could also be highly possible. Thus, the present study was aimed at the assessment of the fraction of total clearance of PM that furnished the primary metabolite CEA to circulation in vivo.

Following intravenous administration of PM to Sprague Dawley rats (Figs. 2, 3; Table 1), mean half-lives of PM and derived CEA were 15.7 and 47.1 min, respectively. CEA showed a longer half-life and was metabolically more stable than PM in vivo. The urinary excretion of the unchanged PM was approximately 7% following administration of PM to rats. The fraction of total clearance of PM that furnished CEA to circulation, f_m, was estimated to be 0.93 based on this urinary excretion data of PM. Moreover, the pharmacokinetic analyses from separate administrations of synthetic CEA and its precursor PM showed that nearly all the PM was biotransformed into CEA in vivo ($f_m = 1.03$). The fraction of total clearance of PM that furnished CEA to circulation was approximately 100%, i.e., virtually complete availability of the metabolite to

Table 2 Mean (±SD values) pharmacokinetic parameters of cyclophosphamide and its metabolites following intravenous administration of cyclophosphamide to Sprague-Dawley rats ($n=6$) at a dose of 20 mg/kg

Pharmacokinetic parameter	CP	HOCP	PM	CEA
C_{max} (μM)	131 ± 77	7.82 ± 2.41	31.1 ± 16.8	1.69 ± 1.34
t_{max} (min)	–	4.98 ± 1.98	34.3 ± 11.7	38.6 ± 15.1
$t_{½}\lambda$ (min)	51.6 ± 17.0	51.1 ± 18.7	91.8 ± 27.6	100 ± 55
V_{SS} (L/kg)	0.671 ± 0.217	–	–	–
MRT (min)	69.0 ± 29.4	65.4 ± 14.4	146 ± 43	176 ± 97
CL_T (ml/min/kg)	11.1 ± 4.7	–	–	–
AUC (μM h)	110 ± 43	20.1 ± 9.8	82.2 ± 29.9	4.70 ± 2.58
% administered dose in 24 h urine	6.20 ± 2.67	0.532 ± 0.312	63.6 ± 9.1	1.33 ± 0.56

circulation once formed (Table 1). A similar result was also found in the animal following simultaneous administration of labeled PM and unlabeled synthetic CEA. The cleavage of P-N bond of aziridinium ion of PM to generate CEA was therefore considered to be the major in vivo metabolic pathway for PM (see Fig. 5). In rats receiving CP (Fig. 4; Table 2), AUC and urinary excretion data indicated that PM was the major circulating metabolite of CP. It is generally believed that PM is ultimate DNA cross-linking agent generated by CP. However, the poor stability and membrane permeability

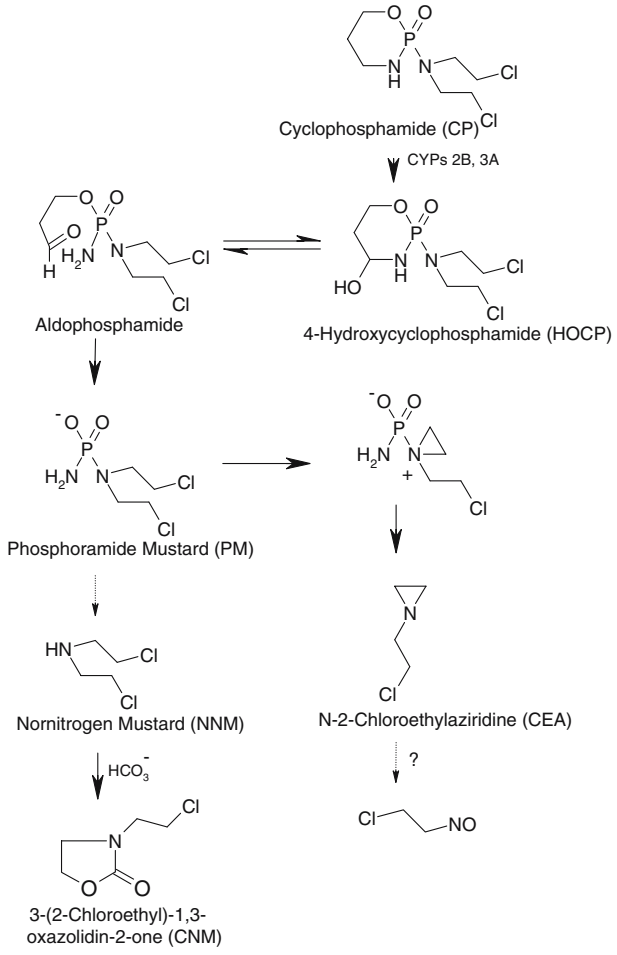

Fig. 5 Major metabolic scheme of cyclophosphamide in vivo

appeared to be the issue for PM to enter the tumor cells and exert its cytotoxic activity. Chan et al reported that the partition of CP, HOCP, and PM between the rat blood cells and plasma were 1, 1, and 0.5, respectively [4]. In this study, the partition of CEA was found to be approximately 1.2, slightly in favor of blood cells. In addition, the volume of distribution of CP and its major metabolites HOCP, PM, and CEA in rats were 671, 936, 419, and 3,680 mL/kg, respectively, indicating that CEA was more widely distributed in tissues compared to its precursors CP, HOCP, and PM. Furthermore, the respective half-life for HOCP, PM, and CEA was 8.1, 15.7, and 44.2 min following the intravenous administration, indicating that CEA was metabolically more stable than HOCP and PM. In light of these pharmacokinetic findings, the role of CEA in the antitumor activity of CP can not be ignored. CEA is observed in both plasma and urine of the rats administered with prodrug CP and it has more favorable pharmacokinetic characteristics than its precursors HOCP and PM. Besides, CEA was the predominant circulating metabolite in rats administered with precursor PM. Thus, the biotransformation pathway leading to the formation of CEA from PM may contribute significantly to the overall antitumor activity of CP since CEA has been shown to have potent in vitro antitumor activity [2, 9, 12].

Metabolism of aziridines and the mechanism of their cytotoxicity have been reviewed in the literature [22]. Hata and Watanabe pointed out that aziridines could be transported across cell membranes and then biotransformed into N-oxide by cellular oxidases followed by fragmentation to produce nitroso compounds. The resultant nitroso metabolites inhibit mitochondrial respiration by decomposition of the ATP-generating system to causes cell death. In addition, the cellular glutathione modulates the cytotoxic process of aziridines by forming glutathione conjugate [22].

In summary, the data from the present study strongly suggested that CEA was an important metabolite for produg CP. Taken together all the in vitro and in vivo findings on CEA reported in literature [2, 9, 11–13, 22] and the present study, we present the major metabolic scheme of CP in Fig. 5. The in vivo biotransformation from PM to CEA rather than NNM is the major metabolic pathway for PM. Although PM showed the

highest in concentration in plasma and urine following intravenous administration of CP to rats and cancer patients, PM may act as a 'prodrug' for CEA under physiological conditions in vivo and CEA may play an important role in the antitumor activity of CP. A study to investigate the subsequent metabolism of CEA and the role of CEA in alkylating DNA, proteins etc. will be very helpful in understanding the mechanism of the overall cytotoxicity of CP.

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