Protective Role of Thiols in Cyclophosphamide-induced Urotoxicity and Depression of Hepatic Drug Metabolism

Michael J. Berrigan, Anthony J. Marinello, Zlatko Pavlic, Cynthia J. Williams, Robert F. Struck, and Hira L. Gurtoo

ABSTRACT

One of the serious toxicities of cyclophosphamide chemotherapy is urotoxicity. In addition to causing leukopenia, high-dose cyclophosphamide caused both depression of hepatic microsomal enzyme activities and extensive urinary bladder damage, suggesting that a common biochemical mechanism may be responsible for both of these effects. Administration of 180 or 200 mg cyclophosphamide per kg to Wistar rats caused 41 to 67% decrease in aryl hydrocarbon hydroxylase activity, a 21 to 54% decrease in aminopyrine demethylase activity, and a 34 to 40% decrease in cytochrome P-450 content. This dose of cyclophosphamide also caused hematuria as well as necrosis and edema in the urinary bladder. Administration of N-acetylcysteine or sodium-2-mercaptoethane sulfonate (mesna) with cyclophosphamide, while not protecting against leukopenia, protected against the enzymatic inactivation and urotoxicity. The biochemical basis of these observations is discussed. The results suggest that a common metabolite of cyclophosphamide, most probably acrolein, is responsible for both of these undesirable effects of cyclophosphamide therapy. Use of combinations including cyclophosphamide and an appropriate thiol may increase the therapeutic index of this drug.

INTRODUCTION

The alkylating agent, cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide), although effective against a variety of tumors, produces a number of toxicities, including hematopoietic depression, alopecia, nausea, vomiting, hemorrhagic cystitis, and others (6, 29). Cyclophosphamide is also used as an immunosuppressive agent in preparation for organ transplantation procedures and in treatment of disease states thought to be of autoimmune etiology (10–12). Patients more susceptible to the toxic effects of this drug include children and persons receiving high-dose i.v. therapy.

Urotoxic effects of cyclophosphamide can be dose limiting (5, 28) and have proven fatal (33). Extensive hydration of patients receiving cyclophosphamide may alleviate urotoxicity; however, this treatment modality has been criticized as being difficult to properly maintain and may provide only partial protection from urinary bladder damage (2, 6).

Cyclophosphamide, ineffective per se, requires metabolic activation by the microsomal mixed-function oxidase system (31), which results in the formation of the alkylating metabolite, phosphoramid mustard, as well as the olefinic aldehyde, acrolein (Chart 1). Hemorrhagic cystitis was initially thought to be due to alkylating metabolites of cyclophosphamide. However, recent reports have provided indirect evidence implicating acrolein, which is devoid of alkylating activity, in the etiology of the urotoxicity of cyclophosphamide (4, 7).

At high doses, cyclophosphamide also causes denaturation of cytochrome P-450, which is an essential component of the mixed-function oxidase system. Marinello et al. (22) demonstrated that acrolein denatures this enzyme and reported that the in vitro addition of sulfhydryl-containing compounds protected from the loss of cytochrome P-450 activity.

Several reports in the literature have indicated that compounds containing free sulfhydryl groups may protect from the urotoxic effects of cyclophosphamide (2, 8, 32). Presumably a result of pharmacokinetic differences, there are great disparities among the efficacies of sulfhydryl-containing compounds with regard to their protective function against cyclophosphamide-induced bladder injury. N-Acetylcysteine has been reported by several investigators to protect from urological toxicities due to cyclophosphamide (2, 19, 32). An isomer of cyclophosphamide, ifosfamide, has hemorrhagic cystitis as its dose-limiting toxicity. Combined therapy with ifosfamide and N-acetylcysteine in patients with lung cancer resulted in decreased hematuria and such treatment had no effect on tumor response compared to patients receiving only ifosfamide (26).

Sodium-2-mercaptoethane sulfonate (ASTA D-7093, mesna) has also been reported to provide protection against urotoxic side effects of cyclophosphamide (17, 30). In a preliminary report, we demonstrated that N-acetylcysteine, administered at a proper dose and schedule, will provide complete protection from the loss of hepatic mixed-function oxidase activity due to high-dose cyclophosphamide administration to rats (1). The interaction of acrolein with critical sulfhydryl groups in the mixed-function oxidase system has been proposed as a potential mechanism for this suicidal inactivation (15).

This report describes parallel toxic effects of cyclophosphamide administration on the integrity of cytochrome P-450 and the urinary bladder and the protection of these deleterious effects of cyclophosphamide by sulfhydryl-containing chemicals.

MATERIALS AND METHODS

Chemicals. Cyclophosphamide, obtained from Southern Research Institute, Birmingham, Ala., was freshly prepared in 0.9% NaCl solution for each experiment. N-Acetylcysteine, NADP, isocitrate, isocitrate...
The protective role of thiols in cyclophosphamide-induced toxicity was studied in male Wistar rats. The rats were treated with cyclophosphamide (CP) and N-acetylcysteine (NAC), and the effects on mixed-function oxidase (MFO) activity were monitored. The metabolic fate of [4-14C]cyclophosphamide was studied, and the detoxicating role of thiols was assessed through the formation of acrolein. The results indicated that NAC protects against cyclophosphamide toxicity by inhibiting MFO activity and reducing acrolein formation.
evaluate 2 sulfhydryl-containing compounds (N-acetylcysteine and mesnum) for their ability to protect against cyclophosphamide-induced loss of cytochrome P-450. In order to use these chemicals, an appropriate dose of N-acetylcysteine was selected from studies designed to investigate the effect of N-acetylcysteine on some mixed-function oxidase activities, namely, hepatic microsomal aminopyrine demethylase and aryl hydrocarbon hydroxylase activities (Table 1). N-Acetylcysteine was not toxic up to a dose of 400 mg/kg; even at 720 mg/kg, N-acetylcysteine only slightly inhibited aminopyrine demethylase activity and essentially had no effect on the aryl hydrocarbon hydroxylase activity. Therefore, from these and other investigations, a dose of 360 mg/kg, divided into 2 equal parts, was selected for application in studies investigating the protection against cyclophosphamide-induced depression of cytochrome P-450 and the urotoxicity of cyclophosphamide. However, for mesnum, which is relatively nontoxic, a dose equalling one-third (wt/wt) that of cyclophosphamide was selected for application in studies investigating the protection against cyclophosphamide-induced depression of cytochrome P-450 and the urotoxicity of cyclophosphamide. Yet, for mesnum, which is relatively nontoxic, a dose equaling one-third (wt/wt) that of cyclophosphamide was selected from the literature (30).

**Effect of Cyclophosphamide on the Mixed-Function Oxidase System and Protection by Sulfhydryl-containing Compounds.** In repeated experiments, when cyclophosphamide was administered at a relatively high dose (180 or 200 mg/kg), hepatic mixed-function oxidase enzymes showed a marked and consistent loss in activity (Tables 2 to 4). Three methods of measuring the activity of this system were used, aryl hydrocarbon hydroxylase and aminopyrine demethylase activities and cytochrome P-450 content. Both the enzyme activities and the cytochrome P-450 content were decreased by cyclophosphamide treatment; aryl hydrocarbon hydroxylase appeared to be affected to the greatest extent. In 4 different experiments, aryl hydrocarbon hydroxylase activity decreased between 41 and 67% of control values; aminopyrine demethylase activity decreased between 21 and 54%; and cytochrome P-450 content decreased 34 to 40%.

In these experiments, treatment of the rats with N-acetylcysteine alone had no noticeable effect either on the enzyme activities or the cytochrome P-450 content. However, in all 4 experiments, the combination of N-acetylcysteine and cyclophosphamide afforded a marked protection against the cyclophosphamide-induced depression of the mixed-function oxidase system.

We also investigated the possibility that another sulfhydryl-containing compound, mesnum, may offer protection from the depressant effects of cyclophosphamide on the mixed-function oxidase system. Mesnum was administered at a lower dose and by a different time schedule than was N-acetylcysteine, following recommendations by Scheef et al. (30). Mesnum, being ionized at physiological pH, is believed to remain in the extracellular space (3); hence, mesnum was administered i.v. Both aryl hydrocarbon hydroxylase and aminopyrine demethylase activities were protected by mesnum from depression caused by the administration of cyclophosphamide (Table 4). Mesnum given alone showed no effects on the enzymatic activities measured.

### Table 1

<table>
<thead>
<tr>
<th>Total N-Acetylcysteine dose (mg/kg)</th>
<th>Aminopyrine demethylase Activity (% of untreated control)</th>
<th>Aryl hydrocarbon hydroxylase Activity (% of untreated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 2</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>240</td>
<td>97 ± 6</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>480</td>
<td>110 ± 5</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>720</td>
<td>89 ± 8</td>
<td>101 ± 2</td>
</tr>
</tbody>
</table>

a. Activities were calculated as nmol HCHO formed per mg protein per hr.  
b. Activities were calculated as pmol equivalent of 3-hydroxybenzo(a)pyrene per mg protein per 10 min.  
c. Mean ± S.D.  
d. Numbers in parentheses, absolute values of untreated controls.
P-450 reductase activity by 21 and 37%. N-Acetylcysteine treatment had no effect on the reductase activity when given by itself but protected almost completely against the depression caused by cyclophosphamide (Table 5).

Effect of Cyclophosphamide and/or N-Acetylcycteine Treatment on Microsomal Metabolism of [4-14C]Cyclophosphamide. As stated above, administration of high doses of cyclophosphamide results in a marked depression of some microsomal oxidase enzymes activities. This decrease in activity should be reflected by a loss in the capacity to metabolize cyclophosphamide, a mixed-function oxidase substrate, in an in vitro system using hepatic microsomes derived from rats pretreated with cyclophosphamide. Hepatic microsomes prepared from rats treated with cyclophosphamide alone (200 mg/kg) had lost their ability to generate protein-binding metabolite(s) by 32%, compared to microsomes from the control group (Table 6). Microsomes from rats treated with both cyclophosphamide (200 mg/kg) and N-acetylcysteine (400 mg/kg) did not differ from control microsomes in their ability to generate protein-binding metabolite(s). Likewise, microsomes from rats treated only with N-acetylcysteine did not differ from control values.

Effect of Sulfhydryl-containing Compounds on Cyclophosphamide-induced Urotoxicity. Leukopenia is an important consequence of cyclophosphamide administration. At the dose level of cyclophosphamide used in these experiments, the total WBC was drastically lowered (Table 7). N-Acetylcysteine by itself showed no effect on the total WBC. No protection from cyclophosphamide-induced leukopenia was observed when N-acetylcysteine was added to the treatment regimen. Combined treatment with mesnum and cyclophosphamide likewise caused no decrease in the leukopenia observed after treatment with cyclophosphamide alone.

Because cyclophosphamide may also cause deformation of WBC which could conceivably cause spurious results when assayed using a Coulter Counter, manual counting of WBC was routinely performed. Both methods gave similar cell counts.

Effect of Sulfhydryl-containing Compounds on Cyclophosphamide-induced Urotoxicity. Histopathology studies of the urinary bladders from rats treated with cyclophosphamide (180 or 200 mg/kg) showed hemorrhagic lesions in the bladder mucosa. The bladders were thickened and spongy. Edema was prominent in perivesical tissues. In ulcerated areas of the bladder, the epithelium was necrotic, and a bloody exudate was seen in the lumen, containing cellular debris, fibrin, and inflammatory cells. Where the mucosa was intact, the epithelium showed various focal changes such as thinning, atypia, and karyorrhexis. Edema and hemorrhage were uniform and diffuse throughout the submucosa and focal in all other bladder tissues (Fig. 1B).

Urinary bladders from rats treated with either N-acetylcysteine or mesnum alone showed no observable pathological changes. Bladders from rats (4 rats/group) receiving both cyclophosphamide and N-acetylcysteine showed, in most cases, no microscopic changes. In one bladder from this group there was evidence of minimal inter- and intracellular edema with slight necrosis (Fig. 1C).

Combination of mesnum with cyclophosphamide allowed for complete reversal of the damage seen in bladders of rats treated with cyclophosphamide (Fig. 1D).

**DISCUSSION**

Cyclophosphamide was originally synthesized because of the erroneous belief that tumor cells, containing phosphoramide with greater activity than that of normal host cells, would allow for a greater degree of activation of cyclophosphamide to cytotoxic metabolites. Although it is now recognized that the selectivity of cyclophosphamide cannot be explained on this basis, its utility as an important anticancer agent is witnessed by its wide use.

It is now known that cyclophosphamide is activated by the mixed-function oxidase system (9, 16). An interesting and clinically relevant aspect of this relationship is that the active metabolites generated by the mixed-function oxidase system may in turn destroy the enzymes responsible for their creation. We provide evidence here supporting preliminary reports (1, 13) that cyclophosphamide administration, at high doses, will depress the activity of the mixed-function oxidase system.

The co-administration of an appropriate thiol compound (N-acetylcysteine, mesnum, or perhaps others) in cyclophospha-
mide therapy effectively blocks mixed-function oxidase destruction. This may have important clinical ramifications, in that higher and/or repeated doses of cyclophosphamide may be given. Furthermore, as this enzyme system is responsible for the metabolism of a wide variety of drugs (21), alterations in the pharmacokinetics of other agents given concurrently with cyclophosphamide may be minimized.

NAPDH-cytochrome P-450 reductase (EC 1.6.2.4) is involved in the transfer of electrons from NADPH to electron acceptors, especially cytochrome P-450, and we have recently demonstrated that cyclophosphamide causes a dose-dependent decrease in hepatic cytochrome P-450 reductase activity (22). This series of experiments displays a readily apparent similarity between the destructive effect of cyclophosphamide on mixed-function oxidase activity and on NAPDH-cytochrome P-450 reductase. Additionally, both activities can be protected from destruction by the inclusion of N-acetylcysteine or mesna into the treatment protocol. The fact that mesna acts in such a manner (providing almost complete protection against microsomal enzyme inactivation) does not support the proposed hypothesis (3) that it will not enter cells, especially hepatic cells.

As discussed above, the urotoxic effects of cyclophosphamide often limit its usefulness. Such toxic results were strikingly manifest in rats treated with cyclophosphamide alone. Gross inspection showed an obvious hematuria and the histopathological studies revealed numerous lesions of the urinary bladder. The histopathological studies demonstrate that combined therapy of cyclophosphamide with an appropriate thiol compound may obviate cyclophosphamide urotoxicity in such a manner that the therapeutic index of cyclophosphamide could be raised.

The parallelism that exists between the effects of cyclophosphamide on hepatic microsomal enzymes and the urinary bladder, combined with the fact that both destructive events can be prevented by a thiol-containing compound, suggests that a similar mechanism may be involved. The metabolite of cyclophosphamide thought to be responsible for its antitumor effect is phosphoramide mustard. Activation of cyclophosphamide releases this moiety, and other metabolites are, of course, generated. One of these metabolites is the electrophilic aldehyde, acrolein. Acrolein has been implicated as the metabolite responsible for hemorrhagic cystitis (4, 7), and there is evidence that acrolein does not participate in the cytotoxic action of cyclophosphamide (34). A report from this laboratory has implicated acrolein as the metabolite responsible for destruction of hepatic microsomal enzymes (23). Studies using various nucleophiles as competitive inhibitors suggest that acrolein interacts with cysteine sulfhydryl groups in these proteins (15). Experiments reported herein are consistent with these observations. It appears that acrolein, a toxic metabolite that apparently has no antitumor activity, may be responsible for both the hemorrhagic cystitis and the destruction of microsomal enzymes seen after cyclophosphamide therapy.

The inability of thiols to protect against myelosuppression suggests that cyclophosphamide-induced myelosuppression is due to metabolites other than acrolein, presumably phosphoramide mustard, which is the proposed alkylating moiety of cyclophosphamide (10). This suggestion is reinforced by several observations demonstrating that a number of alkylating agents are myelosuppressive. Since phosphoramide mustard is also believed to be the ultimate cytotoxic metabolite of cyclophosphamide, our present data would suggest that the cytotoxic effects of cyclophosphamide will most likely be retained in the presence of thiols. Combinations of N-acetylcysteine (or cysteine) and cyclophosphamide have been shown not to interfere with the cytotoxic effects of cyclophosphamide against Walker 256 carcinosarcoma (14, 18). Likewise, the chemotherapeutic effects of ifosfamide against L1210 leukemia were not compromised by cotreatment with N-acetylcysteine (31). Additionally, the urotoxic effects of cyclophosphamide or ifosfamide have been reported to be diminished in humans who also received mesna or N-acetylcysteine (26, 30).

Thus, it appears that a distinct metabolite of cyclophosphamide, acrolein, is responsible for at least 2 types of cyclophosphamide-related toxicities. Appropriate thiol compounds effectively block cyclophosphamide-induced destruction of the mixed-function oxidase system as well as its urotoxic effects. Evidence exists in the literature suggesting that such combinations still retain the desired cytotoxic effects of activated cyclophosphamide. This report corroborates these observations and provides further insight into the mechanism of cyclophosphamide toxicity and how it can be minimized.

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Fig. 1. A, untreated rat urinary bladder. Figures prepared from bladders taken from rats treated with N-acetylcysteine (360 mg/kg) or mesnun (67 mg/kg) alone showed no histopathological changes (not shown). H & E, x 400. B, rat urinary bladder 4 days after treatment with 200 mg cyclophosphamide per kg. Note severe necrosis, edema, and karyorrhexis. The lumen contains cellular debris, fibrin, and inflammatory cells. H & E, x 400. C, rat urinary bladder 4 days following treatment with both cyclophosphamide (180 mg/kg) and N-acetylcysteine (360 mg/kg), showing mild inter- and intracellular edema and slight necrosis in the epithelium. Figures prepared from other rats receiving identical drug treatment showed no histopathological changes (not shown). H × E, x 400. D, rat urinary bladder 4 days after treatment with cyclophosphamide (200 mg/kg) and mesnun (67 mg/kg), demonstrating complete reversal of damage seen in bladders from rats treated with cyclophosphamide alone. H & E, x 200.
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