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# Prevention of cyclophosphamide-induced urotoxicity by reduced glutathione and its effect on acute toxicity and antitumor activity of the alkylating agent

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***Summary.*** *The effect of reduced glutathione on acute lethal toxicity and urotoxicity induced by cyclophosphamide was studied on both mice and rats. The results of this investigation indicate that reduced glutathione is an effective protective agent against bladder damage from treatment with the alkylating agent. The timing of glutathione administration (IV) with respect to cyclophosphamide treatment influenced the uroprotective efficacy of the thiol compound. A schedule-dependent protective effect of glutathione against acute lethal toxicity of the antitumor drug was also observed. This partial protection was accompanied by a reduction in body weight loss following cyclophosphamide treatment. The therapeutic activity of cyclophosphamide on two experimental tumor systems (L1210 and Gross leukemia) was not impaired by combined treatment with glutathione, even at a relatively high dose of glutathione compared with cyclophosphamide.*

## Introduction

Cyclophosphamide (CTX) has a relevant role in the chemotherapeutic treatment of malignant diseases [13]. Urotoxicity is a specific side effect of alkylating agents characterized by the oxazaphosphorine ring [6]. Urotoxic damage is due to the elimination of activated metabolites (particularly acrolein) [14].

In view of the generally recognized difficulty of obtaining new drugs with increased efficacy, it is of relevant importance to enhance the selective toxicity of available antitumor agents against tumor cells by reducing toxic side effects. Protection against toxic effects could allow an increase in drug dose with potential therapeutic advantages.

The protective role that thiol compounds have against toxic effects of alkylating agents has long been recognized [12]. In particular, sulfhydryl compounds have been shown to prevent CTX-induced bladder damage [3, 5, 7, 14]. Since a relevant role of the endogenous tripeptide thiol, glutathione (GSH), in the metabolism-dependent toxicity of CTX has been documented [17], this study was undertaken to determine whether administration of GSH (reduced form) influences bladder damage induced by CTX treatment and the acute lethal toxicity of this alkylating drug. It is well known that GSH has a general role in the inactivation of a number of reactive drugs or drug metabolites [9, 20]. It has recently been reported that GSH provides protection against *cis*-dichlorodiammineplatinum (II) nephrotoxicity without interfering with the chemotherapeutic activity of the antitumor drug [26].

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## Material and methods

*Drugs.* CTX, a product of Asta-Weike AG, was kindly provided by Dr L. Ottolenghi (Berlifarm, Schering SpA, Milan). GSH (reduced form) was a product of Boehringer Biochemica Robin (Milan, Italy). GSH and CTX were freshly dissolved in distilled water. The drugs were injected in a volume of 10 mg/kg body weight.

*Animals.* For antitumor activity experiments, female $BDF_1$ and male C3H/He mice from Charles River Laboratories (Calco, Italy) were used. Toxicity experiments were carried out in male Swiss mice (20–30 g) and male Sprague-Dawley rats (220–240 g) obtained from Nossan (Milan, Italy). The animals were housed in a constant temperature and humidity environment (21° C). Food and water were provided ad libitum.

*Acute toxicity studies.* Swiss mice and Sprague-Dawley rats were divided into experimental groups of 10 animals and were injected IP with CTX at various doses. GSH or solvent solution was administered IV according to different treatment schedules as indicated. In the multiple-treatment experiments the cumulative dose of GSH was identical with that used in the single-treatment experiments. GSH dosage (200 and 500 mg/kg) and treatment schedules were selected after preliminary examination of the protective effect of GSH on the toxicity of a single dose of CTX. The mortality rate of the animals was observed for 21 days, during which the survivors were observed daily. The $LD_{50}$ values were calculated by regression analysis after logit transformation [1].

*Urotoxicity tests.* Groups of 10 Swiss mice received CTX (100, 200, and 400 mg/kg) IP alone or in combination with GSH (100, 250, and 500 mg/kg) administered IV 30 min before and after CTX, unless otherwise indicated. Thus, in these experiments, the cumulative doses of GSH were 200, 500, and 1,000 mg/kg. This treatment schedule was selected on the basis of the protective efficacy against acute lethal toxicity of the alkylating agent. Mice that did not receive GSH were injected, by the same route and schedule of administration, with the same volume of solvent solution. Control animals were treated with identical volumes of solvent solution for each drug. The animals were weighed daily and sacrificed 48 h after treatment.

The assay of bladder toxicity, based on the drug-induced increase in bladder weight [6], was essentially similar to that described by Hacker et al. [18].

The experimental design for evaluation of bladder toxicity in Sprague-Dawley rats was similar to that described above, except that the dosage levels of 400 mg/kg CTX and 1,000 mg/kg GSH were omitted. In addition to the measurement of bladder weight as a criterion of urotoxic damage, CTX-induced injury to the rat bladder was also evaluated by gross assessment (essentially swelling) as described by Brock et al. [6]. Briefly, the extent of swelling was assessed macroscopically and was recorded on a four-point scoring scale, i.e., 0 = no swelling; 1 = slight; 2 = moderate; 3 = severe. The scores recorded for the individual rats of each test group were averaged. Thus, results are presented as mean scores of bladder inflammation ranging between 0 and 3.

The influence of the timing of GSH administration with respect to CTX treatment was examined using a single dose of GSH (500 mg/kg, IV) at various times before and after 140 and 200 mg/kg (IP) CTX for rats and mice, respectively.

*Tumor models and antitumor activity experiments.* To determine the effect of GSH pretreatment on the therapeutic efficacy of CTX, the following experimental models and treatment schedules were used. (a) Experiments with L1210 leukemia were initiated by inoculating female $BDF_1$ mice IP with $10^5$ leukemia cells. CTX was administered IP to tumor-bearing mice in a volume of 10 ml/kg body weight on day 1 after tumor cell transplantation. GSH was administered IV, using different treatment schedules as indicated. (b) The experiments with transplantable Gross leukemia were carried out in female C3H/He mice inoculated IV with $1 \times 10^6$ cells/mouse. Drug treatments consisted of IV injections of CTX given on days 1 and 4 at 30 min after IV administration of GSH.

Each of the experimental groups consisted of ten mice, which were observed daily. Antitumor activity was determined on the basis of two parameters: (a) The fraction of long-term survivors (> 60 days) (cured mice); and (b) % T/C, defined as the median survival time (MST) of dying mice only in the drug-treated group (T), divided by the MST of the untreated control group (C), × 100.

## Results

### *Interference of GSH on acute lethal toxicity of CTX*

The effect of GSH on the acute lethal toxicity of CTX was studied at various dose levels of both antitumor agent and thiol compound. CTX doses were chosen in the range of toxic doses to allow determination of $LD_{50}$ values. The schedule-dependency of the protective effect of GSH on the lethal toxicity of CTX in Swiss mice is shown in Table 1. Whereas a single IV administration of GSH 30 min before or 30 min after CTX treatment provided only marginal protection, multiple GSH doses (30 min before and 30 min after, using the same cumulative dose of GSH given in the single-dose experiments) afforded significant protection (Fig. 1). Indeed, reduction of CTX acute lethal toxicity by GSH is reflected in a consistent (aroung 50%) increase in $LD_{50}$ in separate experiments. A

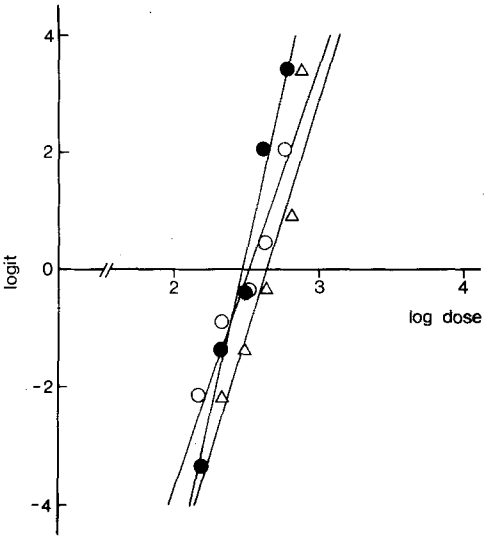


**Fig. 1.** Dose-response relationship for the lethal toxicity of CTX in mice. (●) CTX alone; (○) CTX plus GSH ( 2 × 100 mg/kg); ( △ ) CTX plus GSG ( 2 × 250 mg/kg). CTX was administered IP as a single dose. GSH was administered IV as two fractionated injections 30 min before and 30 min after CTX. The mortality rate was observed for 21 days. The data were analyzed by logit transformation of the response

**Table 1.** Influence of treatment schedule on the protective effect of GSH in acute lethal toxicity of CTX in Swiss mice[a]

| Experiment | GSH dose (mg/kg)[b] | Timing of GSH administration relative to CTX | $LD_{50}$ (mg/kg)[c] |
|---|---|---|---|
| 1 | Solvent only | – | 362 (304–432) |
| | 500 | 30 min before | 380 (309–468) |
| | 500 | 30 min after | 408 (342–487) |
| 2 | Solvent only | – | 299 (256–349) |
| | 2 × 100 | 30 min before | 318 (257–393) |
| | 2 × 250 | and 30 min after | 431 (356–522)* |
| 3 | Solvent only | – | 349 (296–412) |
| | 2 × 250 | 30 min before and 30 min after | 492 (416–624)* |

[a] CTX treatment IP

[b] GSH was administered IV as a single (experiment 1) or fractionated (experiments 2 and 3) dose

[c] The mortality rate was observed for 21 days. $LD_{50}$ values were determined by regression analysis after logit transformation as indicated in Fig. 1. In parentheses, 95% confidence limits

* Significant difference from CTX alone ($P < 0.05$)

**Table 2.** Influence of treatment schedule on the protective effect of GSH on acute lethal toxicity of CTX in Sprague-Dawley rats[a]

| Experiment | GSH dose (mg/kg)[b] | Timing of GSH administration relative to CTX | $LD_{50}$ (mg/kg)[c] |
|---|---|---|---|
| 1 | Solvent only | – | 146 (122–177) |
| | 200 | 30 min before | 189 (164–218) |
| | 500 | | 177 (156–202) |
| 2 | Solvent only | – | 129 (100–168) |
| | 2 × 100 | 30 min before | 212 (180–248)* |
| | 2 × 250 | and 30 min after | 240 (202–283)* |
| 3 | Solvent only | – | 155 (126–190) |
| | 500 | 30 min before | 189 (144–248) |
| | 500 | 30 min after | 215 (150–310) |

[a] CTX treatment IP
[b] GSH was adminstered IV as a single (experiments 1 and 3) or fractionated (experiment 2) dose
[c] The mortality rate was observed for 21 days. $LD_{50}$ were determined by regression analysis after logit transformation. In parentheses, 95% confidence limits
* Significant difference from CTX alone ($P < 0.05$)

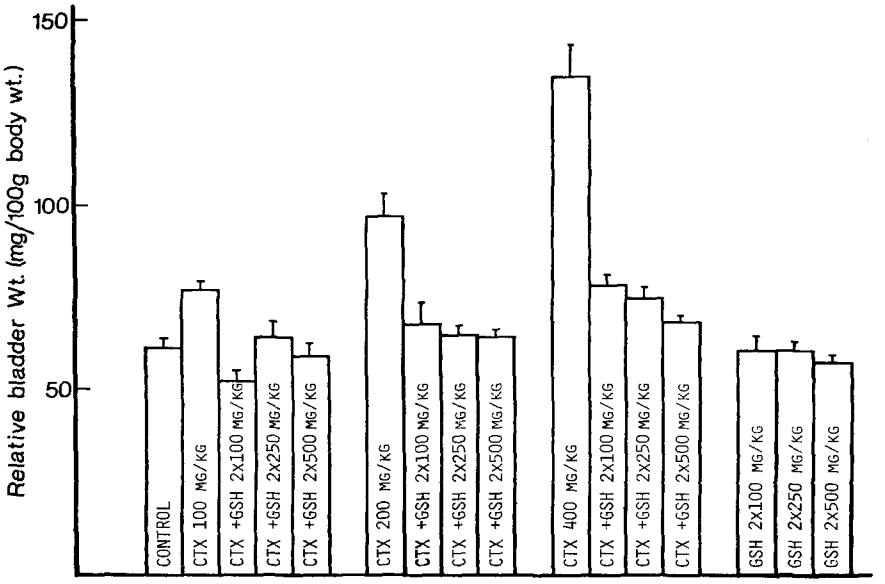


**Fig. 2.** Effect of GSH on CTX-induced increase in bladder weight of male Swiss mice. CTX was administered IP as a single dose; 30 min before and 30 min after CTX, animals received IV injections of GSH. Mice were killed 48 h later; bladders were removed and wet weights were measured. In control (untreated) mice, the average wet weight of the bladder was 15.5 mg. *Bars*, standard error

**Table 3.** Effect of GSH on CTX-induced bladder toxicity and weight loss in Sprague-Dawley rats

| Drug treatment[a] | | Relativ bladder wet weight[b] (mg/100 body wt) | Score[c] | Body weight change[b] (%) |
|---|---|---|---|---|
| CTX (mg/kg) | GSH (mg/kg) | | | |
| Control[d] | – | 30.4 ± 1.6 | 0.2 | + 4.4 |
| 100 | – | 67.9 ± 4.9 | 2.0 | – 5.7 |
| 200 | – | 79.2 ± 5.3 | 2.5 | – 11.1 |
| | 2 × 100 | 30.5 ± 2.0 | 0.1 | + 3.5 |
| 100 | 2 × 100 | 49.2 ± 4.1 | 0.7 | – 1.2 |
| 200 | 2 × 100 | 48.8 ± 2.5 | 1.6 | – 10.4 |
| | 2 × 250 | 28.3 ± 1.3 | 0.1 | + 5.0 |
| 100 | 2 × 250 | 34.3 ± 1.8 | 0.1 | – 1.0 |
| 200 | 2 × 250 | 42.2 ± 2.8 | 0.3 | – 5.0 |

[a] CTX was administered IP as a single dose. Fractionated IV administration was used for GSH (30 min before and 30 min after CTX treatment)
[b] Bladder wet weights and body weights were measured 48 h after CTX administration. In control (untreated) animals the average wet weight of the bladder was 69.6 ± 3.8 mg
[c] Mean scores of gross assessment (6)
[d] Solvents only

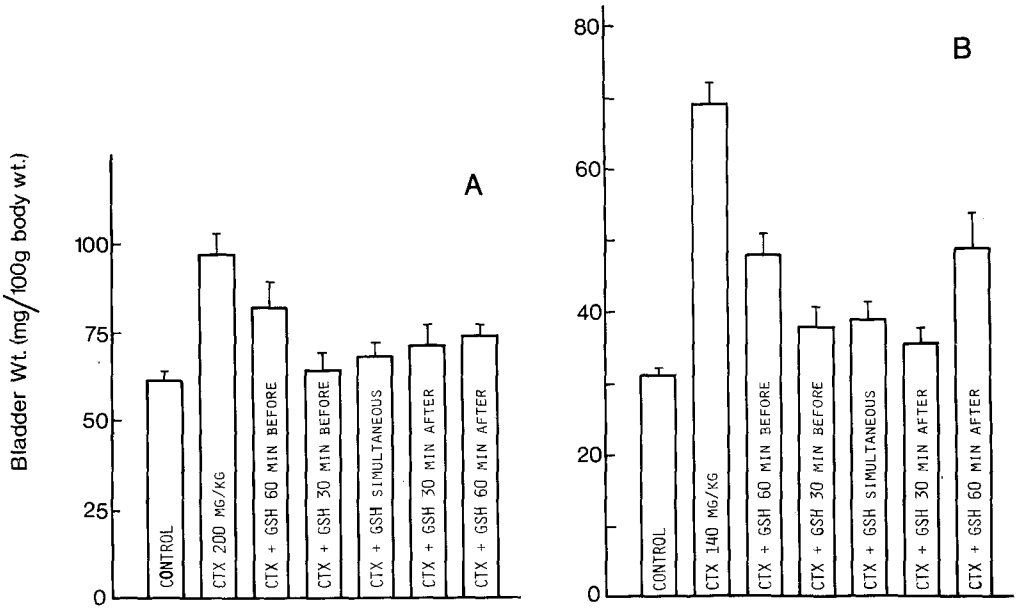


**Fig. 3A, B.** Influence of timing of GSH administration relative to CTX treatment on the protective effect against CTX-induced bladder toxicity. CTX was administered IP as a single dose. GSH was administered IV as a single dose (500 mg/kg) at indicated times with respect to CTX. Bladder weights were measured 48 h after CTX administration. **A** Swiss mice; **B** Sprague-Dawley rats. *Bars,* standard error

**Table 4.** Influence of GSH on the therapeutic activity of CTX on L1210 and Gross leukemia

| Tumor model | Dose and timing of GSH IV administration | CTX dose[a] (mg/kg) | T/C[b] (%) | LTS[c] (> 60 days) | No. of toxic deaths/no. of dead mice[d] |
|---|---|---|---|---|---|
| L1210 | | 100 | 144 | 1/10 | 0/9 |
| | No GSH | 150 | 155 | 3/10 | 2/7 |
| | | 225 | 77 | 7/10 | 3/3 |
| | 500 mg/kg, | 100 | 150 | 0/10 | 0/10 |
| | 30 min before CTX | 150 | 222 | 3/10 | 0/7 |
| | | 225 | 205 | 6/10 | 1/4 |
| | 500 mg/kg, | 100 | 144 | 0/10 | 0/10 |
| | 30 min after CTX | 150 | 166 | 2/10 | 2/8 |
| | | 225 | 194 | 8/10 | 2/2 |
| | 2 × 250 mg/kg, | 100 | 144 | 0/10 | 0/10 |
| | 30 min before and | 150 | 166 | 5/10 | 0/5 |
| | 30 min after CTX | 225 | 361 | 8/10 | 1/2 |
| Gross | No GSH | 2 × 70 | 257 | 2/10 | 0/8 |
| | 500 mg/kg, 30 min before CTX | 2 × 70 | 243 | 1/10 | 0/9 |

[a] L1210 leukemia: CTX given IP on day 1 after tumor transplantation. Gross leukemia: CTX given IV on days 1 and 4 after tumor transplantation

[b] The median survival times (MST) of untreated control mice were 9 and 7 days for L1210 and Gross leukemia, respectively. T/C (%) values are for dying mice only. See *Materials and methods* for details

[c] Number of long-term survivors (> 60 days)/number of treated mice

[d] Deaths that occured in treated animals before the first death of a tumor-bearing untreated animal were attributed to the toxicity of CTX

similar schedule-dependency of the relative protective effect of GSH was also found in the rat (Table 2).

*Effect of GSH on CTX-induced urotoxicity*

CTX caused a marked increase in bladder weight measured 48 h after drug administration at doses of 100 mg/kg or higher [13]. With the same treatment schedule as was effective in protection against acute lethal toxicity (Fig. 1), GSH prevented the CTX-induced increase of bladder weight in Swiss mice at all doses of the antitumor agent tested (Fig. 2). Doses of GSH as low as 200 mg/kg afforded nearly complete protection. GSH provided a protective effect also in Sprague-Dawley rats (Table 3). The uroprotection was also accompanied by a reduction in body weight loss.

Since the uroprotection efficacy of other thiol compounds is critically dependent on the timing of thiol administration [18], further experiments were designed to determine whether

a similar schedule-dependence also occurred for the uroprotective effect of GSH. Figure 3 shows that GSH was effective in both mouse and rat if administered within 30 min before or after CTX. Administration of a single dose of GSH earlier, or later, diminished the protective efficacy. In contrast to the schedule-dependent effect of GSH on CTX-induced mortality, a single dose of GSH seemed to be effective in reducing bladder toxicity.

*Effect of GSH on the antitumor activity of CTX*

The effect of GSH on the chemotherapeutic activity of CTX was examined in two experimental models. The results are summarized in Table 4. In the early ascitic L1210 leukemia, a dose range from 100 to 225 mg/kg of CTX was tested with IP administration of a single dose on day 1 after the transplantation of tumor cells. At the lowest CTX dose of 100 mg/kg, 500 mg/kg GSH did not appreciably influence the therapeutic effectiveness of CTX with any of the treatment schedules. However, at the higher dosages of CTX (150–225 mg/kg), the combination was more effective in increasing the survival of tumor-bearing animals than CTX alone. The therapeutic advantage of the combination was more marked for the fractionated dose of GSH. Protection against host toxicity may account for the increase in survival in the high dose range of the antitumor agent, since fewer toxic deaths occurred in groups receiving the combination treatment with GSH. However, the curative efficacy (i.e., occurrence of long-term survivors) of the antitumor agent appeared unchanged by GSH treatment.

Again, when an intermittent treatment schedule was used, the antitumor effect of CTX on Gross leukemia-bearing mice was not appreciably impaired by GSH administered at a relatively high dosage (500 mg/kg) 30 min before each CTX treatment. GSH alone had no antitumor effect (data not shown) in any of the tumor models used for these studies.

## Discussion

The major problem in cancer chemotherapy is still the lack of selectivity of the available drugs. A promising approach to enhancement of the selective toxicity of anticancer agents against tumor cells is represented by pharmacological attempts to reduce side effects (with particular reference to dose limiting) without interfering with antitumor properties of the cytotoxic agents [12]. Since this approach may require administration of an antidote, the detoxifying interaction between the protective agent and the cytotoxic drug could also produce a partial loss of antitumor activity. Thus, the possibility of preventing toxic side manifestations without any adverse influence on therapeutic effectiveness occurs in two main situations: (a) When differences in the pharmacokinetic behavior between antitumor drug and protective agent are expected to afford a regional detoxification to prevent specific organotoxicity; and (b) when different mechanisms are responsible for antitumor effect and some organ-specific damage.

Hemorrhagic cystitis, which is produced by CTX and related compounds [7], is an example of the latter situation. Indeed, metabolic products of CTX, including acrolein, which is characterized by weak cytotoxic properties, and chloroacetaldehyde have been implicated in this characteristic toxic effect [10, 11, 14, 23], which is a limiting factor in the therapeutic use of the drug, particularly in high-dose chemotherapy regimens [24, 25]. Appropriate sulfhydryl-containing compounds have been reported to provide effective protection against CTX-induced urotoxic effects, probably through deactivation of toxic metabolites, without interfering with the chemotherapeutic activity of the antitumor drug [3, 8]. In the mechanism of the protective action of these thiol compounds, a central role of endogenous GSH has been generally recognized [17, 21]. In particular, a preferential interaction of the endogenous GSH with the electrophilic metabolite, acrolein, has been proposed as a mechanism for protection against some toxic effects of CTX [17]. Our results, which show that exogenously administered GSH is an effective antidode in preventing urotoxic lesions of CTX in two animal models, are consistent with the suggestion that GSH has an important role in the toxicity of this alkylating agent [17], presumably via alkylation of reactive metabolites. Like other thiol compounds, the nucleophilic structure of GSH would enable it to form an adduct with electrophilic drug metabolites [20]; thus, the uroprotective potential of GSH is not surprising.

However, the pharmacological use of GSH as a protective agent against toxicity of alkylating drugs has given disappointing results owing to the lack of protection of exogenous GSH against toxicity of nitrogen mustards [4]. The inability of intact GSH to be taken up by most cells [22] may account, at least in part, for the failure of GSH to protect against highly reactive compounds that exert a direct alkylating action. Indeed, the protection of other sulfhydryl compounds against toxicity of nitrogen mustards has been ascribed to intracellular reaction between thiol and alkylating drug [2, 12].

In contrast, the pharmacokinetic characteristics of GSH may play an important role in the protective action against metabolism-dependent toxicity of CTX. A rapid accumulation of IV-administered GSH in the kidney has been reported [19]. Thus, in the case of oxazaphosphorine mustards, the detoxifying interaction of GSH with the urotoxic metabolite(s) could occur extracellularly (i.e., in kidney tubular fluid). A similar pharmacokinetic mechanism has been reported for the uroprotective efficacy of mesna [21], which has been proposed for selective regional detoxification [5].

The pharmacokinetic features of GSH may represent an advantage over other thiol compounds used as protective agents, since the rapid removal of the tripeptide from plasma [19] enables the administration of large doses without impairment of the therapeutic efficacy of the antitumor agent. As already reported for the combination of GSH and *cis*-dichlorodiammineplatinum (II) [26], the antitumor effects of CTX were not reduced by administration of relatively high doses of GSH. The lack of impairment of antitumor activity of CTX by GSH is in agreement with similar observations showing that other exogenous thiols did not interfere with the antitumor activity of CTX [8, 16, 17]. In addition, GSH is a relatively nontoxic compound, and thus fulfills some essential requirements of effective protective agents [12].

A partial protection of GSH against acute lethal toxicity of CTX was observed only with fractionated administration of the thiol tripeptide (Tables 1 and 2). A plausible explanation for this schedule-dependent protection of GSH against acute lethal toxicity of CTX is its metabolic and pharmacokinetic behavior [19]. The rapid decline in plasma levels of IV-administered GSH [19] may account for the schedule-dependent effect. However, fractionated administration was not necessary for an uroprotective action (Fig. 3). Relevant to this point is the observation that acute lethal toxicity of oxazaphosphorines was unchanged by combination treatment with mesna, an

effective uroprotective agent [8]. Thus, urotoxic damage does not seem to be a toxic effect contributing to mortality following CTX treatment. The mechanism of the schedule-dependent protective effect against acute lethal toxicity of CTX remains unclear. Other toxic events produced by reactive metabolites of CTX [15] might also be alleviated with multiple GSH treatments.

An important role of GSH in the protection against some toxic effects of CTX, especially those linked to the generation of toxic metabolites, has been proposed [17]. The toxicity of CTX reflected by body weight loss in treated rats was somewhat reversed by fractionated administration of GSH (Table 3). Preliminary observations (not shown) indicated that GSH partially protected Sprague-Dawley rats against CTX-induced leukopenia and enhanced the recovery of peripheral blood leukocytes. This finding is of particular interest, since other thiol-containing uroprotective agents, *N*-acetylcysteine and mesna [3], failed to protect against CTX-induced leukopenia.

The protective efficacy of GSH may be relevant in clinical cancer chemotherapy. Indeed, GSH might have a potentially useful role as an adjunct to treatment with alkylating agents of the oxazaphosphorine group: this tripeptide is not toxic after IV administration of relatively high doses in a variety of species; it has been shown not to interfere with antitumor activity of different antitumor agents, including *cis*-platinum [26] and doxorubicin (not shown); and it may have potential advantages over other thiol compounds.

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