Cancer Chemother Pharmacol (2000) 46: 119–127 © Springer-Verlag 2000

ORIGINAL ARTICLE

Alwin D. R. Huitema · Thomas Kerbusch
Matthijs M. Tibben · Sjoerd Rodenhuis · Jos H. Beijnen

Reduction of cyclophosphamide bioactivation by thioTEPA: critical sequence-dependency in high-dose chemotherapy regimens

Received: 31 January 2000 / Accepted: 10 April 2000

Abstract *Purpose*: Cyclophosphamide and thioTEPA are frequently used simultaneously in high-dose chemotherapy regimens. During a pharmacokinetic study of 31 courses in 20 patients of cyclophosphamide and its activated metabolite 4-hydroxycyclophosphamide given in the combination cyclophosphamide–thioTEPA–carboplatin, a sharp decrease in 4-hydroxycyclophosphamide concentration was observed immediately after the start of the thioTEPA infusion. A drug-drug interaction was suspected. This putative interaction was investigated in this study. *Methods*: Possible sequence dependency, due to inhibition of the formation of 4-hydroxycyclophosphamide by thioTEPA, was investigated by altering the sequence of infusion in three patients (four courses) receiving high-dose chemotherapy with cyclophosphamide (1000 or 1500 mg/m^2 per day), thioTEPA (80 or 120 mg/m^2 per day) and carboplatin (265 or 400 mg/m^2 per day) in short infusions for four consecutive days. The pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide were established. Possible inhibition of the metabolism of cyclophosphamide and thioTEPA was investigated in human microsomes. *Results*: A striking sequence dependency of the pharmacokinetics of 4-hydroxycyclophosphamide was observed. Administration of thioTEPA 1 h prior to cyclophosphamide resulted in decreased C_{max} (−62%) and AUC (−26%) values of 4-hydroxycyclophosphamide compared to those of thioTEPA administered 1 h after cyclophosphamide. In human microsomes an inhibition of the conversion of cyclophosphamide to 4-hydroxycyclophosphamide by thioTEPA was observed at clinically relevant concentrations with an IC_{50} of 23 μ*M*. No inhibition of the formation of TEPA by cyclophosphamide was observed. *Conclusions*: ThioTEPA strongly inhibits the bioactivation of cyclophosphamide and this may decrease both efficacy and toxicity. Our results seriously question the practice of the simultaneous continuous infusion of cyclophosphamide and thioTEPA and suggest that the sequencing and scheduling of these two agents in high-dose chemotherapy regimens may be of critical importance.

Key words ThioTEPA · Cyclophosphamide · Pharmacokinetics · Drug-drug interaction · Chemotherapy

A. D. R. Huitema (✉) · T. Kerbusch · M. M. Tibben
Department of Pharmacy and Pharmacology,
The Netherlands Cancer Institute/Slotervaart Hospital,
Louwesweg 6, 1066 EC, Amsterdam, The Netherlands
e-mail: Apahu@slz.nl
Tel.: +31-205124657; Fax: +31-205124753

A. D. R. Huitema · T. Kerbusch · S. Rodenhuis · J. H. Beijnen
Department of Medical Oncology,
The Netherlands Cancer Institute/Antoni van
Leeuwenhoek Hospital, Plesmanlaan 121,
1066 CX, Amsterdam, The Netherlands

J. H. Beijnen
Division of Drug Toxicology, Faculty of Pharmacy,
Utrecht University, PO Box 80082, 3508 TB Utrecht,
The Netherlands

Introduction

Cyclophosphamide and thioTEPA are both alkylating agents and have been used in cancer chemotherapy for many years. The interest in the use of these alkylating agents was renewed by the finding that their dosages can be increased substantially when used in combination with haematological support, such as autologous peripheral blood progenitor cell transplantation. Cyclophosphamide and thioTEPA are the most frequently used cytotoxic agents in high-dose combination chemotherapy regimens [24, 35].

One particularly well-established regimen in high-dose chemotherapy for solid tumours is the CTCb regimen which consists of a combination of cyclophosphamide (6000 mg/m^2), thioTEPA (500 mg/m^2) and carboplatin (800 mg/m^2) administered simultaneously as 96-h continuous infusions [2, 11, 13, 19, 29, 33, 34]. A related regimen, that is commonly used in the

Netherlands, is the CTC regimen which consists of single courses of cyclophosphamide (6000 mg/m^2), thioTEPA (480 mg/m^2) and carboplatin (1600 mg/m^2) divided over 4 days administered in short infusions [28] or multiple courses with the doses of all agents reduced to two-thirds [27]. Cyclophosphamide and thioTEPA are also used simultaneously in many other combinations in high-dose chemotherapy [24, 35].

Cyclophosphamide is a prodrug that requires bioactivation before it exerts cytotoxicity. The first step is the hydroxylation by the cytochrome P450 isoenzymes of cyclophosphamide to 4-hydroxycyclophosphamide, which is in equilibrium with its open-ring tautomeric form aldophosphamide. After β-elimination of acrolein, the ultimate alkylating metabolite phosphoramide mustard is formed. Various deactivation routes exist. Oxidation of cyclophosphamide, 4-hydroxycyclophosphamide and aldophosphamide lead to the formation of the inactive metabolites 2-dechloroethylcyclophosphamide, 4-ketocyclophosphamide and carboxyphosphamide, respectively (Fig. 1) [21]. The cytochrome P450 isoenzymes 2B6 [6], 2C9 [7, 25], 2C19 [7] and 3A4 [25] have been reported to be involved in the formation of 4-hydroxycyclophosphamide, and of these 2B6 has the highest activity, while 3A4 is involved in the formation of 2-dechloroethylcyclophosphamide [25]. Cyclophosphamide metabolism shows autoinduction, which results in an increase in clearance during treatment [18].

ThioTEPA is rapidly metabolized by the cytochrome P450 to TEPA. Both thioTEPA and TEPA have alkylating properties. Only metabolism studies in rat microsomes have been reported, and the rat cytochrome P450 families IIB and IIC have been identified as catalysts for the formation of TEPA [22, 23].

In vitro studies have demonstrated a synergistic activity without cross-resistance for the combination of cyclophosphamide and thioTEPA [12, 31]. However, in these studies 4-hydroxycyclophosphamide was used instead of cyclophosphamide since bioactivation cannot occur in these cell lines. Preclinical in vivo studies have shown that the combination of cyclophosphamide and thioTEPA produces at least an additive cytotoxic effect [31, 32].

During a pharmacokinetic study of 4-hydroxycyclophosphamide in the CTC regimen we observed a sharp decrease in plasma concentration of 4-hydroxycyclophosphamide immediately after the start of the thioTEPA infusion. An interaction between cyclophosphamide and thioTEPA was suspected. In this study, the possible reduction of the bioactivation of cyclophosphamide by thioTEPA was further investigated in cancer patients and in human liver microsomes.

Materials and methods

Chemicals

4-Hydroperoxycyclophosphamide (purity >95%) and cyclophosphamide were kind gifts from Dr. J. Pohl (Asta Medica, Frankfurt, Germany). 4-Hydroperoxycyclophosphamide is a stable precursor of 4-hydroxycyclophosphamide which rapidly liberates 4-hydroxycyclophosphamide in aqueous solution. ThioTEPA (Leder-TEPA) was obtained from AHP Pharma (Hoofddorp, The Netherlands), TEPA (purity >98%) was synthesized at the Faculty of Chemistry, Utrecht University, according to the method described by Craig and Jackson [10]. Ethyl acetate, acetonitrile and methanol (HPLC supra-gradient) were purchased from Biosolve (Valkenswaard, The Netherlands), and potassium dihydrogenphosphate (suprapure grade) was obtained from Merck (Darmstadt, Germany). A 50 m*M* potassium phosphate buffer (pH 7.4) was prepared. Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). A 2 *M* solution of semicarbazide in 50 m*M* potassium phosphate buffer (pH 7.4) was prepared. Stock solutions of cyclophosphamide and thioTEPA were prepared in 50 m*M* potassium phosphate buffer (pH 7.4). Distilled water was used throughout.

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and β-NADP were purchased from Sigma (St. Louis, Mo.). A NADPH-regenerating solution (NRS) was prepared in 50 m*M* phosphate buffer (pH 7.4) containing 0.5 mg/ml β-NADP, 2.0 mg/ml glucose-6-phosphate and 1.5 U/ml glucose-6-phosphate dehydrogenase. Pooled human liver microsomes were purchased from Gentest (Woburn, Mass.) at a concentration of 20 mg/ml protein. This solution was diluted immediately before use with phosphate buffer (pH 7.4) at 4 °C to a concentration of 4 mg/ml.

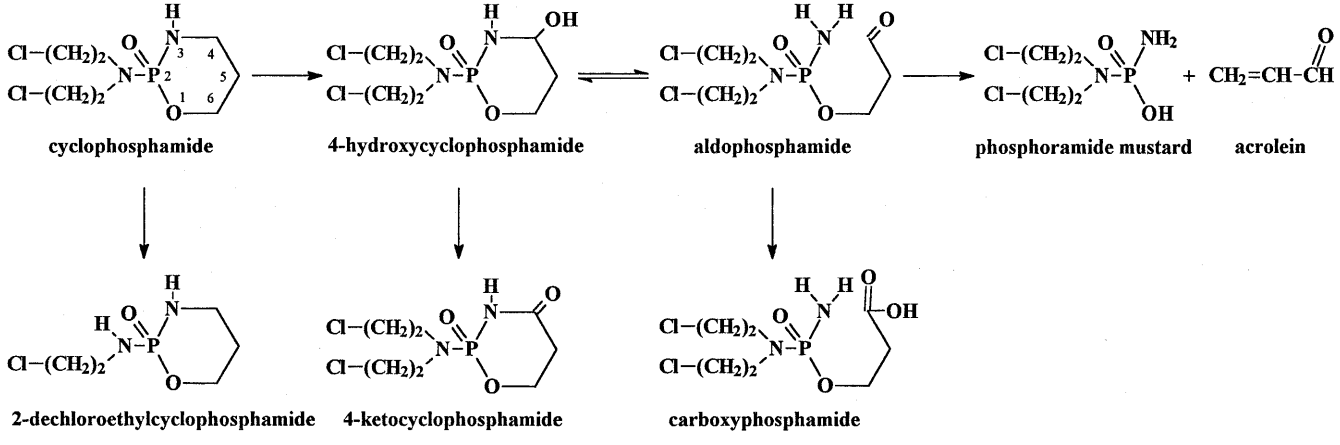

Fig. 1 Metabolism of cyclophosphamide and its main metabolites. The bioactivation of cyclophosphamide is shown in the horizontal direction, and the inactivation processes in the vertical direction

Patients and treatment

All patients took part in high-dose chemotherapy protocols with peripheral blood progenitor cell transplantation and written informed consent was obtained according to institutional policy. All protocols had been approved by the institutional review committee. Patients either had high-risk primary breast cancer and received treatment as part of their adjuvant therapy or had advanced breast cancer. Mobilization of progenitor cells, treatment and inclusion criteria were as described previously [27, 28].

Patients were treated with a single course of full-dose CTC or multiple courses of 'tiny' CTC (tCTC) every 4 weeks. The CTC regimen consisted of cyclophosphamide 1500 mg/m^2 as a daily 1-h infusion, carboplatin 400 mg/m^2 as a daily 1-h infusion and thioTEPA 60 mg/m^2 as twice daily 30-min infusions, over four consecutive days. The tCTC was identical to the CTC regimen except that it involved the use of precisely two-thirds of the doses of each agent. MESNA (500 mg) was administered six times daily for a total of 36 doses, beginning 1 h prior to the first cyclophosphamide infusion. All patients received antiemetics both prophylactically and as indicated, which usually included dexamethasone and granisetron. Patients received prophylactic antibiotics, including ciprofloxacin, itraconazole (twice daily 200 mg) and acyclovir orally, starting 4 days before chemotherapy until 2 weeks after the progenitor cell transplantation.

In order to investigate possible sequence dependency on the pharmacokinetics of cyclophosphamide, thioTEPA, and their metabolites, three patients (four courses) received two different infusion sequences. The standard infusion scheme (scheme A) of the anticancer agents was first cyclophosphamide (1-h infusion) immediately followed by carboplatin (1-h infusion) and thioTEPA (30-min infusion). This scheme was reversed on one of the treatment days in these four courses (scheme B, thiotepa immediately followed by carboplatin and cyclophosphamide). The second thioTEPA dose was administered 12 h after the last thioTEPA infusion. In two courses patients received scheme B on day 2, and in the other courses patients received scheme B on day 1. On all other treatment days, scheme A was used.

Sampling and pharmacokinetic analysis

Samples were collected prior to the start of the infusions on all days of chemotherapy, and on days 1 and 2, 30 min after the start of the infusions (t = 0) and at t = 60, 90, 120, 150, 180, 210, 285, 390 and 660 min after the start of the first infusion. On day 5 an additional sample was collected approximately 22 h after the last cyclophosphamide infusion. A central venous double-lumen catheter was used for the collection of the samples as described previously [15].

Pharmacokinetic parameters calculated for 4-hydroxycyclophosphamide and cyclophosphamide were the maximum concentration (C_{max}) and the area under the concentration-time curve between 0 and 12 h ($AUC_{0–12}$). The $AUC_{0–12}$ was calculated using the trapezoidal rule. After 12 h, the next thioTEPA infusion was started so only the AUC over the first 12 h was used. The mean effects of the reversal of the infusion sequence on the pharmacokinetics of 4-hydroxycyclophosphamide were estimated by calculating the mean percentage change in C_{max} and AUC between day 1 and day 2 for the two patients with the same infusion sequence. The effects of the infusion sequence may be counteracted or amplified by the effects of the autoinduction of cyclophosphamide. Using infusion scheme A on day 1 and scheme B on day 2, changes in C_{max} or AUC (ΔC_{max} and ΔAUC) were caused by the change due to autoinduction (ΔAI) minus the change caused by the reversal of the infusion (ΔRev). For example, the change in C_{max} is represented by the equation $\Delta C_{max} = \Delta AI – \Delta Rev$. For infusion scheme B on day 1 and scheme A on day 2 the percentage change of C_{max} and AUC was estimated by the change due to autoinduction plus the change caused by the reversal of the infusion scheme. Using this sequence, the change in C_{max} is represented by $\Delta C_{max} = \Delta AI + \Delta Rev$. The change in 4-hydroxycyclophosphamide pharmacokinetics on day 2 due to autoinduction and due to reversal of the infusion scheme could thus be estimated by solving these two equations both for ΔAI and ΔRev.

Microsome incubations

The general procedure for the microsome incubations started by mixing 250 µl of the NRS solution with 50–150 µl of the thioTEPA or cyclophosphamide stock solutions and 100–250 µl of 50 m*M* phosphate buffer (pH 7.4) to reach a final volume of 500 µl in a 1.5-ml Eppendorf tube. This mixture was preincubated at 37 °C for 5 min. A 50-µl volume of the 4 mg/ml microsome solution at 4 °C (0.2 mg protein) was added to initiate the reaction. Samples were incubated at 37 °C for 60 min in duplicate. The reaction was terminated by the addition of 500 µl methanol. All experiments were performed in duplicate.

The enzyme kinetics of cyclophosphamide were investigated by the addition of stock solutions of cyclophosphamide to the mixture of NRS and buffer to reach final concentrations of cyclophosphamide between 10 and 200 µ*M*. The Michaelis-Menten constant (Km) and maximum conversion rate (V_{max}) were calculated using a Lineweaver-Burk plot.

The inhibition of the conversion of cyclophosphamide to 4-hydroxycyclophosphamide by thioTEPA was investigated by adding 50 µl of stock solutions of thioTEPA to the mixture of phosphate buffer to reach final concentrations of thioTEPA between 0.5 and 50 µ*M*. Cyclophosphamide stock solution was added to a final concentration of 200 µ*M*. Also inhibition by TEPA (at a concentration of 50 µ*M*) was tested in this system. Possible inhibition of the conversion of thioTEPA to TEPA by cyclophosphamide was investigated using incubation mixtures of 200 µ*M* thioTEPA and cyclophosphamide concentrations between 2 and 200 µM. The IC_{50} was calculated by linear interpolation of the conversion rate versus inhibitor concentration plot.

The conversion rate of the substrate was calculated as amount (picomoles) per unit time (hours) per amount of microsome protein (milligrams). Although 4-hydroxycyclophosphamide is unstable at 37 °C [20], the absolute amount of 4-hydroxycyclophosphamide measured after 60 min incubation reflects the formation rate of this metabolite. Therefore an apparent formation rate ($V_{4–OHCP}$) was calculated for this conversion and used throughout. TEPA is a stable metabolite during a short incubation and therefore the formation rate of TEPA (V_{TEPA}) reflects the true rate of the enzyme reaction.

Assays for cyclophosphamide, thioTEPA and metabolites

Immediately after collection, whole blood samples were centrifuged at 3000 *g* for 3 min at 4 °C. Plasma was separated and stored at −70 °C until analysis. Cyclophosphamide, thioTEPA and TEPA were quantified using a previously described gas chromatographic assay [14]. The accuracy was between 98% and 106%, and between-day and within-day precisions were less than 9% for all drugs over the complete clinical concentration range. For the determination of 4-hydroxycyclophosphamide, 1 ml of plasma was derivatized immediately after collection with 100 µl 2 *M* semicarbazide and stored at −70 °C pending analysis. The plasma layer was extracted with 2.0 ml of ethyl acetate. A 1500 µl volume of the organic layer was removed and evaporated to dryness under air at 30 °C. The sample was reconstituted in 40 µl acetonitrile followed by 125 µl water. Of this solution, 50 µl was injected into the high-performance liquid chromatography (HPLC) system which consisted of a Thermo Separations Products Spectra series (TSP, Fremont, Calif.) model AS3000 automated injector with a 100 µl loop and a model P1000 solvent delivery system. A Spectra 100 variable wavelength UV detector was used (Spectra Physics, Santa Clara, Calif.) set at 230 nm.

Separation was performed using a Prodigy 5 C8 column (250 × 4.6 mm, particle size 5 µm; Phenomenex, Torrance, Calif.) protected with a C8 guard column (Security Guard, Phenomenex). The mobile phase consisted of acetonitrile/0.025 *M* potassium phosphate buffer, pH 6.0 (15:85 v/v). The flow rate was 1.0 ml/min,

and the column was operated at ambient temperature. The analyte was identified to be the semicarbazone derivative of 4-hydroxycyclophosphamide using an electron spray VG Platform II ion spray mass spectrometer (Micromass, Altrincham, UK). The accuracy was between 96% and 103%, and between-day and within-day precisions were less than 7% over a concentration range of 50–5000 ng/ml and TEPA and thioTEPA did not show any interference with the assay [17].

TEPA was determined in the microsome incubate as described for the determination of TEPA in plasma [14]. 4-Hydroxycyclophosphamide was determined in the microsome incubate after adding 100 µl of the 2 *M* semicarbazide solution and 50 µl 4 *M* HCl to the incubate for the derivatization of 4-hydroxycyclophosphamide. After 10 min, the solution was neutralized with 50 µl 4 *M* NaOH and centrifuged at 10,500 *g* for 5 min. Of this solution, 100 µl was injected in the described HPLC system for the determination of the semicarbazone derivative of 4-hydroxycyclophosphamide in plasma.

Results

A total 20 patients (31 courses) were included in the pharmacokinetic study of 4-hydroxycyclophosphamide. Figure 2 shows concentration-time curves of 4-hydroxycyclophosphamide using infusion scheme A (cyclophosphamide followed by carboplatin and thioTEPA) for these patients. Figure 3 shows the concentration-time curves of 4-hydroxycyclophosphamide and thioTEPA on days 1 and 2 of treatment for patient 1 (scheme A on day 1, scheme B on day 2). Since 4-hydroxycyclophosphamide is an unstable metabolite, the elimination of 4-hydroxycyclophosphamide is formation-limited. Therefore, the elimination of 4-hydroxycyclophosphamide follows the elimination of cyclophosphamide [8, 30]. In line with this, during the first 2 h after the start of the cyclophosphamide, infusion 4-hydroxycyclophosphamide concentrations paralleled the cyclophosphamide concentrations. However, immediately after the start of the thioTEPA infusion, the concentration of 4-hydroxycyclophosphamide strongly decreased. This was a consistent finding in all patients included and on all treatment days.

The effects of reversal of the infusion scheme are shown in Fig. 3 and Table 1. The C_{max} of 4-hydroxycyclophosphamide was strongly reduced and no strong decrease 2 h after the start of the infusion was observed using scheme B. In Table 1 the quantitative influences of the infusion sequence on the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide in the three patients treated with two different infusion sequences are summarized. From the data of these four courses the mean changes due to autoinduction (ΔAI) and due to the reversal of the infusion scheme (ΔRev) were calculated. The AUC and C_{max} of 4-hydroxycyclophosphamide after scheme B were 26% and 62% lower, respectively, compared to scheme A. The autoinduction of cyclophosphamide resulted in an increase in AUC and C_{max} of 4-hydroxycyclophosphamide between day 1 and day 2 of 41% and 118%, respectively.

In Fig. 4 the Lineweaver-Burk plot for the conversion of cyclophosphamide to 4-hydroxycyclophosphamide in pooled human microsomes is shown. The Km and V_{max} calculated were 149 µ*M* (40 µg/ml) and 4.7 nmol/h mg, respectively. The inhibition of the conversion of cyclophosphamide by thioTEPA in microsomes is shown in Fig. 5. The IC_{50} value of thioTEPA was 23 µ*M* (4.4 µg/ml). In the patient population included in the pharmacokinetic study, maximum concentrations of thioTEPA between 1.5 and 3.0 µg/ml were found. The conversion of thioTEPA to TEPA was not inhibited by cyclophosphamide (Fig. 6) and the conversion of cyclophosphamide was not inhibited by TEPA in microsomes.

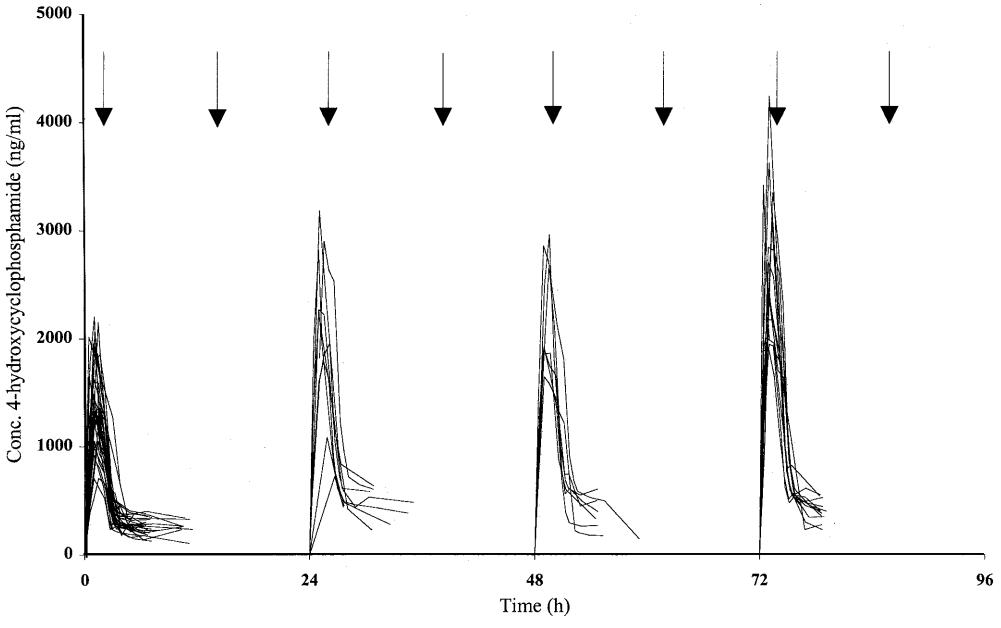

Fig. 2 Concentration-time curves of 4-hydroxycyclophosphamide of the patients included in the pharmacokinetic study with the standard infusion sequence (scheme A: cyclophosphamide → carboplatin → thioTEPA). The *arrows* indicate the start of the thioTEPA infusions

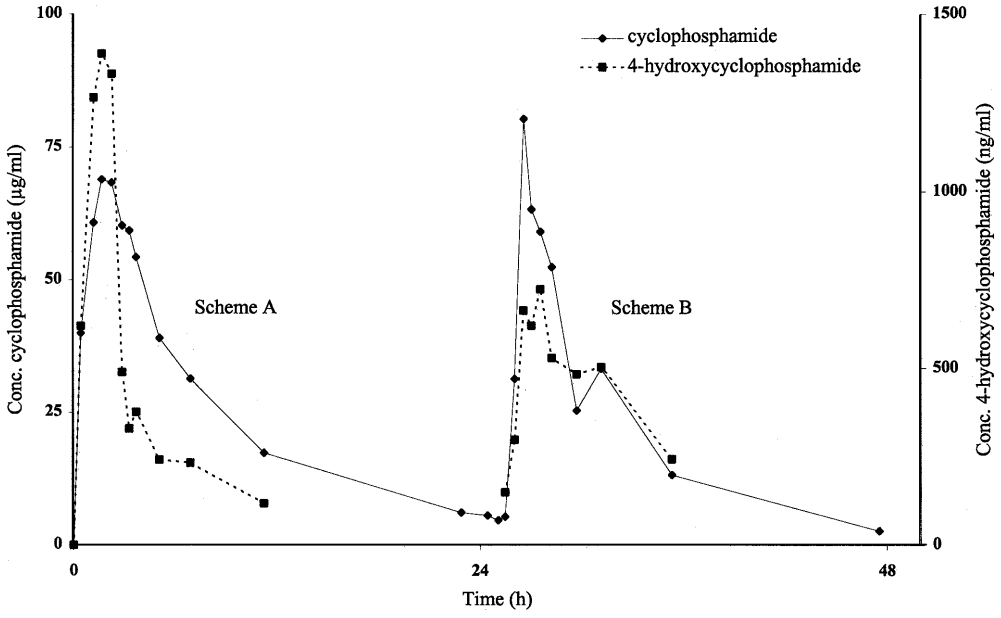

Fig. 3 Concentration-time curves of 4-hydroxycyclophosphamide and cyclophosphamide for patient 1 on day 1 (infusion scheme A: cyclophosphamide → carboplatin → thioTEPA) and 2 (infusion scheme B: thioTEPA → carboplatin → cyclophosphamide) of treatment

Table 1 Sequence dependency of cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics (*scheme A* cyclophosphamide → carboplatin → thioTEPA, *scheme B* thiotepa → carboplatin → cyclophosphamide; *AUC* area under the concentration time curve between 0 and 12 h after the start of the cyclophosphamide infusion)

Patient	Course	Dose cyclophosphamide (mg/m^2)	Parameter	Day 1 4-hydroxy-cyclophosphamide	Cyclophosphamide	Day 2 4-hydroxy-cyclophosphamide	Cyclopho-sphamide
				Scheme A		Scheme B	
1	1	1500	AUC (h µg/ml)	4.8	451	4.8	588
			C_{max} (µg/ml)	1.4	69	0.66	80
2	1	1000	AUC (h µg/ml)	4.0	173	4.5	150
			C_{max} (µg/ml)	1.2	31	0.81	27
				Scheme B		Scheme A	
2	2	1000	AUC (h µg/ml)	4.2	253	6.5	200
			C_{max} (µg/ml)	0.59	51	2.0	50
3	1	1000	AUC (h µg/ml)	3.7	288	7.3	123
			C_{max} (µg/ml)	0.48	43	2.0	52

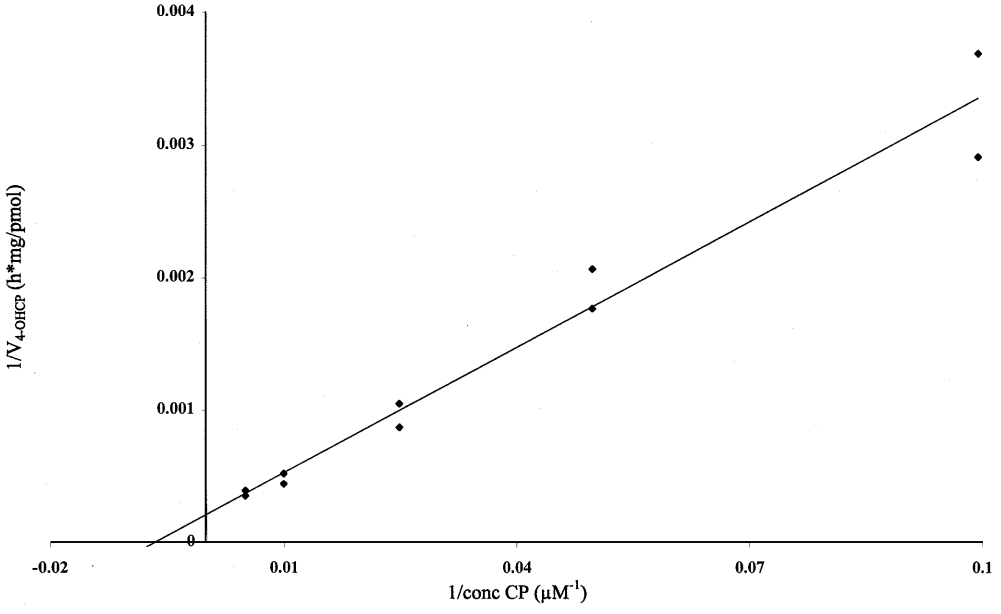

Fig. 4 Lineweaver-Burk plot for the conversion of cyclophosphamide to 4-hydroxycyclophosphamide in pooled human microsomes. The calculated Km and V_{max} values were 149 µ*M* (40 µg/ml) and 4.7 nmol/h mg, respectively

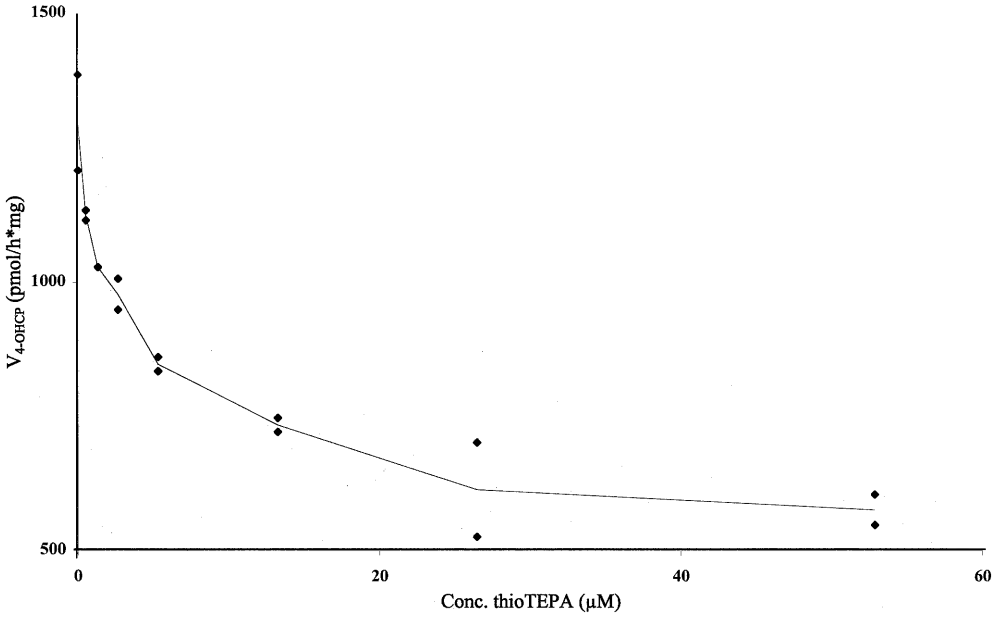

Fig. 5 Inhibition of the conversion of cyclophosphamide (200 μ*M*) by thioTEPA (IC_{50} 23 μ*M*). The solid line represents the mean value for each duplicate

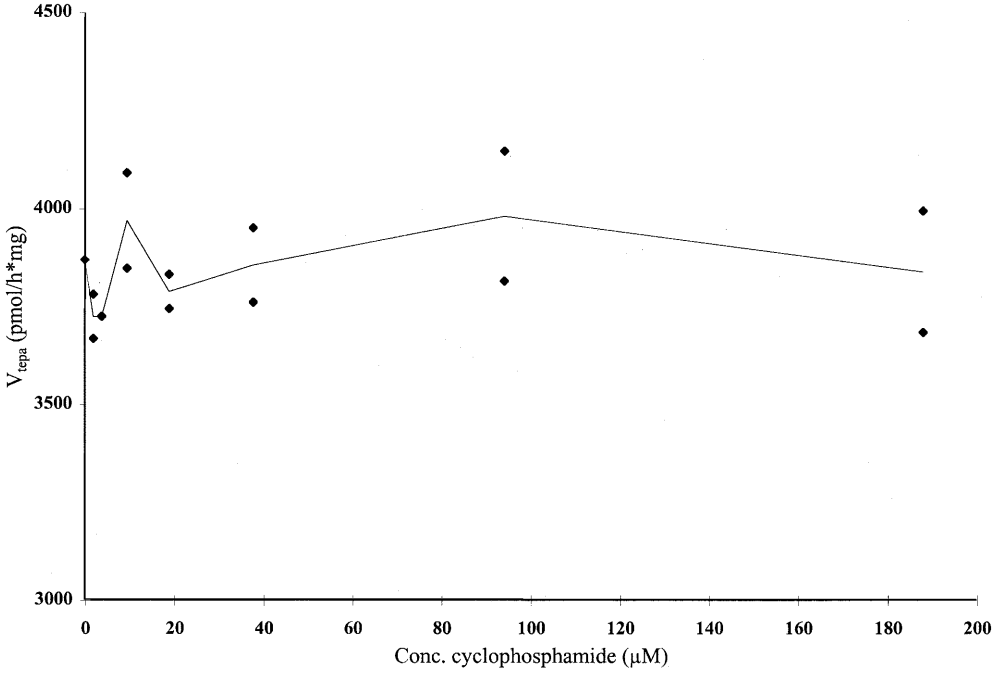

Fig. 6 Influence of cyclophosphamide on the conversion rate of thioTEPA (200 μ*M*) by cyclophosphamide. The solid line represents the mean value for each duplicate

Discussion

Due to the sharp decrease in 4-hydroxycyclophosphamide concentrations immediately after the start of the thioTEPA infusion, an interaction between cyclophosphamide and thioTEPA was suspected. Since cyclophosphamide and thioTEPA are both drugs extensively metabolized by cytochrome P450 an inhibition of the liver metabolism of cyclophosphamide by thioTEPA was the most likely mechanism.

The elimination of 4-hydroxycyclophosphamide is formation-limited and therefore a decrease in the formation rate of this metabolite is immediately represented by a sharp decrease in plasma concentration of this metabolite. From Table 1 and Fig. 3 it can be concluded that the formation of 4-hydroxycyclophosphamide was sequence-dependent due to the inhibition of the formation of 4-hydroxycyclophosphamide by thioTEPA. Cyclophosphamide shows autoinduction, which causes a decreased exposure to cyclophosphamide but an increased exposure to 4-hydroxycyclophosphamide during treatment [16, 26]. The AUC and C_{max} of 4-hydroxycyclophosphamide were strongly reduced after infusion scheme B (thioTEPA → cyclophosphamide) compared to scheme A (cyclophosphamide → thio-

TEPA), whereas the autoinduction of cyclophosphamide metabolism resulted in an increased exposure to 4-hydroxycyclophosphamide during treatment. The mean overall effects calculated from the patient data showed that simultaneous administration of thioTEPA resulted in decreased exposure to 4-hydroxycyclophosphamide.

An increase in the AUC of 4-hydroxycyclophosphamide was accompanied by a decrease in the AUC of cyclophosphamide (Table 1). This was expected since the 4-hydroxylation of cyclophosphamide is the major route of elimination of cyclophosphamide. Cyclophosphamide is, however, also metabolized to 2-dechloroethylcyclophosphamide. Furthermore, a considerable amount (17–29%) of cyclophosphamide is eliminated unchanged in the urine [5, 9, 26]. The elimination of cyclophosphamide through 2-dechloroethylcyclophosphamide accounts for approximately 5% of the total elimination of cyclophosphamide [4, 26]. Chen et al. have found a slight but not significant increase in urinary excretion of cyclophosphamide when combined with thioTEPA [9]. A significant increase in renal clearance and in 2-dechloroethylcyclophosphamide formation in high-dose cyclophosphamide therapy compared to conventional dose chemotherapy has been found by Busse et al. [4]. This may indicate that the side-chain oxidation of cyclophosphamide and the renal elimination of cyclophosphamide may serve as escape pathways for the elimination of cyclophosphamide.

All patients received itraconazole, a known inhibitor of CYP3A4, starting 4 days before chemotherapy. The timing of the strong decrease in 4-hydroxycyclophosphamide levels was related to thioTEPA administration but not to the administration of itraconazole or any of the other coadministered drugs. Furthermore, CYP2B6 is considered the main isoenzyme responsible for the formation of 4-hydroxycyclophosphamide. Therefore, these drugs were not considered to be related to the observed interaction.

In a preclinical study, without pharmacokinetic monitoring, the activity of the combination of cyclophosphamide and thioTEPA was compared to that of single-agent treatment. Both the sequence of administration and the interval between administration largely influenced the efficacy of the combination [32]. These findings can be explained by the demonstrated pharmacokinetic sequence-dependency of the combination.

From the experiments with the pooled human microsomes, it can be concluded that a strong inhibition of the bioactivation of cyclophosphamide occurs at clinically relevant concentrations of thioTEPA. For the inhibition of the formation of 4-hydroxycyclophosphamide by thioTEPA an IC_{50} of 23 μM was calculated. Anderson et al. have reported IC_{50} values between 1.0 and 40 μM in human microsomes, which is in accordance with our data [1]. This proves the hypothesis of an inhibition of the cytochrome P450 enzymes responsible for the conversion of cyclophosphamide by thioTEPA. Cyclophosphamide and thioTEPA are both metabolized by enzymes from the 2B and 2C classes [6, 7, 18, 22, 23, 25]. However, it remains unclear which specific isoenzymes are involved.

The interaction described may be very relevant for the treatment of patients with high-dose chemotherapy with combinations of thioTEPA and cyclophosphamide. As shown, a strong sequence dependency exists in the hydroxylation of cyclophosphamide. In the very frequently used CTCb regimen the anticancer agents are administered simultaneously as a 96-h continuous infusion [2, 11, 13, 19, 29, 33, 34]. Instead of a sharp decrease in bioactivation as shown in our study, a continuous infusion of cyclophosphamide and thioTEPA will lead to a continuous decreased exposure to 4-hydroxycyclophosphamide. Chen et al. and Anderson et al. have observed a decrease in cyclophosphamide clearance when cyclophosphamide is combined with thioTEPA [1, 9]. This finding can now be explained by an interaction of these agents as demonstrated in our study.

Ayash et al. have found an inverse relationship between the AUC of cyclophosphamide and cardiotoxicity and tumor response in the CTCb regimen [3]. This relationship is explained by the hypothesis that the bioactivation of cyclophosphamide is inversely related to the AUC of cyclophosphamide. Therefore, a reduction in the bioactivation due to a drug-drug interaction could have a large impact on the efficacy of the therapy.

In summary, this study showed that the bioactivation of cyclophosphamide is strongly reduced by thioTEPA both in vitro and in cancer patients. Administration of the complete cyclophosphamide dose more than 24 h (approximately three times the $t_{1/2}$ of cyclophosphamide) before thioTEPA will result in a noncompromised bioactivation of cyclophosphamide. Moreover, this may result in more predictable pharmacokinetics. This is a very relevant finding since thioTEPA- and cyclophosphamide-based regimens are used very frequently in high-dose chemotherapy.

Our results seriously question the practice of the simultaneous continuous infusion of cyclophosphamide and thioTEPA and suggest that the sequencing and scheduling of these two agents in high-dose chemotherapy regimens may be of critical importance. Differences in toxicity and efficacy between centres and regimens may be caused by these factors.

Acknowledgements This research was supported by a grant from the Dutch Cancer Society (project NKI 97-1439).

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