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Antitumor efficacy and pharmacokinetic analysis of 4-hydroperoxycyclophosphamide in comparison with cyclophosphamide ± hepatic enzyme effectors

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Abstract 4-Hydroperoxycyclophosphamide is an oxazaphosphorine which is readily converted without enzymatic involvement to 4-hydroxycyclophosphamide – a key intermediate in the antitumor activity of this class of drugs. The efficacy of 4-hydroperoxycyclophosphamide as a systemically administered antitumor drug was examined in mice bearing EMT-6 mammary carcinoma and in rats bearing 13762 mammary carcinoma in comparison with other oxazaphosphorines. 4-Hydroperoxycyclophosphamide was a more potent tumor cell killing agent than cyclophosphamide or ifosfamide in animals bearing the EMT-6 tumor. There were no significant differences in the toxicity to bone marrow amongst the three oxazaphosphorines. 4-Hydroperoxycyclophosphamide (90 mg/kg) on days 7, 9 and 11 produced 11.5 days of tumor growth delay compared with 10.4 days and 7.1 days for cyclophosphamide (150 mg/kg) and ifosfamide (150 mg/kg) administered on the same schedule, respectively. 4-Hydroperoxycyclophosphamide was tolerated at 90 mg/kg daily for 5 days and at 75 mg/kg twice daily for 4 days producing tumor growth delays of 14.4 days and 16.6 days, respectively. In rats bearing 13762 tumors, 4-hydroperoxycyclophosphamide (90 mg/kg) on days 8, 10 and 12 produced a tumor growth delay of 14.5 days compared with 8.9 days for cyclophosphamide (100 mg/kg) administered on the same schedule. Treatment of 13762 tumor-bearing rats with phenobarbital, pentobarbital or etanidazole increased the tumor growth delay produced by cyclophosphamide while treatment with cimetidine decreased the tumor growth delay produced by cyclophosphamide but not significantly. Administration of 4-hydroperoxycyclophosphamide (90 mg/kg) produced blood concentrations of 4-hydroxycyclophosphamide three-fold higher than those produced by administration of cyclophosphamide (100 mg/kg) at 15 min after drug injection. Treatment with phenobarbital or pentobarbital increased 4-hydroxycyclophosphamide blood concentration while pretreatment with cimetidine decreased 4-hydroxycyclophosphamide blood concentration from cyclophosphamide. 4-Hydroperoxycyclophosphamide is an effective antitumor agent worthy of further investigation.

Key words 4-Hydroperoxycyclophosphamide · Oxazaphosphorines · Cytochrome P-450 inducers

Introduction

Cyclophosphamide is one of the oldest, most widely used and most generally efficacious of the antitumor alkylating agents [25]. Although cyclophosphamide may be the most active component of many combination chemotherapy regimens, it is not curative in human neoplastic disease. Cyclophosphamide undergoes a well-documented complex metabolism to active and inactive species in the host [25, 29]. Among the strategies that have been used to improve the efficacy of cyclophosphamide are analog synthesis and modulation of specific steps of the drug's metabolic pathway.

4-Hydroperoxycyclophosphamide (4-HC), an analog of cyclophosphamide first prepared in the early 1970s [28, 30], has proven to be a very useful research tool because it is converted to the key metabolite 4-hydroxycyclophosphamide in cell culture media and thus has provided a reproducible means of studying the

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cytotoxicity, DNA binding characteristics, etc. of "cyclophosphamide" in vitro. As the supply of 4-HC increased and the frequency of the use of bone marrow transplantation in clinical cancer treatment increased, 4-HC proved useful in selectively killing tumor cells in bone marrow ex vivo [17].

In applying 4-HC as a systemic anticancer agent, a major question is whether the therapeutic index of cyclophosphamide would be maintained. Similar questions arise regarding the use of agents which alter the metabolism of cyclophosphamide by inducing or inhibiting specific enzymes, primarily in the liver, involved in that drug's activation or catabolism [1, 7, 8, 23–25, 27]. Several early studies with cyclophosphamide, 4-HC and related congeners such as 4-hydroperoxyifosfamide, 4-hydroxycyclophosphamide, 4-hydroxyifosfamide, and 4-peroxycyclophosphamide in the treatment of murine L1210 and/or P388 leukemia suggest that on the basis of the ratio of effective dose to lethal dose, cyclophosphamide has the greatest therapeutic index [11, 20].

This study compared 4-HC with cyclophosphamide alone and in combination with modifiers in two rodent mammary carcinoma models relating circulating levels of 4-hydroxycyclophosphamide to antitumor activity in vivo.

Material and methods

Drugs

4-HC was obtained as a gift from MGI Pharma, (Minneapolis, Minn). Cyclophosphamide and cimetidine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Etanidazole was a gift from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, Md). Ifosfamide, pentobarbital and phenobarbital were purchased from the Dana-Farber Cancer Institute pharmacy. For standards, 4-hydroxycyclophosphamide was prepared from 4-HC as previously reported [13].

Tumors

EMT-6 *tumor*

The EMT-6 mouse mammary carcinoma grown as a solid tumor subcutaneously in the flanks of female Balb/C mice (Taconic Farms, Germantown, N.Y.) has been used widely in radiobiology and chemotherapy studies. The EMT-6 murine mammary carcinoma is an in vivo – in vitro tumor system. In vivo the EMT-6 tumor grows to 100 mm^3 in about 7 days after subcutaneous implantation of 2×10^6 cells [39].

Rat 13762 *tumor*

The rat mammary adenocarcinoma 13762 is a carcinogen-induced (DMBA) tumor of the female Fischer 344 rat. The tumor can metastasize to the lungs and abdominal organs. The tumor is composed of epithelial tissue in folds and acini. The tumor grows to 100 mm^3 in about 14 days when implanted subcutaneously in the hind legs of female rats [37, 38, 40].

Tumor cell survival assay

EMT-6 murine mammary tumor cells (2×10^6) were implanted in each hind leg of female Balb/C mice. When the tumors had reached a volume of approximately 100 mm^3 (about 1 week after tumor cell implantation), the animals were treated with various doses of cyclophosphamide, ifosfamide or 4-HC administered by intraperitoneal injection as single doses or multiple doses (three injections over 9 h). Mice were killed at 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised and single-cell suspensions were prepared as previously described [31, 32]. The plating efficiency of untreated tumor cell suspensions ranged from 10% to 16%. The results are expressed as the surviving fraction ($\pm$ SE) of cells from treated groups as compared with untreated controls [32].

Bone marrow toxicity

Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle, and a colony-forming unit–granulocyte macrophage (CFU-GM) assay was carried out as previously described [32]). Colonies of at least 50 cells were scored manually. The results of three experiments, in which each group was measured in duplicate at three cell concentrations, were averaged. The results are expressed as the surviving fraction of treated groups as compared with untreated controls.

Tumor growth delay

EMT-6 *tumor*

When the tumors had reached a volume of approximately 100 mm^3 (day 7 after tumor cell implantation), cytotoxic therapy was initiated. Cyclophosphamide (150 mg/kg), ifosfamide (150 mg/kg) or 4-HC (75 or 100 mg/kg) was administered by intraperitoneal injection on days 7, 9 and 11. 4-HC (50, 75 or 90 mg/kg) was also administered daily on days 7 through 11 by intraperitoneal injection or 4-HC (50 or 75 mg/kg) was administered twice daily on days 7 through 10 by intraperitoneal injection.

Rat 13762 *tumor*

When the tumors had reached a volume of approximately 50 mm^3 (day 8 after tumor cell implantation), cytotoxic therapy was initiated. 4-HC (50, 75 and 90 mg/kg) or cyclophosphamide (100 mg/kg) was administered by intraperitoneal injection on days 8, 10 and 12. In some groups, the animals were treated with phenobarbital (36 mg/kg) or pentobarbital (36 mg/kg) by intraperitoneal injection on days 4 through 8 along with cyclophosphamide on days 8, 10 and 12 or were treated with etanidazole (500 mg/kg) or cimetidine (100 mg/kg) by intraperitoneal injection on days 8, 10 and 12 along with cyclophosphamide.

The progress of each tumor was measured thrice weekly until it had reached a volume of 1500 mm^3. Tumor growth delay was calculated as the number of days required for each treated tumor to reach a volume of 500 mm^3 as compared with untreated control tumors. Each treatment group comprised five mice or four rats, and

the experiment was repeated three times. Tumor growth delay was taken as the mean value ± SE calculated for the treatment group as compared with the control group [33, 36].

Determination of blood concentrations of 4-hydroxycyclophosphamide.

Blood was drawn via the ocular sinus from the rats described above in the tumor growth delay studies on day 8 at 0.25, 0.5, 1, 4 and 8 h after 4-HC or cyclophosphamide administration.

The aliquot of whole blood (150 µl) collected for the 4-hydroxycyclophosphamide assay was immediately added to a 10-ml glass test-tube containing 1.2 ml aqueous 12% perchloric acid, 220 µl 10 mg/ml 3-aminophenol, and 12 mg/ml hydroxylamine hydrochloride in 1 *M* HCl, 25 µl aqueous 10% sodium tungstate [26], and 40 µl aqueous 10 µ*M* methyl vinyl ketone, the internal standard [3]. The blood and reagent were mixed, brought to the laboratory on ice, and centrifuged. The supernatant was transferred to another 10-ml glass test-tube, heated at 100 °C in a boiling water bath for 15 min and cooled. From this point the samples were protected from light.

The derivatized samples were analyzed by high performance liquid chromatography (HPLC) on a 4-µm Waters Nova-pak phenyl cartridge (Millipore Corp., Milford, Mass.) with an isocratic mobile phase consisting of 0.45 *M* ammonium acetate, pH 4.0, and 11% acetonitrile. The column was protected by a Waters µ-Bondapak phenyl guard-pak. The sample supernatants and a set of standards prepared fresh daily were injected from a Waters Model 712 WISP autoinjector. The eluent was delivered by a Waters Model 510 HPLC pump at a flow rate of 1 ml/min. Postcolumn effluent was acidified in a 3.1-µl Upchurch mixing tee (Bodman, Aston, Pa.) with 0.3 *M* trifluoroacetic acid delivered at 0.5 ml/min by another Waters Model 510 HPLC pump. The fluorescent hydroxyquinoline derivatives were quantitated with a Waters Model 470 fluorescence detector at excitation and emission wavelengths of 350 and 500 nm, respectively. Standards were prepared by adding 4-hydroxycyclophosphamide to blood taken prior to cyclophosphamide administration. Final concentrations of 4-hydroxycyclophosphamide standards were 0, 10, 25, 50, 75, 100, 150, and 200 µ*M*. The standards were derivatized and quantitated by HPLC as described above. Peak height ratios (7-hydroxyquinoline/4-methyl-7-hydroxyquinoline) were calculated. The linear equation for peak height ratio as a function of standard concentration was determined by least-squares analysis. Sample concentrations were calculated by reference to this equation. The linearity of detector response was evaluated by correlation (r^2) analysis of the least-squares data. Blood concentrations at times after drug administration were plotted as a function of time [41].

Determination of plasma levels of cyclophosphamide

Plasma cyclophosphamide concentrations were measured by the method of Juma et al. [12] with some modification. Briefly, to 50-µl aliquots of each sample or standard was added 5 µl 80 µg/ml ifosfamide as an internal standard, 40 µl 1 *N* sodium hydroxide and 50 µl distilled water. The resulting mixture was extracted with 600 µl ethyl acetate. A 400-µl aliquot of the extract was evaporated to dryness under nitrogen at 60 °C in a 1.5-ml conical tube (Sarstedt No. 70.692, Numbrecht, Germany). The residue was treated with 10 µl of trifluoroacetic anhydride and 20 µl of ethyl acetate, the tubes were tightly capped and heated for 20 min at 70 °C. This was evaporated as described above and reconstituted with 50 µl ethyl acetate. A 1-µl portion was analyzed on a Hewlett-Packard model 5890A gas chromatograph with a nitrogen phosphorus (N-P) selective detector. The carrier gas was helium flowing at 21 ml/min. The column was a 530-µm 10 × 10 m long quartz capillary (Hewlett-Packard model HP-1) heated to 200 °C. Quantitation was on the basis of sample : internal standard peak height ratios.

Results

The survival of EMT-6 tumor cells and bone marrow granulocyte macrophage progenitor cells (CFU-GM) from animals treated with single doses of cyclophosphamide, ifosfamide or 4-HC is shown in Fig. 1. Each of these three antitumor alkylating agents killed tumor cells in a log-linear manner. Over the same dosage range in this tumor, 4-HC was the most potent of the three drugs and ifosfamide was the least potent. The difference between tumor cell killing by 4-HC and cyclophosphamide was dose modifying, that is there was an increasing differential between tumor cell killing by 4-HC with increasing drug dose compared with cyclophosphamide. There were no significant differences in the toxicity of the three drugs toward bone marrow CFU-GM with a trend toward ifosfamide being less toxic to the bone marrow than to the tumor.

4-HC has a relatively short circulating half-life (~30 min); therefore, 4-HC might be expected to be more effectively administered by continuous infusion than by bolus injection. As a continuous infusion model [34], the tumor cell and bone marrow CFU-GM killing by 4-HC administered in three intraperitoneal injections over 9 h. was compared with that produced by 4-HC to the same total dose administered in a single intraperitoneal injection (Fig. 2). There was no significant

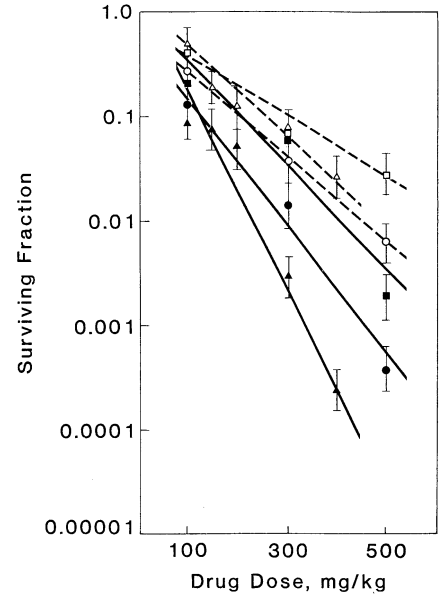

Fig. 1 Survival of EMT-6 cells (*solid symbols*) and bone marrow CFU-GM (*open symbols*) from EMT-6 tumor-bearing female Balb/C mice treated with single intraperitoneal doses of 4-HC (▲, △), cyclophosphamide (●, ○) or ifosfamide (■, □). Points are the means of three independent determinations; *bars* SEM

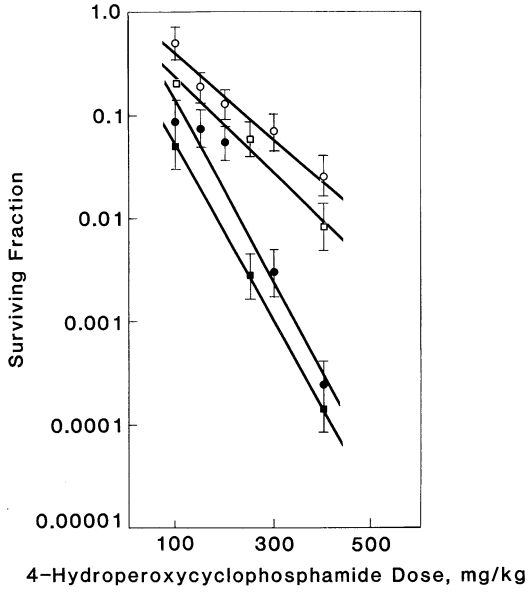

Fig. 2 Survival of EMT-6 cells (*solid symbols*) and bone marrow CFU-GM (*open symbols*) from EMT-6 tumor-bearing animals treated with single intraperitoneal doses (●, ○) or three doses over 9 h. to the same total doses (■, □) of 4-HC. Points are the means of three independent determinations; *bars* SEM

Table 1 Growth delay of the EMT-6 mammary carcinoma produced by 4-hydroperoxycyclophosphamide (4-HC) and related oxazaphosphorines

Treatment group	Tumor growth delay[a] (days)
Schedule: days 7, 9, 11	
Cyclophosphamide (150 mg/kg)	10.4 ± 1.2
Ifosfamide (150 mg/kg)	7.1 ± 0.8
4-HC (75 mg/kg)	3.8 ± 0.4
4-HC (100 mg/kg)	11.5 ± 1.3
Schedule: Days 7 → 11	
4-HC (50 mg/kg)	6.4 ± 0.7
4-HC (75 mg/kg)	9.6 ± 0.9
4-HC (90 mg/kg)	14.4 ± 1.3
Schedule: 2 × per day, days 7 → 10	
4-HC (50 mg/kg)	9.0 ± 1.0
4-HC (75 mg/kg)	16.6 ± 1.7

[a]Tumor growth delay is the difference in days for treated tumors to reach a volume of 500 mm^3 as compared with untreated control tumors. Untreated control tumors reached 500 mm^3 in about 14 days. Values are the means ± SE for 15 animals

difference between the tumor cell and bone marrow CFU-GM killing by 4-HC administered by the multiple-dose and single-dose regimens. There was a trend toward more cytotoxicity toward both the tumor cells and the bone marrow CFU-GM when 4-HC was administered by the multiple-dose continuous infusion model.

The EMT-6 murine mammary carcinoma is a chemotherapy-responsive tumor. Cyclophosphamide (150 mg/kg) administered by intraperitoneal injection on alternate days (days 7, 9 and 11) post-tumor cell implantation resulted in a tumor growth delay of about 10 days in this tumor (Table 1). Ifosfamide (150 mg/kg) administered on the same schedule produced a tumor growth delay of about 7 days. 4-HC was more toxic than cyclophosphamide and ifosfamide. On the three-dose alternate day schedule (days 7, 9 and 11), 4-HC doses of 150 mg/kg and 125 mg/kg were lethal. When 4-HC (100 mg/kg) was administered on alternate days on (days 7, 9 and 11), a tumor growth delay of about 11.5 days was obtained. Reduction in the 4-HC dose to 75 mg/kg administered on the same alternate-day schedule resulted in a lower tumor growth delay of about 3.8 days. 4-HC was tolerated when administered daily by intraperitoneal injection at a dose of 90 mg/kg daily for 5 days on days 7 through 11, producing a tumor growth delay of about 14.4 days. When 4-HC (75 mg/kg) or 4-HC (50 mg/kg) was administered daily for 5 days on days 7 through 11, tumor growth delays of about 9.6 days and 6.4 days resulted, respectively. On a twice-daily schedule for 4 days on days 7 through 10, 4-HC (75 mg/kg) was tolerable and produced 16.6 days of tumor growth delay. 4-HC (50 mg/kg) administered twice daily for 4 days on days 7 through 10 resulted in a tumor growth delay of about 9 days.

In order to carry out tumor response studies in a second tumor system and to carry out pharmacokinetic studies in the same animals, Fischer 344 female rats bearing the 13762 mammary carcinoma were chosen as the experimental model system. 4-HC (90 mg/kg) administered by intraperitoneal injection on alternate days (days 8, 10 and 12) post-tumor cell implantation was tolerable and produced a tumor growth delay of about 14.5 days (Table 2). There was a clear dose response effect of 4-HC in this tumor with doses of 75 mg/kg and 50 mg/kg administered on the same alternate-day schedule resulting in about 6.3 days and 3.6 days of tumor growth delay, respectively. In comparison, cyclophosphamide (100 mg/kg) administered by intraperitoneal injection on alternate days (days 8, 10 and 12) produced a tumor growth delay of about 8.9 days. Cyclophosphamide is a prodrug which undergoes activation by hepatic microsomal enzymes [25]. Formation of 4-hydroxycyclophosphamide by a microsomal cytochrome P-450 may be a limiting step in cyclophosphamide efficacy [23–25, 29]. Several biocompatible agents have been described which induce increased production of hepatic microsomal cytochrome P-450. Phenobarbital and pentobarbital are two of these [23–25, 27]. Cimetidine, on the other hand, may inhibit the catabolism of cyclophosphamide [1, 7, 8, 25]. Etanidazole is a nitroimidazole chemosensitizer that has been shown to increase the antitumor activity of cyclophosphamide [10, 35]. When animals were treated with phenobarbital (36 mg/kg) or pentobarbital (36 mg/kg) by intraperitoneal injection daily on days 4 through 8 along with cyclophosphamide on days 8,

Table 2 Growth delay of the rat 13762 mammary carcinoma produced by 4-hydroperoxycyclophosphamide or cyclophosphamide alone or along with hepatic enzyme effectors

Treatment group	Tumor growth delay[a] (days)
4-Hydroperoxycyclophosphamide	
90 mg/kg, days 8,10,12	14.5 ± 1.1*
75 mg/kg, days 8,10,12	6.3 ± 0.6
50 mg/kg, days 8,10,12	3.6 ± 0.4
Cyclophosphamide	
100 mg/kg, days 8,10,12	8.9 ± 1.1
+Phenobarbital, 36 mg/kg, days 4–8[b]	11.0 ± 0.9
+Pentobarbital, 36 mg/kg, days 4–8	15.5 ± 1.6*
+Etanidazole, 500 mg/kg, days 8,10,12	13.9 ± 1.3*
+Cimetidine, 100 mg/kg, days 8,10,12	7.9 ± 0.8

*$P < 0.001$ vs cyclophosphamide (D) unn multiple comparison test)
[a] Tumor growth delay is the difference in days for treated tumors to reach a volume of 500 mm^3 as compared with untreated control tumors. Untreated control tumors reached 500 mm^3 in about 21 days. Values are the means ± SE for 12 animals
[b]The tumor growth delays produced by the hepatic enzyme effectors were: (1) phenobarbital (36 mg/kg, days 4–8), 0.6 ± 0.3 days; (2) pentobarbital (36 mg/kg, days 4–8), 0.3 ± 0.3 days; (3) etanidazole (500 mg/kg, days 8, 10, 12), 1.2 ± 0.4 days; and (4) cimetidine (100 mg/kg, days 8, 10, 12), 0.5 ± 0.3 days. The hepatic enzyme effectors were administered by intraperitoneal injection. When they were administered on the same day, the hepatic enzyme effector was injected 5 min prior to the cyclophosphamide

10 and 12, tumor growth delays of about 11 days and about 15.5 days resulted, respectively. Treatment with etanidazole (500 mg/kg) by intraperitoneal injection 5 min prior to each injection of cyclophosphamide produced about 13.9 days of tumor growth delay while treatment with cimetidine (100 mg/kg) in the same manner resulted in a tumor growth delay of about 7.9 days.

Blood was collected from the rats described in Table 2 over a time course after administration of the first dose of 4-HC or cyclophosphamide. Whole blood was analyzed for 4-hydroxycyclophosphamide concentration and serum was analyzed for cyclophosphamide concentration in animals receiving that drug. Shortly (15 min) after intraperitoneal injection of 4-HC (90, 75 or 50 mg/kg) relatively high blood concentrations of 4-hydroxycyclophosphamide were detected (Fig. 3). The blood concentrations of 4-hydroxycyclophosphamide corresponded to the dose of 4-HC administered and dropped very rapidly so that by 1 h post- drug administration the blood concentrations were only about 35% of those seen at 15 min post-drug administration. Administration of cyclophosphamide (100 mg/kg) resulted in a plasma concentration of 4-hydroxycyclophosphamide 33 μ*M* by 15 min post-drug administration. The plasma concentration of 4-hydroxycyclophosphamide from cyclophosphamide decreased relatively slowly and at 1 h post-drug administration was 70% of the 15-min level.

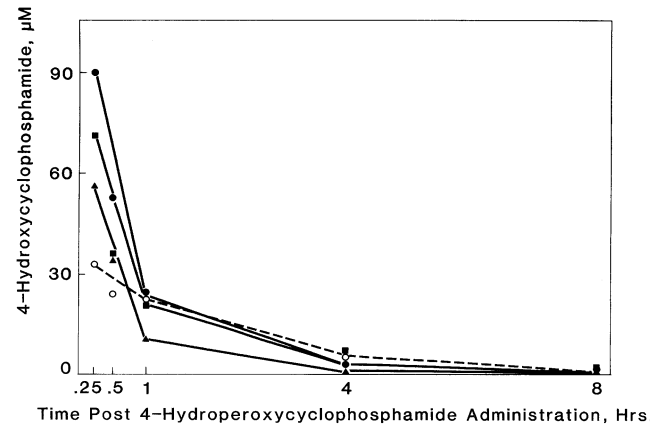

Fig. 3 Blood concentrations of 4-hydroxycyclophosphamide in 13762 mammary carcinoma-bearing female Fischer 344 rats over 8 h after a single dose of 4-HC (90 mg/kg) (●), 4-HC (75 mg/kg) (■), 4-HC (50 mg/kg) (▲) or cyclophosphamide (100 mg/kg) (○). Points are the means of three to five independent experiments

Administration of a hepatic microsomal cytochrome P-450 inducer, phenobarbital or pentobarbital, markedly altered the blood concentrations of 4-hydroxycyclophosphamide after cyclophosphamide injection (Fig. 4). At 15 min after cyclophosphamide injection, blood concentrations of 4-hydroxycyclophosphamide were 85 μ*M* and 70 μ*M* and were very similar to the blood concentrations of 4-hydroxycyclophosphamide obtained 15 min after a dose of 90 mg/kg or 75 mg/kg of 4-HC, respectively. Under these cyctochrome P-450-induced conditions, the blood concentrations of 4-hydroxycyclophosphamide decreased at a rate very similar to that of 4-hydroxycyclophosphamide produced from 4-HC so that by 1 h post-cyclophosphamide

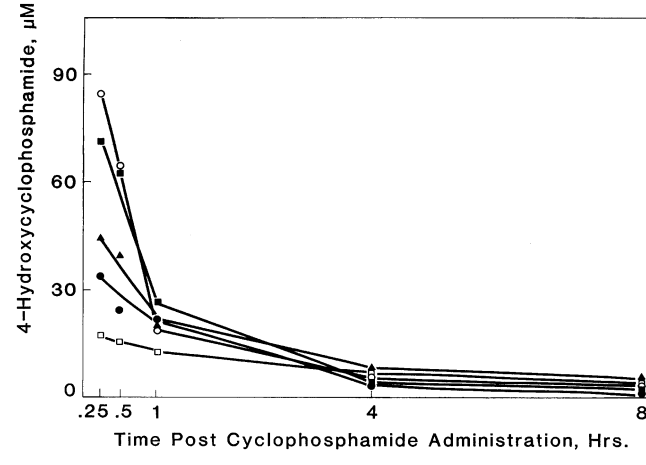

Fig. 4 Blood concentrations of 4-hydroxycyclophosphamide in 13762 mammary carcinoma-bearing female Fischer 344 rats over 8 h after a single dose of cyclophosphamide alone (●), after pretreatment with phenobarbital (○), after pretreatment with pentobarbital (■), along with cimetidine (□) along with etanidazole (▲). Points are the means of three to five independent experiments

administration the blood concentrations of 4-hydroxycyclophosphamide were about 25% of those detected at 15 min after drug injection. Treatment with etanidazole along with cyclophosphamide increased the blood concentration of 4-hydroxycyclophosphamide from the parent drug to a lesser degree than did the hepatic microsomal cytochrome P-450 inducers. Treatment with cimetidine along with cyclophosphamide appeared to decrease the blood concentration of 4-hydroxycyclophosphamide from the parent drug to about 50% of that seen in the absence of cimetidine. In these same animals 15 min after administration of cyclophosphamide (100 mg/kg) the plasma concentration of cyclophosphamide was 533 μM after the drug alone, 316 μM and 193 μM after pretreatment with phenobarbital and pentobarbital, respectively, and 693 μM after pretreatment with cimetidine.

Discussion

The availability of 4-HC, an oxazaphosphorine that is converted to the key intermediate species 4-hydroxycyclophosphamide in circulation without enzymatic intervention, for systemic administration has allowed several questions regarding the potency and toxicity of such a molecule to be addressed [25]. 4-HC was, on a milligram per kilogram basis, a more active antitumor agent and more toxic than cyclophosphamide in both tumor/host systems studied. In the female Balb/C mouse bearing the EMT-6 murine mammary carcinoma on the alternate-day $\times$ 3 schedule, the animals tolerated 300 mg/kg 4-HC and 450 mg/kg cyclophosphamide or ifosfamide total dose. The 450 mg/kg dose of cyclophosphamide resulted in a tumor growth delay equivalent to 285 mg/kg 4-HC. The daily $\times$ 5 and twice daily $\times$ 4 schedules of 4-HC allowed higher total doses of the drug to be administered but were less efficient anticancer regimens on a total drug dose basis than the alternate-day schedule. In other words, a 50 mg/kg increase in total dose of 4-HC resulted in about 4.5 days of tumor growth delay when administered on the alternate-day $\times$ 3 schedule while the same increase in total drug dose administered on either the daily or twice-daily schedules resulted in about 2 days of tumor growth delay. Despite being less efficient in terms of antitumor activity, the twice-daily $\times$ 4 schedule of 4-HC produced the greatest tumor growth delay because a total dose of 600 mg/kg of 4-HC was tolerated by the animals on the twice-daily regimen. 4-HC was also a more active antitumor agent in the female Fischer 344 rat bearing the 13762 mammary carcinoma. In that tumor a total dose of 300 mg/kg of cyclophosphamide was equivalent to the tumor growth delay produced by a total dose of 250 mg/kg of 4-HC.

Although 4-HC was less toxic to murine bone marrow CFU-GM on a milligram per kilogram basis than either cyclophosphamide or ifosfamide, 4-HC was lethal at a lower total single dose than either cyclophosphamide or ifosfamide, indicating that cardiac or CNS toxicity may be limiting in 4-HC administration. 4-HC was apparently converted much more efficiently to 4-hydroxycyclophosphamide than cyclophosphamide; however, the rate of disappearance of 4-hydroxycyclophosphamide upon administration of 4-HC was more rapid than from cyclophosphamide. It is likely that the apparent rate of 4-hydroxycyclophosphamide disappearance was slower in the case of cyclophosphamide because plasma levels of 4-hydroxycyclophosphamide in that case reflect both the rate of the formation of 4-hydroxycyclophosphamide and its rate of removal. The rate of formation of 4-hydroxycyclophosphamide is slower from cyclophoshamide than from 4-HC. The area under the blood 4-hydroxycyclophosphamide concentration versus time curve over the period from 15 min to 4 h after drug administration for 4-HC (90 mg/kg) was 1.6-fold greater than for cyclophosphamide (100 mg/kg). Treatment of 13762 mammary carcinoma-bearing rats with the microsomal cytochrome P-450 inducers, phenobarbital or pentobarbital, markedly increased the blood concentration at early time-points of 4-hydroxycyclophosphamide after cyclophosphamide administration. Pentobarbital plus cyclophosphamide treatment produced a tumor growth delay of 15.5 days with a total dose of 300 mg/kg of cyclophosphamide. Extrapolating the 4-HC data to the same total dose would result in a computed tumor growth delay of 18 days (Table 2). Because treatment with a cytochrome P-450 inducer may result in the increased expression of these enzymes in many tissues, especially the lung, the use of these agents may not result in an increase in the therapeutic index of cyclophosphamide [5, 18, 25].

Prior treatment of rabbits with cimetidine has been shown by Anthony et al. [2] to prolong the plasma half-life of cyclophosphamide, presumably by inhibiting the hepatic enzyme(s) responsible for metabolizing the drug. Cimetidine pretreatment of mice bearing P-388 leukemia has been shown to increase the antitumor activity of cyclophosphamide in these animals [1, 7, 8]. The cimetidine treatment prolonged the circulating half-life of cyclophosphamide in these animals and prolonged the time over which alkylating metabolites were produced from the cyclophosphamide [7, 8]. The alteration in cyclophosphamide metabolism in these animals may be responsible for the increased efficacy of the treatment combination compared with cyclophosphamide alone. However, cimetidine is also known to be an immunomodulator which may act by inhibiting suppressor T cell activity, thus enhancing immune system activity against the tumor [9, 14, 16, 19]. Treatment of rats bearing 13762 mammary carcinoma with cimetidine prior to each dose of cyclophosphamide did not significantly alter the tumor growth delay produced by the drug although the trend was toward

less tumor growth delay with cimetidine present. At early time-points (15 min to 1 h) lower plasma blood concentrations of 4-hydroxycyclophosphamide were measured in animals pretreated with cimetidine; however, by 4 h post-cyclophosphamide administration there was no difference between the blood concentrations of 4-hydroxycyclophosphamide in animals receiving cimetidine and cyclophosphamide and in those receiving cyclophosphamide only.

Etanidazole, a 2-nitromidazole radio- and chemosensitizer, has been shown to decrease thiol levels in tumor and normal tissues [10, 35]. Administration of etanidazole to rats bearing the 13762 mammary carcinoma prior to each dose of cyclophosphamide resulted in significantly increased tumor growth delay compared with cyclophosphamide and resulted in a small increase in 4-hydroxycyclophosphamide levels at early times after cyclophosphamide administration.

In general terms, this report describes potential means of increasing the antitumor efficacy of the oxazaphosphorine, cyclophosphamide. This includes analog development and modulator development. Of the treatment regimens described only the combination of etanidazole and cyclophosphamide has undergone clinical trial [4, 6, 15, 21, 22]. These early clinical trials focused on dose finding for etanidazole and found that adequate serum levels for etanidazole to predict alkylating agent sensitization should be possible [15, 21, 22]. 4-HC has undergone clinical trial for ex vivo purging of tumor cells from the bone marrow for transplant regimens [17]. Systemic administration of 4-HC was efficacious in two preclinical mammary carcinoma systems and warrants further investigation and consideration for clinical trial.

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