Therapeutic Efficacy of Cyclophosphamide as a Function of Inhibition of Its Metabolism

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SUMMARY

Experiments were designed to determine the Michaelis-Menten kinetic constants of cyclophosphamide metabolism in vitro in the presence of known inhibitors of rat hepatic microsomal mixed-function oxidase activity, the rate of cyclophosphamide metabolism in vivo in the presence of the most effective inhibitors of rat hepatic microsomal cyclophosphamide metabolism, the antitumor efficacy of cyclophosphamide as a function of its rate of activation, and the toxicity of cyclophosphamide as a function of its rate of activation. The hepatic microsomal mixed-function oxidase activation of cyclophosphamide was inhibited by all of the following compounds: 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525A), ethylmorphine, norcodeine, 7,8-benzoflavone, acetophenetidin, metyrapone, meperidine, testosterone, progesterone, estradiol-17β, deoxycorticosterone, quinine, and probenecid. Compounds that did not inhibit cyclophosphamide activation included caffeine, folic acid, methotrexate, guanine, adenine, 6-mercaptopurine, 8-azaguanine, and 6-azauracil. Nitrogen mustard, chlorambucil, and bis(2-chloroethyl)amine hydrochloride did not inhibit hepatic microsomal mixed-function activity. The most effective competitive inhibitor was SKF 525A. Among the most effective noncompetitive inhibitors were the steroid hormones. In vivo cyclophosphamide metabolism in male rats paralleled in vitro metabolism. Thus, at early time points, blood levels of alkylating activity were decreased when SKF 525A nor metyrapone had an effect on tumor growth or blood leukocyte levels. The data further demonstrate the futility of attempting to improve the therapeutic efficacy of cyclophosphamide by pretreatment with drugs that alter its rate of activation, and they also provide a rational basis for the ineffectiveness of such an effort.

INTRODUCTION

Cyclophosphamide is an antineoplastic agent thought to exert its therapeutic effect via alkylation. However, cyclophosphamide itself is virtually without alkylating or cytotoxic activity and must be activated before it can exert its biological effect. Activation of cyclophosphamide in vitro has been demonstrated to be catalyzed by the same hepatic microsomal mixed-function oxidase system that is functional in the oxidative metabolism of many other drugs (2, 4, 5, 7, 8, 12, 23, 24).

With the knowledge that cyclophosphamide is activated by the hepatic microsomal mixed-function oxidase system and with the wealth of information available as to the stimulation, depression, and inhibition of this enzyme activity (17), experiments can be readily designed to regulate the rate of cyclophosphamide activation. In fact, previous investigations have demonstrated that the rate of cyclophosphamide activation can be stimulated by phenobarbital pretreatment (2, 7, 18, 20, 24) or be depressed with cobalt chloride or 3-methylcholanthrene pretreatment (24), and that male rats activate cyclophosphamide at a much faster rate than do female rats (24). Altered blood levels of alkylating activity observed after pretreatment with stimulators and depressors of cyclophosphamide activation appeared to be due solely to altered cyclophosphamide activation rates, precluding an effect of these compounds on those factors, e.g., enzyme concentration and blood flow, which contribute to kinetic constants describing rates of excretion, inactivation, and/or distribution (24). Furthermore, the therapeutic response to cyclophosphamide observed in female rats and in control male rats was similar, and remained quantitatively unchanged when the rate of cyclophosphamide activation in male rats was stimulated by phenobarbital pretreatment or depressed by cobalt chloride pretreatment (24). The principle providing a rational explanation for this observation has been presented previously (24) and expanded upon under “Discussion.” Similarly, significant quantitative changes in the undesired response, leukopenia, were not observed, as expected, if

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undesired, as well as desired toxicities result from the same action of the drug (24). Thus, the therapeutic index of cyclophosphamide also was not changed by an increase or decrease in its rate of activation.

All of the aforementioned drugs are thought to increase or decrease hepatic microsomal mixed-function oxidase activity by increasing or decreasing the amount of enzyme present. Another way to regulate enzyme activity is to introduce activators and inhibitors of existing enzyme. Thus, a commonly used inhibitor of hepatic microsomal mixed-function oxidase activity, SKF 525A, inhibited the conversion of cyclophosphamide in vivo to its alkylating and cytotoxic metabolite(s) (4). However, SKF 525A had no effect on the antitumor (Leukemia L1210) efficacy of cyclophosphamide (15), although it did reduce the lethality of cyclophosphamide to mice and rats (15), as did 2 other inhibitors of hepatic microsomal mixed-function oxidase activity, chloramphenicol and 2,4-dichloro-6-phenylphenoxyethyldiethylamine (11, 15). In contrast, the administration of SKF 525A enhanced the teratogenic effect of cyclophosphamide (13).

Experiments described in this paper were undertaken to determine (a) the kinetic characteristics of hepatic microsomal cyclophosphamide metabolism in the presence of drugs known to inhibit hepatic microsomal mixed-function oxidase activity, (b) whether drugs that inhibit the metabolism of cyclophosphamide in vitro also inhibit its metabolism in vivo, (c) whether the antitumor efficacy of cyclophosphamide in an experimental tumor system (Walker 256 carcinosarcoma) is a function of its rate of activation, and (d) whether the undesired toxicity of cyclophosphamide is a function of its rate of activation.

MATERIALS AND METHODS

Animals and Tissue Preparation. Male Holtzman rats (230 to 270 g) obtained from The Holtzman Co., Madison, Wis., were used in all experiments except those in which 170- to 180-g male Holtzman rats were given i.m. injections of Walker 256 carcinosarcoma ascites cells (obtained from Cancer Chemotherapy National Service Center via Arthur D. Little, Inc., Cambridge, Mass.). In all experiments, the animals were fed a standard chow diet ad libitum. All animals were sacrificed between 8 and 9 a.m. Hepatic microsomal preparations were obtained as previously described (23).

Estimate of Cyclophosphamide Metabolism In Vitro. The incubation mixture and colorimetric estimation of metabolites capable of alkylation were as previously described (23), except that semicarbazide hydrochloride (37.5 μmoles/reaction flask) was used in all experiments. The following compounds were tested, at the concentrations given, as potential inhibitors of cyclophosphamide activation: ethylmorphine hydrochloride (0.5 and 1.0 mM), SKF 525A (10 and 20 μM), norcodeine (0.5 and 1.0 mM), meperidine (0.25 and 0.5 mM), quinine hydrobromide (20 and 40 μM), acetophenetidin (0.5 and 1.0 mM), probenecid (0.5 and 1.0 mM), 7,8-benzoflavone (10 and 20 μM), metyrapone (20 and 40 μM), testosterone (20 and 40 μM), progesterone (20 and 40 μM), β-estradiol-3-benzoate (20 and 40 μM), deoxycorticosterone (20 and 40 μM), folic acid (0.1 mM), methotrexate (0.1 mM), guanine (0.1 mM), adenine hydrochloride (0.1 mM), 6-mercaptothione (0.1 mM), 8-azaguanine (0.1 mM), 6-azauracil (0.1 mM), and caffeine (1.0 mM). Testosterone, progesterone, β-estradiol-3-benzoate, deoxycorticosterone, and 7,8-benzoflavone were introduced to the incubation mixture in 10 μl of ethanol. Preliminary experiments revealed that this amount of ethanol had no effect on the kinetic constants of cyclophosphamide metabolism. All of the other compounds were introduced in water.

Estimate of Cyclophosphamide Metabolism In Vivo. Male rats were given i.p. injections of 0.9% NaCl solution, metyrapone (50 μmoles/kg), or SKF 525A (10 or 50 μmoles/kg) in a volume of about 0.5 ml. Cyclophosphamide (400 mg/kg, i.p.) in a volume of 2.6 ml was injected 45 min later. Blood samples were taken, and alkylating activity that was present before and after extraction with methylene chloride was determined as previously described (24). The latter was determined because methylene chloride extracts cyclophosphamide from water without extracting a significant amount of the metabolites (24).

Dose Response and ED₅₀ Determinations. One million Walker 256 carcinosarcoma ascites cells, in a volume of 0.1 ml, were injected i.m. into the hindlegs of male rats. Then (23.25 hr later), 0.9% NaCl solution, metyrapone (50 μmoles/kg), or SKF 525A (10 or 50 μmoles/kg) was injected i.p. in a volume of about 0.5 ml. Exactly 45 min later, freshly prepared cyclophosphamide in 0.9% NaCl solution was injected i.p. at dosage levels of 0, 0.25, 0.5, 1.0, and 2.0 mg/kg. Four to 6 rats were used at each dosage level. Tumor growth and inhibition thereof were determined 7 days after the introduction of tumor cells. Dose-response curves were determined, and ED₅₀ values were calculated as previously described (24). The rationale on which the foregoing protocol is based has been described in detail (24).

Estimate of Cyclophosphamide Toxicity. Cyclophosphamide (50 mg/kg) in about 0.5 ml 0.9% NaCl solution, was injected i.p. 45 min after the i.p. injection of 0.9% NaCl solution, metyrapone (50 μmoles/kg), or SKF 525A (10 or 50 μmoles/kg). Blood was obtained from the tail vein at various times thereafter, and leukocyte counts were determined as previously described (24).

Data Analyses. Data describing reaction velocities and dose-response curves were analyzed as previously described (23, 24). Inhibition was interpreted as being competitive when the 1/ν intercepts (no inhibitor versus inhibitor) were not significantly different (p > 0.05), and as being noncompetitive when the 1/S intercepts (no inhibitor versus inhibitor) were not significantly different (p > 0.05).
RESULTS

Initial experiments with known substrates for hepatic microsomal mixed-function oxidases were designed to find drugs that would inhibit cyclophosphamide activation in vitro; the most effective inhibitors then were to be used in subsequent in vivo experiments. Since cyclophosphamide is often used clinically in combination with analgesics, steroids, and other antineoplastic agents, the ability (or lack thereof) of some of these compounds to inhibit cyclophosphamide activation was also investigated.

The results of these experiments are shown in Table 1. The most effective inhibitor of cyclophosphamide activation was SKF 525A. Other competitive inhibitors included metyrapone, quinine, ethylmorphine, acetophenetidin, and norcodeine. Noncompetitive inhibitors included 7,8-benzoflavone, progesterone, deoxycorticosterone, estradiol-17β, testosterone, meperidine, and probenecid. Compounds that did not inhibit cyclophosphamide activation included caffeine, folic acid, methotrexate, guanine, adenine, 6-mercaptopurine, 8-azaguanine, and 6-azauracil (data not presented).

In the current experiments, metyrapone and steroid hormones inhibited cyclophosphamide activation by hepatic microsomal preparations competitively and noncompetitively, respectively. In contrast, metyrapone inhibited ethylmorphine N-demethylation noncompetitively (Ref. 16; N. E. Sladek, unpublished observations), and the steroid hormones inhibited N-demethylation of ethylmorphine competitively (Ref. 25; N. E. Sladek, unpublished observations). These observations are difficult to reconcile with the conclusion reached previously (23) that ethylmorphine, cyclophosphamide, and probably many other drugs are biotransformed by the same hepatic microsomal mixed-function oxidase system or by several enzyme systems with a common rate-limiting component.

An evaluation of hepatic microsomal mixed-function oxidase activity with the use of ethylmorphine as substrate revealed that concentrations of 1 mM chlorambucil, nitrogen mustard, or nor-HN2 had no inhibitory effect (data not presented). Thus, it is unlikely that these compounds inhibit cyclophosphamide activation or that the first-formed metabolites of cyclophosphamide inhibit metabolism of the parent compound by alkylating and inactivating the enzyme system.

Experiments were next designed to determine whether the inhibition of metabolism observed in vitro could be effected in vivo. In general, compounds that distribute in total body water and inhibit cyclophosphamide metabolism with a $K_i$ of about 50 µM or higher in vitro would not be expected to be very effective inhibitors of cyclophosphamide activation in vivo at the dosage levels "normally" used. For example, the administration of 250 mg of a potential inhibitor, with a molecular weight of 250, to a 72-kg man calculates out to a dose of about 14 µmoles/kg; and, assuming that 70% of total body weight is water, a maximum concentration in body water of 20 µM.

SKF 525A and metyrapone were chosen for use in vivo as representative competitive inhibitors of cyclophosphamide activation because they are water soluble and, of the various compounds tested in vitro, they exhibited the most favorable $K_i$'s.

Total alkylating activity (Chart 1) and alkylating activity that remained after methylene chloride extraction (Chart 2) were determined as described in "Materials and Methods." As previously reported (4, 24), peak blood levels of alkylating activity were reached at about 1 hr after the injection of cyclophosphamide. Blood levels of alkylating activity were markedly depressed at all time periods measured, when male rats were given injections of SKF 525A (50 µmoles/kg) 45 min prior to the injection of cyclophosphamide, in accordance with previous reports (4, 19). Pretreatment with metyrapone (50 µmoles/kg) or SKF 525A (10 µmoles/kg) caused blood
levels of alkylating activity to be somewhat depressed for the first 2 hr after cyclophosphamide injection but, by 3 hr postinjection, no differences remained. No alkylating activity was found in the blood at 24 hr after the administration of cyclophosphamide, regardless of how the animals were pretreated. In all cases, qualitatively similar results were obtained when blood was first extracted with methylene chloride before analysis for alkylating activity, indicating that the results reflect differences in metabolite(s) rather than cyclophosphamide levels. Thus, at the dosage levels used, inhibitors of cyclophosphamide metabolism in vitro were also effective inhibitors of cyclophosphamide activation in vivo, and inhibition of activation was dose dependent.

Cyclophosphamide inhibited tumor growth in untreated male rats with an ED$_{50}$ of 0.76 mg/kg (Table 2). Although they inhibited cyclophosphamide activation, the administration of metyrapone (50 μmoles/kg) or SKF 525A (10 μmoles/kg) did not significantly alter the therapeutic response to cyclophosphamide, in that the ED$_{50}$'s obtained were essentially the same as those observed in control rats. However, a higher dose of SKF 525A (50 μmoles/kg) did alter the therapeutic response to cyclophosphamide, in that the ED$_{50}$ obtained was 2.11 mg/kg.

As is the case with virtually all antineoplastic agents, 1 of the major undesired effects of cyclophosphamide is leukopenia. Therefore, as a quantitative estimate of toxicity, leukocyte counts in peripheral blood were made at various times after the injection of cyclophosphamide into male rats that had received injections (45 min earlier) of 0.9% NaCl solution, SKF 525A, or metyrapone. Preliminary experiments (data not presented) established that leukopenia due to cyclophosphamide and/or its metabolites is log-dose dependent, with a 50% depression in leukocyte counts achieved at a dose of 13 mg/kg.

Following the administration of 1 dose of cyclophosphamide, 50 mg/kg, to otherwise untreated male rats, the leukocyte count decreased steadily, reaching a nadir 4 days postinjection (Chart 3). At this time, the leukocyte count was about 18% of the initial count. Nine days postinjection, the leukocyte count had returned to about 65% of that of the control. These findings are substantially in agreement with previous reports (10, 24, 26). Essentially the same response was obtained if male rats preinjected with metyrapone (50 μmoles/kg) or SKF 525A (10 μmoles/kg) were used. In rats

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μmoles/kg)</th>
<th>Slope</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>95% confidence interval (mg/kg)</th>
<th>Validity analysis</th>
</tr>
</thead>
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<td>0.9% NaCl solution</td>
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<td></td>
<td>0.76</td>
<td>0.54 – 1.05</td>
<td></td>
</tr>
<tr>
<td>SKF 525A</td>
<td>50</td>
<td>-22.3</td>
<td>2.11</td>
<td>1.44 – 3.41</td>
<td></td>
</tr>
<tr>
<td>SKF 525A</td>
<td>10</td>
<td>-27.6</td>
<td>0.92</td>
<td>0.65 – 1.33</td>
<td></td>
</tr>
<tr>
<td>Metyrapone</td>
<td>50</td>
<td>-33.6</td>
<td>0.74</td>
<td>0.52 – 1.06</td>
<td></td>
</tr>
</tbody>
</table>

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Cyclophosphamide Metabolism
Points, mean of 3 animals. •, 0.9% NaCl solution; ., SKF 525A, 50 µmoles/kg; @, metyrapone. Leukocyte counts at zero time averaged 15,860/cu mm. Mmoles/kg), or metyrapone (50 µmoles/kg), was injected i.p. 45 min post-cyclophosphamide injection, rather than at 4 days, as was depression was less and the nadir was reached 3 days before the injection of cyclophosphamide (50 mg/kg). At various times thereafter, blood was removed from the tail vein, and leukocyte counts were made. Leukocyte counts at zero time averaged 15,860/cu mm. Points, mean of 3 animals. •, 0.9% NaCl solution; ., SKF 525A, 50 µmoles/kg; @, SKF 525A, 10 µmoles/kg; *, metyrapone.

††† treated previously with injections of SKF 525A, 50 µmoles/kg, the rates of leukocyte depression and recovery following cyclophosphamide administration were similar to those observed in control rats, but the magnitude of leukocyte depression was less and the nadir was reached 3 days post-cyclophosphamide injection, rather than at 4 days, as was the case in control rats. Differential counts of granulocytes (neutrophils) and agranulocytes (lymphocytes) showed essentially the same pattern, although there was great variability in the neutrophil counts (data not presented).

DISCUSSION

With the increased use of combination chemotherapy in the treatment of neoplastic disease, deserved attention has been and should be given to potential “drug interactions,” including those involving hepatic microsomal mixed-function oxidase biotransformation, since these interactions may significantly alter therapeutic and toxic responses. Virtually all of the experimental work relating rate of drug biotransformation to therapeutic response has been conducted with drugs that effect “graded,” reversible responses (1, 17), and the generalization that “acceleration or deceleration of drug biotransformation will affect the duration and intensity of action of these drugs” has been made (17). Little attention has been paid to the relationship between rates of biotransformation and therapeutic efficacy of chemotherapeutic or other drugs that are expected to produce “all-or-none” (1) irreversible responses.

The response produced by drugs that produce in all-or-none effect, e.g., tumor cell kill, depends on both concentration of and contact time with the drug. Thus, if the drug itself is active, stimulation of its inactivation should decrease tumor cell kill by decreasing concentration and contact time; depression or inhibition of its inactivation should increase tumor cell kill by increasing concentration and contact time.

Predictions are more difficult if the parent drug is inactive. Since tumor cell kill is a function of drug concentration and contact time, it might be predicted that stimulation, depression, or inhibition of drug activation would have no effect on therapeutic response, provided that (a) absorption rates are not a factor; (b) rates of activation follow 1st-order kinetics; (c) the metabolite is without unique distribution patterns, i.e., more or less distributed in total body water; (d) receptor sensitivity remains unaltered; (e) the drug is not cell-stage specific, i.e., all tumor cells of a given type must be essentially equally sensitive to the drug, and tumor cell kill must be a function of mass-action kinetics; (f) rates of excretion and/or inactivation of the metabolite follow 1st-order kinetics and the kinetic constants describing them remain unaltered; and (g) the active metabolite relative to the parent compound is readily excreted, which is to be expected since microsomal biotransformation typically results in the production of metabolites that are less lipid soluble and more water soluble, enabling them to be excreted more rapidly (17).

Under these conditions, the probability that a given cell will be exposed to the lethal action of the drug remains unchanged. This prediction proved to be the case in previous experiments in that, relative to untreated male rats, a stimulated rate of cyclophosphamide activation in male rats pretreated with phenobarbital as well as depressed rates of cyclophosphamide activation in female rats and in male rats pretreated with cobalt chloride, did not result in quantitatively altered therapeutic or toxic responses to cyclophosphamide administration.

The current experiments demonstrated that cyclophosphamide activation could be inhibited by other drugs which are substrates for hepatic microsomal mixed-function oxidase(s). As expected, inhibition was dose dependent. As before, blood levels of alkylating activity following cyclophosphamide administration i.p. appeared to be governed by 1st-order activation and 1st-order disappearance kinetics, and the altered blood levels of alkylating activity observed after (essentially) concurrent administration of cyclophosphamide and various inhibitors of hepatic microsomal mixed-function oxidase activity can probably be accounted for solely by altered cyclophosphamide activation rates, precluding an effect of these inhibitors on those factors that contribute to kinetic constants describing rates of excretion, inactivation, and/or distribution (3, 14). As predicted, administration of metyrapone (50 µmoles/kg) or SKF 525A (10 µmoles/kg) with cyclophosphamide resulted neither in an
altered therapeutic response, i.e., ED50, nor in an altered toxic response, i.e., leukopenia, to cyclophosphamide. Thus, the therapeutic index describing the therapeutic efficacy of cyclophosphamide as an antineoplastic agent in an experimental tumor system remained unchanged. However, SKF 525A, 50 μmoles/kg, did depress both the therapeutic and toxic responses to cyclophosphamide, although the therapeutic index remained essentially unchanged. An explanation for the unexpected responses may be that, at the dosage level used, cyclophosphamide activation in vivo was sufficiently depressed, such that significantly greater amounts of the parent compound were excreted, violating one of the conditions, mentioned above, necessary to obtain the predicted responses.

It has been hypothesized that the marked differences in cyclophosphamide antitumor activity between species, e.g., mice and humans, and within species, e.g., among persons bearing Burkitt’s lymphoma, have as their basis inter- and intraspecies differences in rates of cyclophosphamide activation. However, in patients bearing Burkitt’s lymphoma, a response or lack thereof could not be correlated with the rate of cyclophosphamide activation (9), and the present and previous (24) experiments demonstrate why, theoretically, such correlations might not be expected. Thus, it seems probable that inter- and intraspecies differences in cyclophosphamide antitumor activity are related to differences in tumor sensitivity, although differences in the spectra of metabolites produced could account for some of the interspecies differences.

Unlikely, then, is the improvement of the therapeutic efficacy of cyclophosphamide in the treatment of human neoplasias by alteration of its rate of activation. However, human hepatic microsomal mixed-function oxidase activity may proceed at a comparatively slow rate relative to that observed in commonly used laboratory animals (3, 6, 9, 18). If a significant percentage of a given dose of the parent compound is excreted unchanged in the “untreated” patient, stimulation of cyclophosphamide activation, e.g., with phenobarbital pretreatment, may result in a greater percentage of the same dose of parent compound converted to active drug, and thus greater cell kill might be achieved, although attempts to do so have been negative (9). However, an improved therapeutic index would be unexpected. An additional factor to consider in extrapolating the data and conclusions reached herein to the management of human neoplastic disease with cyclophosphamide is that in these experiments cyclophosphamide was administered only once. Obviously, the situation is much more complex when the drug is repeatedly administered over a prolonged period of time, as is the case clinically.

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