Effects of co-medicated drugs on cyclophosphamide bioactivation in human liver microsomes

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The alkylating agent cyclophosphamide (CP) is a prodrug requiring cytochrome P-450-mediated bioactivation to form the active 4-hydroxycyclophosphamide (4OHCP). Modifications in the rate of CP bioactivation may have implications for the effectiveness of CP therapy, especially in high-dose regimens. In this study, agents frequently co-administered with CP in high-dose chemotherapy regimens were tested for their possible inhibition of the bioactivation of CP in human liver microsomes. The K_m and V_{max} values for the conversion of CP to 4OHCP were 93 μM and 4.3 nmol/h·mg, respectively. No inhibition was observed for aciclovir, carboplatin, ciprofloxacine, granisetron, mesna, metoclopramide, ranitidine, roxitromycin and temazepam. Inhibition was observed for amphotericin B, dexamethasone, fluconazole, itraconazole, lorazepam, ondansetron and thiotepa, with IC_{50} values of 50, >100, >50, 5, 15, >100 and 1.25 μM, respectively. For all but thiotepa, these IC_{50} values were higher than the therapeutic drug levels and thus considered of no clinical relevance. We conclude that of the tested co-medicated agents, only thiotepa inhibited metabolism of CP to 4OHCP at clinically relevant concentrations, and may thereby influence therapeutic and toxic responses of CP therapy. *Anti-Cancer Drugs* **16:331–336 © 2005 Lippincott Williams & Wilkins.**

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Introduction

The alkylating agent cyclophosphamide (CP) is an oxazaphosphorine prodrug that requires bioactivation by cytochrome P-450 (CYP) enzymes to form 4-hydroxycyclophosphamide (4OHCP) (Fig. 1). Multiple CYP isoenzymes are capable of activating CP *in vitro*, including CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18 and 2C19, with 2B6 displaying the highest 4-hydroxylase activity [1–6]. Approximately 70–80% of the administered CP dose is bioactivated. CP is capable of inducing its own metabolism, resulting in an increased rate of 4-hydroxylation after multiple administrations [7]. 4OHCP is unstable and spontaneously decomposes into phosphoramide mustard (PM), the ultimate alkylating metabolite. In contrast to 4OHCP, PM cannot enter target cells and therefore only the intracellularly formed PM fraction is considered to be cytotoxic. Plasma concentrations of 4OHCP may be a good marker of the alkylating activity of CP [6]. An inactivation route of CP is *N*-dechloroethylation of the parent drug, mediated by CYP3A4, to form 2-dechloroethylcyclophosphamide and chloroacetaldehyde [5,6,8]. This route only accounts for 5% of the administered CP dose. Moreover, the aldehyde dehydrogenase (ALDH) enzymes, especially ALDH1A1 and 3A1, are involved in the detoxification of CP, with the formation of inert carboxyphosphamide [9]. Approximately 20% of administered CP is excreted unchanged in the urine [6].

Modifications in the activation, inactivation and detoxification processes of CP have implications for the effectiveness of CP. Pharmacokinetic interactions with CP have been demonstrated for several compounds. In most cases, the underlying mechanism involves induction or inhibition of CYP enzymes. Compounds for which inhibition of CP metabolism has been shown in humans are allopurinol [10,11], busulphan [12], chloramphenicol [13], chlorpromazine [10], ciprofloxacine [14], fluconazole [15,16] (although other authors report no effect of fluconazole [10]) and thiotepa [17–20]. Examples of compounds which have been shown to induce CP metabolism in humans are dexamethasone [4,10,21,22], ondansetron [23,24], phenobarbital [4,25–28], phenytoin [28–31], prednisone/prednisolone [11,32,33] and rifampicin [4]. The clinical significance of most drug–drug interactions with CP remains unclear.

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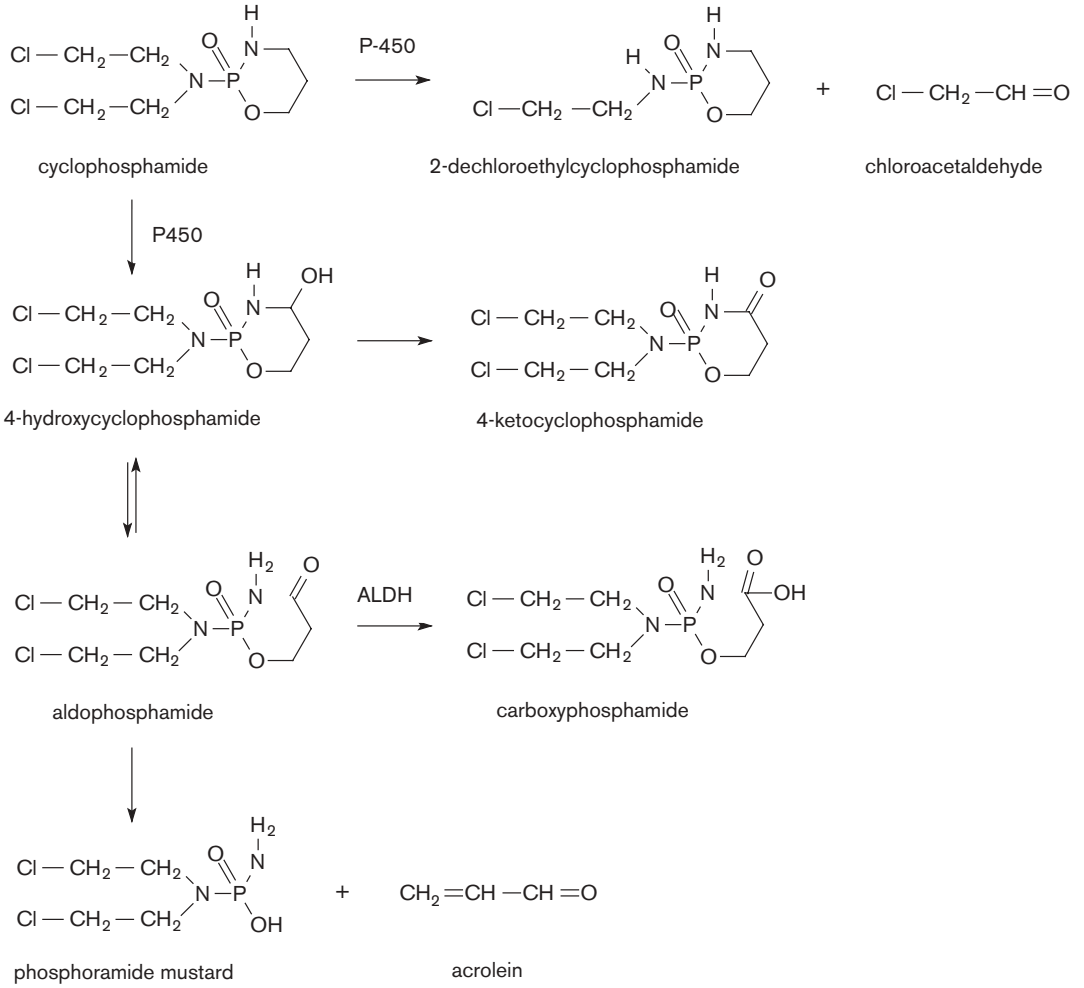
Fig. 1



Metabolism of CP. The inactivation pathways are depicted horizontally, while CP activation is shown vertically.

CP is frequently employed in combination chemotherapy regimens, both in conventional as well as in high doses. High-dose chemotherapy followed by stem cell transplantation is only possible when various drugs are co-administered to prevent or treat unwanted side-effects, including antibiotics, antivirals, antimycotics, anti-emetics, antianxiety drugs or H_2-antihistamines. Previous and/or concomitant administration of microsomal enzyme-inducing agents, but especially enzyme-inhibiting agents, with CP may alter the extent of CP bioactivation and may thereby influence therapeutic and toxic responses of high-dose CP therapy. We investigated possible inhibitory effects of co-medicated drugs on CP bioactivation *in vitro* in human liver microsomes. We focused on drugs regularly co-administered in the high-dose CTC chemotherapy regimen in which CP is administered in combination with carboplatin and thiotepa, as applied in our institute [34].

Materials and methods

Chemicals

CP and 4-hydroperoxycyclophosphamide (4OOHCP) were a generous gift of Dr Niemeyer (Baxter Oncology, Frankfurt, Germany) (purity $>95\%$). 4OOHCP is a stable precursor of 4OHCP, which is liberated in aqueous solutions. Co-medicated drugs were aciclovir, amphotericin B, carboplatin, ciprofloxacine, dexamethasone, fluconazole, granisteron, itraconazole, lorazepam, mesna, metoclopramide, ondansetron, ranitidine, roxitromycine, temazepam and thiotepa, which originated from the Department of Pharmacy (Slotervaart Hospital, Amsterdam). Potassium dihydrogenphosphate and potassium hydrogenphosphate originated from Merck (Darmstadt, Germany). A 50 mM phosphate buffer (pH 7.4) was prepared. Semicarbazide hydrochloride (analytical reagent grade) was purchased from Acros (Geel, Belgium). A 2 M solution of SCZ in 50 mM potassium phosphate

buffer was prepared. Magnesium chloride was purchased from Sigma (Zwijndrecht, The Netherlands). A 20 mg/ml magnesium chloride solution was prepared in 50 mM potassium phosphate buffer. Stock solutions of CP and co-medications were prepared in 50 mM potassium phosphate buffer. Stock solution of itraconazole was prepared in DMSO and also diluted with DMSO to obtain the final concentration in the incubation system. Distilled water was used throughout.

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and β-NADP were obtained from Sigma. A NADPH regenerating solution (NRS) was prepared in 50 mM phosphate buffer consisting of 500 µg/ml β-NADP, 2 mg/ml glucose-6-phosphate and 1.5 U/ml glucose-6-phosphate dehydrogenase. Pooled human liver microsomes (20 mg/ml in 250 mM sucrose) were obtained from Gentest (Woburn, MA). Immediately before use, the pooled human liver microsomes were diluted with potassium phosphate buffer at 4°C, to reach a final protein concentration of 4 mg/ml solution.

Microsome incubations

A mixture of 250 µl NRS, 50 µl CP stock solution, 25 µl magnesium chloride solution and 125 µl potassium phosphate buffer was pre-incubated at 37°C for 5 min. When co-medication was tested, 50 µl potassium phosphate buffer was replaced by 50 µl of a co-medication stock solution. Since itraconazole stock solutions were prepared in DMSO, the effect of DMSO was also tested. Reactions were initiated by addition of 50 µl of the 4 mg/ml microsome solution (stored at 4°C). Samples were incubated for 1 h at 37°C. The reaction was terminated by the addition of 400 µl of cold methanol. To this mixture 100 µl of a 2 M semicarbazide solution was added to stabilize the formed 4OHCP. To initiate the reaction of semicarbazide with 4OHCP, 50 µl of 4 M HCl was added. After 10 min, the solution was neutralized with 50 µl of 4 M NaOH solution, as validated previously [19]. Conversion of CP to 4OHCP was tested at CP concentrations of 0–200 µM. Any effect of co-medicated drugs was studied at a CP concentration of 200 µM and a drug concentration of 0.5–200 µM.

Assay for 4OHCP

A 100 µl volume of the final microsome incubates was analyzed for CP and 4OHCP using a HPLC-MS/MS assay recently developed in our laboratory [35]. Sample pretreatment of the microsome incubates was similar to that described for plasma samples. 4OHCP was quantified as the stable semicarbazone derivative. The coefficients of variation of the assay were less than 12% for both intra-day and inter-day precisions for each compound, and mean accuracies were within ± 15%.

Calculation of kinetic parameters

The conversion rate of the substrate, or 4OHCP formation rate (V_{4OHCP}), was calculated as amount (nmol) per unit time (h) per amount microsome protein (mg). Although 4OHCP is unstable at 37°C [36], the absolute amount of 4OHCP measured after 60 min incubation reflects the formation rate of this metabolite. Therefore, V_{4OHCP} was calculated as apparent formation rate for this conversion.

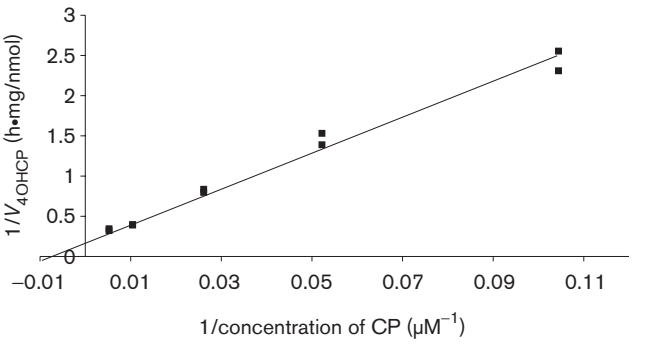
The Michaelis–Menten constant (K_m) and maximum conversion rate (V_{max}) were calculated using a Lineweaver–Burk plot. Using the graph with V_{4OHCP} plotted versus inhibitor concentration, the 50% inhibitory concentration (IC_{50}) was the concentration causing half-maximal inhibition.

Results

The Lineweaver–Burk plot for the conversion of CP to 4OHCP in pooled human liver microsomes is shown in Figure 2. The K_m and V_{max} calculated were 93 µM (24 µg/ml) and 4.3 nmol/h·mg, respectively.

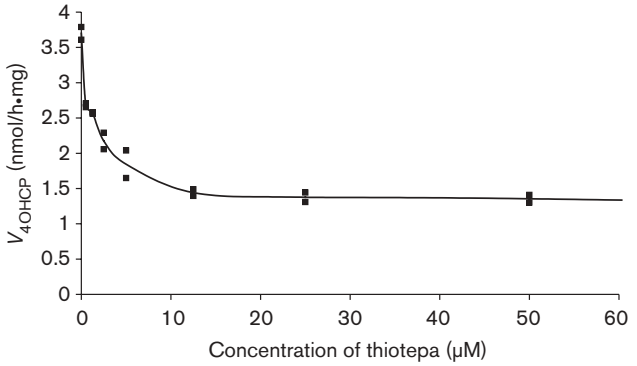
After testing the inhibitory activity of all co-medicated agents (at concentrations of 200 µM) on the bioactivation of CP, inhibition was seen for amphotericin B, dexamethasone, fluconazole, itraconazole, lorazepam, ondansetron and thiotepa. For these compounds the inhibition was tested at concentrations of 0, 0.5, 1.25, 2.5, 5, 12.5, 25, 50 and 200 µM. The inhibition of the bioactivation of CP is shown in Figures 3–5 for thiotepa, itraconazole and lorazepam, respectively. The IC_{50} values for all seven agents showing inhibitory activity are listed in Table 1. For all but thiotepa, these IC_{50} values were higher than the therapeutic drug levels and thus considered of no clinical relevance.

Fig. 2



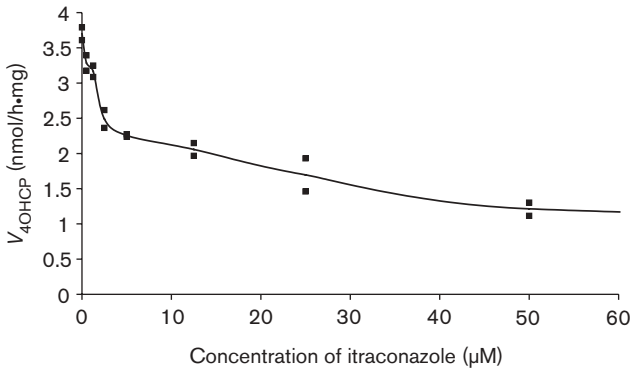
Lineweaver–Burk plot for the conversion of CP to 4OHCP in pooled human microsomes. The K_m and V_{max} calculated were 93 µM (24 µg/ml) and 4.3 nmol/h·mg, respectively.

Fig. 3



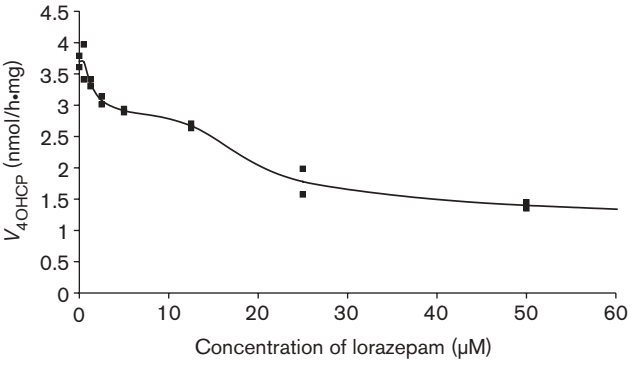
Inhibition of the bioactivation of CP (200 μM) by thiotepa (IC_{50} = 1.25 μM). The solid line represents the mean value for each data point.

Fig. 4



Inhibition of the bioactivation of CP (200 μM) by itraconazole (IC_{50} = 5 μM). The solid line represents the mean value for each data point.

Fig. 5



Inhibition of the bioactivation of CP (200 μM) by lorazepam (IC_{50} = 5 μM). The solid line represents the mean value for each data point.

Table 1 Inhibitory effects of co-medicated drugs on the bioactivation of cyclophosphamide

Co-medicated agent	IC50 (mM)	IC50 (μg/ml)	Therapeutic levels (μg/ml)
Amphotericin B	50	46	<2
Dexamethasone	>100	>39	<0.5
Fluconazole	>50	>15	<15
Itraconazole	5	3.4	<2
Lorazepam	15	4.8	<0.2
Ondansetron	>100	>29	<0.2
Thiotepa	1.25	0.24	<2

Discussion

This study provided information on the inhibitory action of co-medicated agents on the CYP-mediated bioactivation of CP. Only thiotepa appeared to inhibit the bioactivation of CP in clinically relevant concentrations. This has already been demonstrated in previous studies [17–20]. The IC_{50} value found in our study (1.25 μM) and reported by others (1.4–41 μM [18] and 23 μM [19]) is within the range of thiotepa plasma concentrations in high-dose regimens, and therefore of clinical relevance. This result is not surprising since CP bioactivation is mainly mediated by CYP2B6 and thiotepa is one of the few compounds known to specifically inhibit this isoenzyme [20].

With this study in human liver microsomes, no information could be garnered on the CYP enzyme-inducing properties of drugs. Interactions of CYP inducers with CP are, however, less interesting since already up to 80% of the administered CP dose is 4-hydroxylated. Accelerating this process will only result in a minor increase in 4OHCP exposure. Delayed CP metabolism due to CYP inhibition, however, may result in increased fractional urinary excretion of the parent compound and therefore significantly lower exposure to its activated metabolite. From this study, no information was available on drug–drug interactions other than those mediated by CYP, e.g. interactions on the level of the enzyme aldehyde dehydrogenase activity.

The azole antimycotics itraconazole and fluconazole are inhibitors of CYP enzymes [37]. For both compounds we observed inhibition of CP bioactivation; however, not in clinically relevant concentrations. Other authors have found conflicting results on the inhibitory capacity of fluconazole on CP metabolism [10,15,16] in patients. Ciprofloxacin is a compound for which we have not found any inhibitory capacity in our study. Carlens *et al.* [14], however, reported an increased relapse risk in bone marrow transplant patients treated with ciprofloxacin, which may indicate decreased exposures to the bioactivated metabolite 4OHCP. Moreover, Xie *et al.* [38] demonstrated a reduction in CP biotransformation in

rats when ciprofloxacine was co-administered, which was explained by significant suppressed gene expression of CYP2C11 and CYP3A1. While dexamethasone [4,10,21,22] and ondansetron [23,24] have been shown to induce CP metabolism in humans, we have found slight inhibiting properties of these agents at not clinically relevant concentrations. Lorazepam is not known to inhibit CYP enzymes, while our results show inhibition of CP biotransformation at high concentrations.

The clinical effects of inhibition of CP metabolism are unclear. Exposure to 4OHCP has been both associated with therapeutic response as well as the occurrence of veno-occlusive disease of the liver [34]. Therefore, drug–drug interactions may have a strong impact on the therapeutic outcome of CP containing therapy.

In summary, this study demonstrated no clinically relevant effects of co-medicated agents other than thiotepa on the biotransformation of CP. Therefore, co-administration of these agents before or during high-dose CTC regimen is not expected to affect the therapeutic efficacy of CP chemotherapy.

References

1 Chang TKH, Weber GF, Crespi CL, Waxman DJ. Differential activation of cyclophosphamide and ifosfamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Res* 1993; **53**:5629–5637.

2 Chang TKH, Yu L, Goldstein JA, Waxman DJ. Identification of the polymorphically expressed CYP2C19 and the wild-type CYP2C9-ILE^{359} allele as low-K_m catalysts of cyclophosphamide and ifosfamide activation. *Pharmacogenetics* 1997; **7**:211–221.

3 Roy P, Yu LJ, Crespi CL, Waxman DJ. Development of a substrate activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. *Drug Metab Disp* 1999; **27**:655–666.

4 Chang TKH, Yu L, Maurel P, Waxman DJ. Enhanced cyclophosphamide and ifosfamide activation in primary human hepatocyte cultures: response to cytochrome P-450 inducers and autoinduction by oxazaphosphorines. *Cancer Res* 1997; **57**:1946–1954.

5 Ren S, Yang JS, Kalhorn TF, Slattery JT. Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes. *Cancer Res* 1997; **57**:4229–4235.

6 Yu L, Waxman DJ. Role of cytochrome P450 in oxazaphosphorine metabolism. Deactivation via *N*-dechloroethylation and activation via 4-hydroxylation catalyzed by distinct subsets of rat liver cytochromes P450. *Drug Metab Disp* 1996; **24**:1254–1262.

7 Moore MJ. Clinical Pharmacokinetics of cyclophosphamide. *Clin Pharmacokinet* 1991; **20**:194–208.

8 Bohnenstengel F, Hofmann U, Eichelbaum M, Kroemer HK. Characterization of the cytochrome P450 involved in side-chain oxidation of cyclophosphamide in humans. *Eur J Clin Pharmacol* 1996; **51**:297–301.

9 Moreb J, Schweder M, Suresh A, Zucali JR. Over-expression of the human aldehyde dehydrogenase class I results in increased resistance to 4-hydroperoxycyclophosphamide. *Cancer Gene Ther* 1996; **3**:24–30.

10 Yule SM, Boddy AV, Cole M, Price L, Wyllie R, Tasso MJ, *et al.* Cyclophosphamide pharmacokinetics in children. *Br J Clin Pharmacol* 1996; **41**:13–119.

11 Bagley CM, Bostick FW, DeVita VT. Clinical pharmacology of cyclophosphamide. *Cancer Res* 1973; **33**:226–233.

12 Hassan M, Ljungman P, Ringdén O, Hassan Z, Oberg G, Nilsson C, *et al.* The effect of busulphan on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite: time interval influence on therapeutic efficacy and therapy-related toxicity. *Bone Marrow Transplant* 2000; **25**:915–924.

13 Faber OK, Mouridsen HT, Skovsted L. The effect of chloramphenicol and sulphaphenazole on the biotransformation of cyclophosphamide in man. *Br J Clin Pharmacol* 1975; **2**:281–285.

14 Carlens S, Ringden O, Aschan J, Hagglund H, Ljungman P, Mattsson J, *et al.* Risk factors in bone marrow transplant recipients with leukaemia. Increased relapse risk in patients treated with ciprofloxacin for gut decontamination. *Clin Transplant* 1998; **12**:84–92.

15 Yule SM, Walker D, Cole M, McSorley L, Cholerton S, Daly AK, *et al.* The effect of fluconazole on cyclophosphamide metabolism in children. *Drug Metab Disp* 1999; **27**:417–421.

16 Marr KA, Leisenring W, Crippa F, Slattery JT, Corey L, Boeckh M, *et al.* Cyclophosphamide metabolism is affected by azole antifungals. *Blood* 2004; **103**:1557–1559.

17 Chen TL, Passoscoelho JL, Noe DA, Kennedy MJ, Black KC, Colvin OM, *et al.* Nonlinear pharmacokinetics of cyclophosphamide in patients with metastatic breast-cancer receiving high-dose chemotherapy followed by autologous bone-marrow transplantation. *Cancer Res* 1995; **55**:810–816.

18 Anderson LW, Chen T, Colvin OM, Grochow LB, Collins JM, Kennedy MJ, *et al.* Cyclophosphamide and 4-hydroxycyclophsophamide/aldophosphamide kinetics in patients receiving high-dose cyclophosphamide chemotherapy. *Clin Cancer Res* 1996; **2**:1481–1487.

19 Huitema ADR, Kerbusch T, Tibben MM, Rodenhuis S, Beijnen JH. Reduction of cyclophosphamide-bioactivation by thiotepa: critical sequence-dependency in high-dose chemotherapy regimens. *Cancer Chemother Pharmacol* 2000; **46**:119–127.

20 Rae JM, Soukhova NV, Flockhart DA, Desta Z. Triethylenethiophosphoramide is a specific inhibitor of cytochrome P450 2B6:implications for cyclophosphamide metabolism. *Drug Metab Disp* 2002; **30**:525–530.

21 Lindley C, Hamilton G, McCune JS, Faucette S, Shord SS, Hawke RL. The effect of cyclophosphamide with and without dexamethasone on cytochrome P450 3A4 and 2B6 in human hepatocytes. *Drug Metab Disp* 2002; **30**:814–821.

22 Busse D, Busch FW, Bohnenstengel F, Eichelbaum M, Fischer P, Opalinska J, *et al.* Dose escalation of cyclophosphamide in patients with breast cancer: consequences for pharmacokinetics and metabolism. *J Clin Oncol* 1997; **15**:1885–1896.

23 Gilbert CJ, Petros WP, Vredenburgh J, Hussein A, Ross M, Rubin P, *et al.* Pharmacokinetic interaction between ondansetron and cyclophosphamide during high-dose chemotherapy for breast cancer. *Cancer Chemother Pharmacol* 1998; **42**:497–503.

24 Cagnoni PJ, Matthes S, Day TC, Bearman SI, Shpall EJ, Jones RB. Modification of the pharmacokinetics of high-dose cyclophosphamide and cisplatin by antiemetics. *Bone Marrow Transplant* 1999; **24**:1–4.

25 Jao JY, Jusko WJ, Cohen JL. Phenobarbital effects on cyclophosphamide pharmacokinetics in man. *Cancer Res* 1972; **32**:2761–2764.

26 Cohen JL, Jao JY, Jusko WJ. Pharmacokinetics of cyclophosphamide in man. *Br J Pharmacol* 1971; **43**:677–80.

27 Sladek NE, Priest J, Doeden D, Mirocha CJ, Pathre S, Krivit W. Plasma half-life and urinary excretion of cyclophosphamide in children. *Cancer Treat Rep* 1980; **64**:1061–1066.

28 Sladek NE, Doeden D, Powers JF, Krivit W. Plasma concentrations of 4-hydroxycyclophosphamide and phosphoramide mustard in patients repeatedly given high doses of cyclophosphamide in preparation for bone marrow transplantation. *Cancer Treat Rep* 1984; **68**:1247–1254.

29 Williams ML, Wainer IW, Embree L, Barnett M, Granvil CL, Ducharme MP. Enantioselective induction of cyclophosphamide metabolism by phenytoin. *Chirality* 1999; **11**:569–574.

30 Slattery JT, Kalhorn TF, McDonald GB, Lambert K, Buckner CD, Bensinger WI, *et al.* Conditioning regimen-dependent disposition of cyclophosphamide and hydroxycyclophosphamide in human marrow transplantation patients. *J Clin Oncol* 1996; **14**:1484–1494.

31 De Jonge ME, Huitema ADR, Van Dam SM, Beijnen JH, Rodenhuis S. Significant induction of cyclophosphamide and thiotepa metabolism by phenytoin. *Cancer Chemother Pharmacol* 2004; in press.

32 Faber OK, Mouridsen HT, Skovsted L. The biotransformation of cyclophosphamide in man: influence of prednisone. *Acta Pharmacol Toxicol* 1974; **35**:195–200.

33 Boddy AV, Furtun Y, Sardas S, Sardas O, Idle JR. Individual variation in the activation and inactivation of metabolic pathways of cyclophosphamide. *J Natl Cancer Inst* 1992; **84**:1744–1748.

34 Huitema ADR, Spaander M, Mathôt RAA, Tibben MM, Holtkamp MJ, Beijnen JH, *et al.* Relationship between exposure and toxicity in high-dose chemotherapy with cyclophosphamide, thioTEPA and carboplatin. *Ann Oncol* 2002; **13**:374–384.

35 De Jonge ME, Van Dam SM, Hillebrand MJX, Rosing H, Huitema AD, Rodenhuis S, *et al.* Simultaneous quantification of cyclophosphamide, 4-hydroxycyclophosphamide, *N,N′,N′*-triethylenethiophosphoramide (thiotepa) and *N′.N′.N′*-triethylenephosphoramide (tepa) in human plasma

by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-MS/MS). *J Mass Spectrom* 2004; **39**:262–271.

36 Low JE, Borch RF, Sladek NE. Further studies on the conversion of 4-hydroxyoxaphosphorines to reactive mustards and acrolein in inorganic buffers. *Cancer Res* 1983; **43**:5815–5820.

37 Albengres E, Le Louet H, Tillement JP. Systemic antifungal agents. Drug interactions of clinical significance. *Drug Safety* 1998; **18**:83–97.

38 Xie HJ, Broberg U, Griskevicius L, Lundgren S, Carlens S, Meurling L, *et al.* Alteration of pharmacokinetics of cyclophosphamide and suppression of the cytochrome P450 genes by ciprofloxacin. *Bone Marrow Transplant* 2003; **31**:197–203.