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The effect of cimetidine on cyclophosphamide metabolism in rabbits*

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Summary. Six female rabbits were given 20 mg/kg cyclophosphamide (containing 100 μCi [^{3}H-chloroethyl]-cyclophosphamide) alone or 1 h following 100 mg/kg cimetidine. Serial plasma and urine specimens were collected and levels of cyclophosphamide and its metabolites (4-hydroxycyclophosphamide, 4-ketocyclophosphamide, phosphoramide mustard, and carboxyphosphamide) were measured. 4-Ketocyclophosphamide was the major metabolite present in rabbit plasma and urine, with lesser amounts of 4-hydroxycyclophosphamide, carboxyphosphamide, and phosphoramide mustard also being identified. Cimetidine pretreatment resulted in prolongation of cyclophosphamide's half-life from 24.3±7.3 to 33.5±9.5 min (mean ± SD; P = 0.036) but did not significantly alter the $AUC_{0-8\,h}$ for the latter drug. Cimetidine pretreatment resulted in a significantly greater $AUC_{0-8\,h}$ for 4-hydroxycyclophosphamide (189.4±77 vs 364.6±126.7 μmol min/l^{-1}; P = 0.016) as compared with control values. A higher $AUC_{0-8\,h}$ value for phosphoramide mustard (53.7±69.2 vs 95.7±34.7 μmol min/l^{-1}) was also observed after cimetidine dosing but the difference was not significant (P = 0.21). Kinetics of 4-ketocyclophosphamide and carboxyphosphamide were not significantly affected by cimetidine treatment. Cimetidine was added to hepatic microsomes isolated from phenobarbital-treated rabbits; it did not inhibit cyclophosphamide's metabolism in vitro, suggesting that its in vivo effect may be mediated through mechanisms other than cytochrome P-450 inhibition. Cimetidine pretreatment increases exposure to cyclophosphamide and its major activated metabolite, 4-hydroxycyclophosphamide. Potentiation rather than inhibition of cyclophosphamide's pharmacodynamic effect is to be predicted when cimetidine is given concomitantly with the former. Alterations in hepatic blood flow or mechanisms other than microsomal inhibition by cimetidine may explain this potentiation.

Introduction

Cyclophosphamide (CPA) is a frequently used anticancer and immunosuppressive drug requiring metabolism for its therapeutic effect. Oxidation by hepatic microsomal mixed-function oxidase enzymes produces 4-hydroxycyclophosphamide, which exists in equilibrium with aldophosphamide hydrate (see Fig. 1) [3, 4, 7, 8, 11, 12, 14]. Spontaneous decomposition of aldophosphamide yields phosphoramide mustard, the active alkylating species, and acrolein, which may be responsible for bladder toxicity [5, 6]. An aldehyde oxidase converts 4-hydroxycyclophosphamide to 4-ketocyclophosphamide, an inactive product, and oxidation of the 4-hydroxy metabolite by aldehyde dehydrogenase yields carboxyphosphamide [2, 22].

The pharmacology of CPA is potentially susceptible to drugs or clinical conditions that alter the microsomal enzymic systems. Most inducers of cytochrome P-450 metabolism such as phenobarbital or phenytoin result in a shorter CPA half-life [13, 18], whereas inhibitors of P-450 metabolism lengthen half-life. However, CPA is only a prodrug and its antineoplastic activity resides in 4-hydroxycyclophosphamide and phosphoramide mustard. The effect of microsomal P-450 inducers on these important metabolites is less clear. Alberts et al. [1] found that induction of P-450 by phenobarbital resulted in a reduction in the exposure to active CPA metabolites and a decrease in animal survival in a mouse leukemia model. Conversely, Sladek [18] found that phenobarbital did not change either the AUC of 4-hydroxycyclophosphamide or phosphoramide mustard or the dose of CPA required to inhibit the growth of Walker 256 carcinosarcoma. Other studies evaluating the effect of mi-

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Table 1. CPA clearance, partial metabolic clearance to metabolites, and renal clearance before and after cimetidine treatment

	Clearance (ml/min)		
	Control	Cimetidine	*P* value
CPA clearance	117.7 ±64.4	100.1 ±70.4	0.66
Partial metabolic clearance:			
4-Keto-CPA	21.9 ±16.2	10.6 ± 5.4	0.14
PM	0.043 ± 0.03	0.043 ± 0.04	1.00
Carboxyphosphamide	0.25 ± 0.20	0.29 ± 0.29	0.79
Renal clearance:			
CPA	2.7 ± 2.6	2.3 ± 1.4	0.74
4-Keto-CPA	11.2 ± 7.4	7.2 ± 3.1	0.24
PM	2.8 ± 4.1	1.7 ± 1.7	0.56
Carboxyphosphamide	26.5 ±39.7	23.5 ±31.3	0.89

Data represent the mean ± SD

crosomal enzyme inducers on CPA toxicity have similarly produced conflicting results [19].

Cimetidine is an H_2-antagonist that is frequently used in cancer patients to prevent or treat gastritis and/or ulcers. It has been shown to be a nonspecific inhibitor of cytochrome P-450 and to alter the kinetics of several drugs that undergo hepatic microsomal metabolism. Previous studies by Dorr et al. [9] have indicated that cimetidine increases the total alkylating activity of CPA and enhances its antileukemic effect in a mouse model. However, the effect of cimetidine on the rate and degree of CPA metabolite formation has not been assessed.

To determine the effects of cimetidine on CPA metabolism, we used a system in which individual CPA metabolites can be determined and multiple blood samples can be obtained from the same animal following CPA administration. [^{3}H]-CPA, labeled in the bis(2-chloroethyl)amino moiety, was used to enable specific measurement of CPA and each of its major metabolites except acrolein. The rabbit was employed as a model since it is a large enough animal to enable repeated blood sampling yet is commonly available and relatively inexpensive.

In addition to evaluating the effects of cimetidine on CPA metabolism, this study is the first to report both the simultaneous quantitation of CPA and its tumor-active metabolites (4-hydroxycyclophosphamide and phosphoramide mustard) in the plasma and urine of rabbits and the identification and quantitation of CPA metabolites in this species.

Table 2. Effect of cimetidine on the half-life of CPA

Terminal $t_{1/2}$ (min)		
Control	Cimetidine	*P* value
24.3±7.3	33.5±9.5	0.036

Data represent the mean ± SD

Materials and methods

Cyclophosphamide. CPA was purchased as commercial material from Bristol-Myers (Evansville, Ind.) and was dissolved in sterile water to the desired concentration. Tritium side-chain-labeled CPA was obtained from Amersham Corp. and was 98% pure as determined by thin-layer chromatography (specific activity, 57.5 mCi/mg).

Rabbits. Female white New Zealand rabbits weighing 3–4 kg were obtained from Myrtle Rabbitry (Eagleville, Tenn.) and housed in clean, individual cages in the animal quarters at the Nashville Veterans Administration Medical Center. The animals were certified to be *Pasteurella*- and disease-free upon receipt and were kept in a clean room separate from other rabbits. The quarters are supervised by the local Animal Care Committee under the supervision of a veterinarian as required by United States federal regulations. Study procedures were reviewed by the supervising veterinarian and the local Research and Development Committee prior to the study. Care was taken to minimize animal discomfort.

Drug treatment and blood collection. Six rabbits were given 20 mg/kg CPA spiked with 100 μCi [^{3}H]-CPA intravenously (i. v.) via a marginal ear vein on two separate occasions. On one occasion the animals received CPA alone as a bolus infusion, and on the other they received CPA 1 h following a 5-min i. v. administration of 100 mg/kg cimetidine. This dose of cimetidine has been used in other studies [9] to produce definite microsomal enzyme inhibition. Four rabbits received CPA alone, followed by cimetidine plus CPA 1 month later, whereas two animals were initially given CPA and cimetidine, followed 1 month later by CPA alone. Cimetidine was purchased as commercial material from Smith Kline Beecham Pharmaceuticals (Philadelphia, Pa.).

A 3-ml blood sample was collected from the ear contralateral to that used for drug administration both prior to CPA dosing and at 5, 15, 30, 45, and 60 min thereafter. Either ear was used to obtain samples at 90, 120, 180, 240, 360, and 480 min after CPA treatment. Each blood sample was collected in iced heparin tubes containing 0.5 ml 0.4 M semicarbazide hydrochloride (pH = 7.0) to trap 4-hydroxycyclophosphamide as aldophosphamide semicarbazone [20]. The samples were shaken and plasma was isolated and immediately frozen. Urine was collected using a stainless-steel metabolic cage with 10 ml 0.4 M semicarbazide hydrochloride (pH = 7.0) in the collection flask. The urinary volume was measured and an aliquot, stored. All samples were kept at –20° C for analysis, which was performed within 1 week after specimen collection.

CPA and metabolite assay. Plasma and urine samples were assayed for CPA and metabolites according to the method of Struck et al. [21].

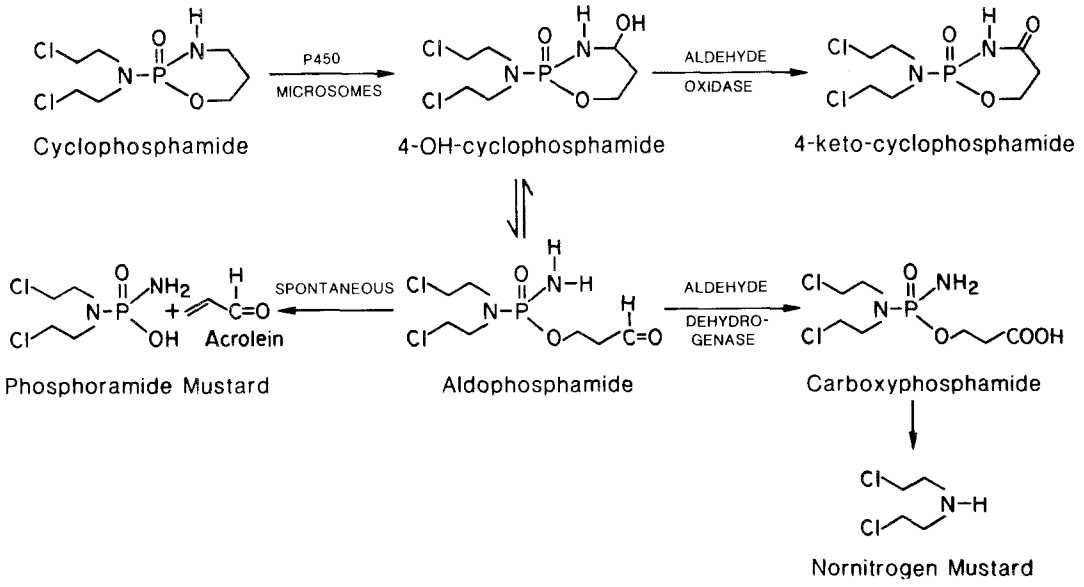

Fig. 1. Metabolic pathways of CPA

Plasma samples (1.5 ml) were allowed to melt and were immediately extracted with chloroform (3 × 10 ml) in a separatory funnel. The samples were rinsed from the separatory funnel with water and then lyophilized. The residues were titrated with 10 ml methanol and treated with 5 ml ethereal diazomethane (prepared from *N*-nitroso-*N*-methyl-urea; ICN Biomedicals, Inc. K K Labs, Plainview, N.Y.). The mixture was stirred for 10 min at room temperature and centrifuged, and the supernate was evaporated to dryness under vacuum pressure. The chloroform extracts were dried over sodium sulfate and then filtered, the sodium sulfate was washed with chloroform and then filtered, and the combined filtrate and wash from each sample was evaporated under vacuum. Chloroform- and methanol-extract residues were stored at –20° C for thin-layer chromatography (TLC).

The above extracts were dissolved in a small volume of acetone and analyzed in triplicate on Analtech (Newark, Del.) precoated silica-gel G plates (thickness, 250 μm) using acetone:chloroform (3 : 1, vol/vol) for the chloroform extracts and chloroform: methanol (9 : 1, vol/vol) for the methanol extracts. Plates were activated by heating for 1 h at 100° C followed by storage in a desiccating container. Methanol extracts were analyzed on plates that had been additionally heated for 15 min at 140° C just prior to analysis; plates were allowed to cool to room temperature prior to sample application. Following separation of metabolites on the TLC plate using cochromatography with synthetic standards, the plates were scraped and the radioactivity present as each metabolite was counted with correction for quench.

Pharmacokinetics. Half-life ($t_{1/2}$) measurements were estimated using linear regression analysis of the logarithm of the plasma CPA concentration plotted against time. The volume of distribution (V_d) was estimated from the dose divided by the plasma concentration back-extrapolated to time 0. The area under the plasma-decay curve (AUC) for CPA and its metabolites was calculated by the trapezoidal rule from 0 to 480 min and by the log trapezoidal rule to infinity. Clearance (*C*) was calculated from the formula *C* = D/AUC, where D represents the delivered dose. Partial metabolic clearance was estimated as the ratio of the amount of drug excreted in the urine as a given metabolite (corrected for the metabolite's molecular weight) to the delivered dose, multiplied by the total drug clearance. Renal clearance was calculated by measuring the amount of CPA or metabolite recovered in the urine and dividing by the AUC for that compound.

Microsomal assay. In vitro CPA metabolism was measured by the method of Marinello et al. [16]. Liver samples were obtained from rabbits that had been given 500 mg/l phenobarbital in their drinking water for 7 days. Microsomes were prepared as previously described [23]. Protein [15] and cytochrome P-450 [17] concentrations were determined and microsomal samples were stored at –80° C until the time of the study. Initial incubation mixtures consisted of microsomes equivalent to 0.3 nmol cytochrome P-450, 50 μM to 1 mM CPA, 0.1 μCi [^{3}H]-CPA, 50 mM magnesium chloride, 10 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate dehydrogenase, 50 mM phosphate buffer (pH 7.4), and either 0 or 50 mM cimetidine, SKF-525 A, or metyrapone in a total volume of 0.9 ml (all chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.). After a 3-min preincubation period at 37° C, 100 mM glucose-6-phosphate (0.1 ml) was added and the mixture was incubated for a further 60 min using a shaking water bath. Next, 1 ml 90 mM phosphate buffer was added and metabolism was terminated by the addition of 6 ml chloroform. After vigorous

Table 3. $AUC_{0-8\,h}$ data for CPA and its major metabolites in rabbits before and after cimetidine administration

	$AUC_{0-8\,h}$ (μmol min l^{-1})		
	Control	Cimetidine	*P* value
CPA	3,683 ± 2,574	4,694 ± 2,817	0.53
4-keto-CPA	5,480 ± 2,775	5,818 ± 2,532	0.83
4-hydroxy-CPA	189.4 ± 77	364.6 ± 126.7	0.02
PM	53.7 ± 69.2	95.7 ± 34.7	0.21
Carboxyphosphamide	176.8 ± 117.1	71.9 ± 31.6	0.35

Data represent the mean ± SD

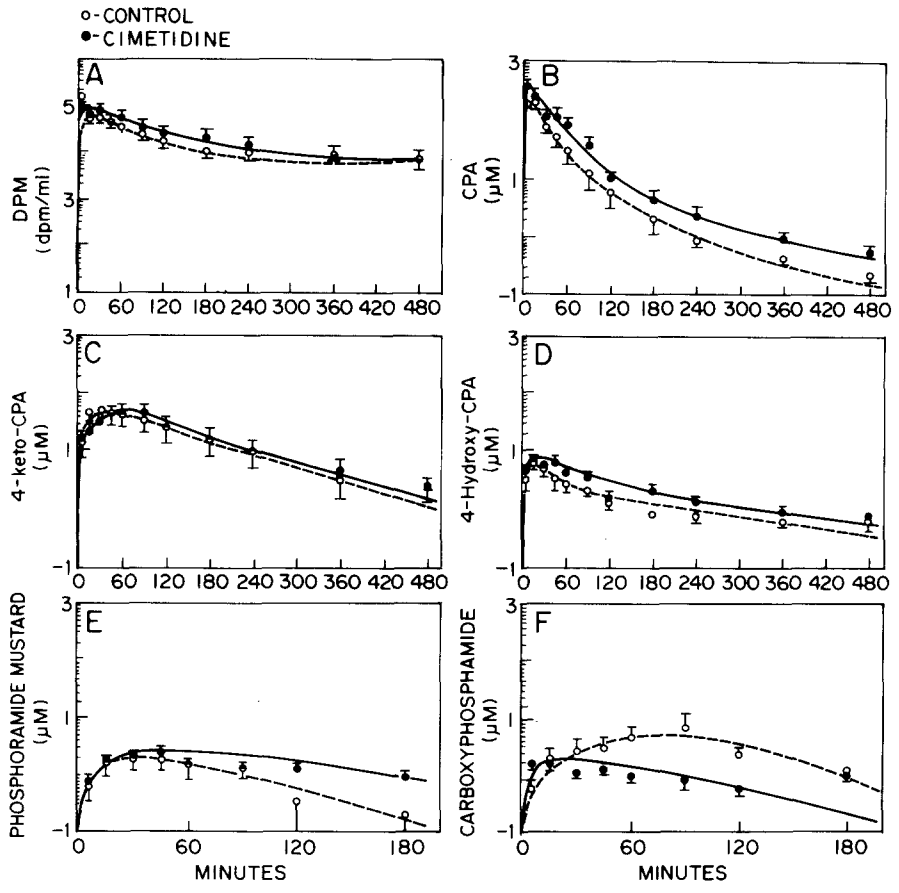

Fig. 2 A–F. Plasma levels of CPA and its metabolites over 8 h before and after cimetidine treatment (100 mg/kg). **A** Plasma radioactivity. **B** CPA. **C** 4-Keto-CPA. **D** 4-Hydroxy-CPA. **E** Phosphoramide mustard. **F** Carboxyphosphamide. O– – – –O, control; ●——●, cimetidine

mixing, the aqueous layer was removed following centrifugation. Then, 1 ml aqueous layer was counted in a scintillation counter and the formation of aqueous metabolites of [^{3}H]-CPA was determined. After subtraction of the background, the enzymatic activity was expressed as nanomoles of [^{3}H]-CPA-related aqueous metabolites formed per milligram of protein. The linearity of the assay was verified by varying P-450 concentrations, incubation time, and substrate concentrations.

Statistical methods. The paired *t*-test was used to detect significant differences ($P < 0.05$).

Results

Plasma CPA clearance was 117 ± 64.4 ml/min in the rabbit, with a terminal half-life of 24.3 ± 7.3 min. Metabolism appeared to be the major route of elimination, with renal clearance being measured at only 2.7 ± 2.6 ml/min. CPA's volume of distribution was 7.2 ± 3.1 l. 4-Ketocyclophosphamide was the major metabolite measured in rabbit plasma and urine. The partial metabolic clearance to 4-ketocyclophosphamide accounted for 20% of the delivered dose (Table 1). This observation caused concern because this metabolite is not typically found at such high levels in studies using other animals species and humans [1, 21]. Consequently, representative samples of plasma and urine were carefully analyzed for 4-ketocyclophosphamide and nornitrogen mustard, a likely contaminating metabolite, by cochromatography using synthetic standards. These experiments confirmed that 4-ketocyclophosphamide is indeed the major metabolite in both of these biological fluids. For example, thin-layer chromatographic analysis of plasma collected 90 min after drug treatment revealed that 79.2% of the radioactivity in a chloroform extract was 4-ketocyclophosphamide, with 3.7% occurring as nornitrogen mustard; 8.1%, as unchanged drug; 5%, as the minor metabolites *N*-dechloroethyl-cyclophosphamide and/or alcophosphamide; and 2.6%, as 4-hydroxycyclophosphamide. 4-Hydroxycyclophosphamide was also identified in the plasma and urine. Other measurable metabolites included phosphoramide mustard and carboxyphosphamide, typically the major urinary metabolite in animals and humans [2, 22].

Cimetidine pretreatment resulted in prolongation of CPA's half-life from 24.3 ± 7.3 to 33.5 ± 9.5 min ($P = 0.036$, Table 2). In addition, greater exposure to 4-hydroxycyclophosphamide ($AUC_{0-8\,h} = 189.4 \pm 77$ vs 364.6 ± 126.7 μmol min l^{-1}; $P = 0.016$, Table 3) was observed in cimetidine-treated rabbits. As noted in Fig. 2, the terminal half-life of 4-hydroxycyclophosphamide is long, which makes the estimation of this parameter difficult. To ensure that the changes in 4-hydroxycyclophosphamide AUC measurements seen after cimetidine treatment were not due to variability in half-life estimates, the AUC for 4-hydroxycyclophosphamide in each rabbit was also calculated from time 0 until the end of the 8-h data collection. No significant change in measurements was noted when the $AUC_{0-8\,h}$ (189.4 ± 77) was compared with the $AUC_{0-\infty}$ (189.7 ± 75.9) in the control group or in the

Table 4. Urinary recovery of CPA and its major metabolites in rabbits before and after cimetidine administration

	Urinary recovery (%)		
	Control	Cimetidine	*P* value
CPA	2.2 ± 1.5	3 ±2.4	0.50
4-hydroxy-CPA	16.2 ± 6.8	14 ±9.9	0.66
4-keto-CPA	1 ± 0.5	0.5 ±0.3	0.07
PM	0.03 ± 0.002	0.11 ±0.01	0.08
Carboxyphosphamide	0.25 ± 0.12	0.59 ±0.50	0.14
DPM	18.4 ±11.8	14.7 ±7.8	0.54

cimetidine group ($AUC_{0-8\,h}$ = 364.6 ± 126.7; $AUC_{0-\infty}$ = 392.5 ± 137.8). Higher $AUC_{0-8\,h}$ values for phosphoramide mustard (53.7 ± 69.2 vs 95.7 ± 34.7 μmol min l^{-1}; Table 3, Fig. 2) were also seen in cimetidine-treated animals, but the difference was not significant (P = 0.34). No differences in renal clearance (Table 1), urinary recovery (Table 4), or volumes of distribution for CPA or its metabolites occurred after cimetidine treatment. Moreover, cimetidine did not significantly change the partial metabolic clearances to 4-ketocyclophosphamide, carboxyphosphamide, or phosphoramide mustard.

To determine whether the prolongation of the half-life of CPA by cimetidine was the result of a direct effect of this agent on hepatic microsomes, CPA was incubated with hepatic microsomes in vitro in the presence of cimetidine and other microsomal inhibitors. The addition of 50 mM SKF-525A and 50 mM metyrapone resulted in a significant inhibition of CPA metabolism, as previously reported [16]. The addition of up to 50 mM cimetidine to the microsomal enzyme system did not produce inhibition of CPA metabolism (Table 5).

Discussion

The pharmacology of CPA has been studied in several species and was summarized in a recent review [19]. Although many studies have evaluated the kinetics of the parent drug, few pharmacokinetic data are available for CPA metabolites. Because of the absence of a strong UV-absorbing chromophore in CPA and the reactivity of its metabolites, which make analytical measurements difficult, CPA's metabolites are usually quantitated in terms of plasma alkylating activity using the colorimetric NBP assay [6]. The use of side-chain-radiolabeled CPA enables quantitative measurements of all individual ring-oxidation metabolites except acrolein [22]. This CPA assay technique is specific but tedious.

Table 5. In vitro inhibition of the metabolism of 1 mM CPA

Inhibitor (50 mM)	Metabolites formed (% control)
Metyrapone	12 ± 8
SKF-525A	52 ± 12
Cimetidine	95 ± 6

This study is the first to identify and quantify metabolites of CPA in rabbits. The rabbit metabolizes CPA in a manner qualitatively similar to that of other species, with 4-hydroxycyclophosphamide, 4-ketocyclophosphamide, phosphoramide mustard, and car boxyphosphamide occurring in the plasma and urine. Peak plasma metabolite concentrations occur within 30–90 min following i.v. dosing, although the shapes of the concentration-time curves vary for the different metabolites. Advantages of the rabbit model include the ability of each animal to serve as its own control and provide serial plasma sampling. Since measurable 4-hydroxycyclophosphamide and other CPA metabolite concentrations are found in the plasma and urine of rabbits as in man, this model can be used for studying environmental factors affecting the cytochrome P-450 enzyme, which activates CPA.

However, differences in the proportions of CPA metabolites make this model imperfect for comparisons with man. 4-Ketocyclophosphamide is the major metabolite in the rabbit, accounting for 20%–30% of the metabolized drug. The larger volume of distribution for CPA, the greater partial metabolic clearance to 4-ketocyclophosphamide, and the lower rate of CPA renal clearance are other potential disadvantages of using the rabbit as a model for studying CPA pharmacology.

Despite differences between the rabbit and man in CPA metabolism, the type of metabolism is qualitatively similar in these two species, which enables some evaluation of the effects of cimetidine on CPA metabolism. The data we obtained using this model are consistent with a decrease in the rate of conversion of CPA to 4-hydroxycyclophosphamide due to cimetidine. Surprisingly, we could not detect in vitro inhibition by cimetidine of microsomal P-450 CPA metabolism. This suggests that CPA can be activated by microsomal proteins that are not affected by cimetidine and that delayed conversion of CPA to 4-hydroxycyclophosphamide may result from alterations in hepatic blood flow, another consequence of cimetidine administration [10].

Sladek [18] has marshaled a strong case supporting the concept that 4-hydroxycyclophosphoramide is the pharmacologically important circulating (transport) form of CPA. Increased exposure to this metabolite would increase the cytotoxicity of CPA. Our results demonstrate an increase in the AUC for 4-hydroxycyclophosphoramide following cimetidine administration and support the findings of Dorr et al. [9], who have shown increased potentiation of CPA in mice given cimetidine concomitantly with CPA. The

mechanism underlying this increase should not necessarily change the AUC for this metabolite but only alter the profile of the 4-hydroxycyclophosphoramide concentration vs time curve. Changing the rate of conversion of 4-hydroxycyclophosphoramide to a subsequent metabolite or altering its renal excretion would change its AUC. The rate of conversion of CPA to 4-ketocyclophosphamide (the partial metabolic clearance) was slightly decreased in cimetidine-treated animals, but this was not statistically significant ($P = 0.14$). No differences in the partial metabolic clearances to carboxyphosphamide or phosphoramide mustard were noted. It is possible that cimetidine inhibits the aldehyde oxidase that converts 4-hydroxycyclophosphoramide to 4-ketocyclophosphamide but that our model was not sufficiently sensitive to detect such changes. Further in vitro studies of this enzyme could address this question.

Both CPA and cimetidine are commonly used in cancer patients; thus, an interaction between these agents is potentially a frequent problem. We are unaware of any clinical trials evaluating CPA-cimetidine interaction; however, a patient with lung cancer who received daily CPA and cimetidine doses was observed to experience grade IV toxicity 1 month after CPA initiation (R. Livingston, personal communication). Factors affecting a CPA-cimetidine interaction would include the doses used, the duration of cimetidine administration, and the interval between cimetidine and CPA treatments. The results of our study, together with the data of Dorr et al. [9], suggest potentiation of CPA's effect when this drug is combined with cimetidine. The mechanism of this interaction appears to involve factors other than microsomal enzyme inhibition.

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