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Effect of Disulfiram (Tetraethylthiuram Disulfide) and Diethyldithiocarbamate on the Bladder Toxicity and Antitumor Activity of Cyclophosphamide in Mice[1]

Miles P. Hacker,[2] William B. Ershler, Robert A. Newman, and Richard L. Gamelli

Departments of Pharmacology [M. P. H., R. A. N.], Medicine [W. B. E., R. A. N.], and Surgery [R. L. G.], Vermont Regional Cancer Center, College of Medicine, University of Vermont, Burlington, Vermont 05405

ABSTRACT

Cyclophosphamide is the most commonly prescribed alkylating agent in clinical medicine. The usefulness of cyclophosphamide is often limited, however, by its propensity to cause hemorrhagic cystitis especially in children or patients receiving concomitant radiotherapy. Administration i.p. of cyclophosphamide at doses of 100 mg/kg or more to mice produced a significant increase in urinary bladder weight within 48 hr of treatment. The present studies demonstrate that disulfiram prevented cyclophosphamide-induced bladder damage when administered p.o. within 1 hr of cyclophosphamide treatment. Diethyldithiocarbamate, a sulfhydryl-containing metabolite of disulfiram, had identical uroprotective activity. Unlike disulfiram, diethyldithiocarbamate was effective only when administered 2 to 4 hr after cyclophosphamide. Disulfiram augmented slightly the antitumor activity of cyclophosphamide against L1210 murine leukemia *in vivo* when administered 30 min prior to cyclophosphamide. In contrast, diethyldithiocarbamate had no effect on the antitumor activity of cyclophosphamide when administered 4 hr after cyclophosphamide.

INTRODUCTION

CTX[3] is the most commonly prescribed alkylating agent in clinical medicine. Unlike most other alkylating agents, CTX requires metabolic activation to attain significant cytotoxic and alkylating activity. The proposed pathways of CTX metabolism have been investigated extensively and have been the subject of several review articles (2, 12, 20, 27). Initially, CTX is hydroxylated by the hepatic mixed-function oxidase system to 4-hydroxycyclophosphamide, which is in equilibrium with its open-ring tautomer aldophosphamide. At this stage, aldophosphamide can either undergo further metabolism by aldehyde dehydrogenase to noncytotoxic moieties, such as carboxyphosphamide, 4-ketophosphamide, or alcophosphamide, or it can undergo hydrolysis to the toxic moieties phosphoramide mustard and acrolein. Phosphoramide mustard can then be further hydrolyzed to bis(2-chlorethyl)amine, yet another cytotoxic metabolite.

An important dose-limiting toxicity encountered in 10 to 40% of patients receiving CTX therapy is hemorrhagic cystitis (31). This toxicity is most prevalent in children, patients receiving concomitant radiotherapy or high-dose CTX for bone marrow transplantation. The causative agent of this untoward effect has recently been identified to be acrolein (3, 6). Cox (6) has suggested that acrolein formation occurs within the urinary bladder primarily because acrolein has an extremely short biological half-life but also because acrolein administered by injection is rapidly converted by the liver to 3-hydroxypropylmercapturic acid, a nonreactive metabolite.

A number of techniques, including high-volume diuresis (29), surgical intervention (14), and chemical cauterization (9, 23), have been used to treat CTX-induced cystitis. More recently, the parenteral administration of certain sulfhydryl-containing compounds such as NAC (28), 2-mercaptoethanesulfonate, mesnum (22), and dimercaptosuccinic acid (7) have been reported to prevent hemorrhagic cystitis.

DSF (tetraethylthiuram disulfide) has been reported to decrease the toxicity of several alkylating agents (15) and to inhibit the carcinogenicity of various *N*-nitroso compounds (16, 21). Inasmuch as DSF is rapidly hydrolyzed *in vivo* to DDTC, a sulfhydryl-containing compound, and is excreted in the urine primarily as a DDTC or as a DDTC conjugate, we proposed that DSF, or its metabolite DDTC, could also prevent CTX-induced cystitis in a manner similar to that of NAC or mesnum. The present report describes the effect of DSF and DDTC on the toxicological and oncolytic activities of CTX in mice. Portions of this work have been reported previously in preliminary communications (17, 18).

MATERIALS AND METHODS

Clinical-formulation CTX was purchased from Mead Johnson Co., Evansville, Ind. DSF and DDTC were obtained from Sigma Chemical Co. (St. Louis, Mo.). Male albino CD mice (20 to 25 g) were purchased from Canadian Breeders Laboratory, Montreal, Quebec, Canada. Male C57BL/6 × DBA/2 F_1 mice (hereafter referred to as $BD2F_1$) mice, weighing 18 to 22 g, were obtained from The Jackson Laboratory, Bar Harbor, Maine. Animals were housed in our central animal facilities, which maintain an environment of controlled temperature, relative humidity, and 12-hr light and 12-hr dark cycles. Food and water were available *ad libitum*.

The assay of CTX-induced urinary bladder toxicity has been described previously (18). Briefly, CD mice were given CTX i.p. and killed by cervical dislocation 48 hr later. The bladders were excised immediately, and the residual urine was expressed by mild external pressure. The bladders were then blotted dry, and wet weight was measured. Bladder weights were expressed as mg bladder weight per 100 g body weight. To correlate bladder weight changes with CTX-induced bladder damage, bladders were fixed in 10% neutral buffered formalin and prepared for histopathological examination.

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[2] To whom requests for reprints should be addressed, at Vermont Regional Cancer Center, University of Vermont, 1 South Prospect Street, Burlington, Vt. 05401.

[3] The abbreviations used are: CTX, cyclophosphamide; NAC, *N*-acetylcysteine; DSF, disulfiram; DDTC, diethyldithiocarbamate; ILS, increased life span; MST, mean survival time.

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To assess the effect of DSF or DDTC on the oncolytic activity of CTX, BD2F$_1$ mice were inoculated i.p. with 1 million L1210 murine leukemia cells. CTX was administered i.p. 24 hr later, and DSF or DDTC were administered p.o. at selected times with respect to CTX. Antitumor activity was expressed as the percentage of ILS of treated mice compared to that of nontreated controls by comparison of the MST of both groups using the formula

$$\% \text{ ILS} = \left(\frac{\text{MST}_{\text{treated}}}{\text{MST}_{\text{controls}}} - 1\right) \times 100$$

Data were analyzed for statistical significance using Student's *t* test, and a level of significance of $p < 0.05$ was chosen.

RESULTS

Mice were given various doses of CTX i.p., and bladder weights were measured 48 hr later. As depicted in Chart 1, CTX produced a significant increase in bladder weight when administered at doses of 100 mg/kg or greater. Since 100 mg CTX per kg consistently caused an increase in urinary bladder weight without producing concomitant life-threatening toxicity, CTX was administered routinely at 100 mg/kg in subsequent toxicity studies.

When administered simultaneously with CTX, DSF effectively prevented CTX-induced increases in bladder weight at doses as low as 125 mg/kg (Chart 2). The uroprotective effect was further substantiated by histopathological examination of bladders removed from mice treated with 100 mg CTX per kg, CTX plus 125 mg DSF per kg, or 0.9% NaCl solution. CTX administration resulted in edema of the lamina propria and muscularis, mild inflammatory reaction, and disruption of the epithelial lining. In contrast, mice given DSF or DSF plus CTX had no histological evidence of bladder damage.

The uroprotective potential of NAC is highly dependent upon the timing of NAC administration with respect to CTX (28). To determine whether a similar schedule dependency existed for DSF-mediated uroprotection, mice were given DSF at selected times preceding or subsequent to CTX treatment. DSF effectively prevented CTX-induced bladder damage when administered within 1 hr of CTX (Chart 3). However, the DSF-mediated uroprotection diminished significantly if administered either prior to or following this time.

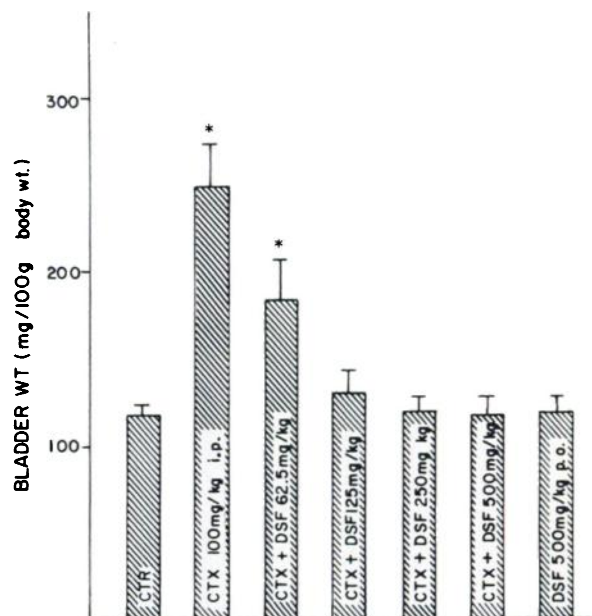

Chart 2. Prevention of CTX-induced urinary bladder weight by DSF. Mice were given 100 mg CTX i.p. simultaneously with various p.o. doses with DSF. Forty-eight hr later, bladders were removed, and wet weights were measured. Values are means for 10 mice. *Bars*, S.E. *, values significantly different from controls ($p < 0.05$).

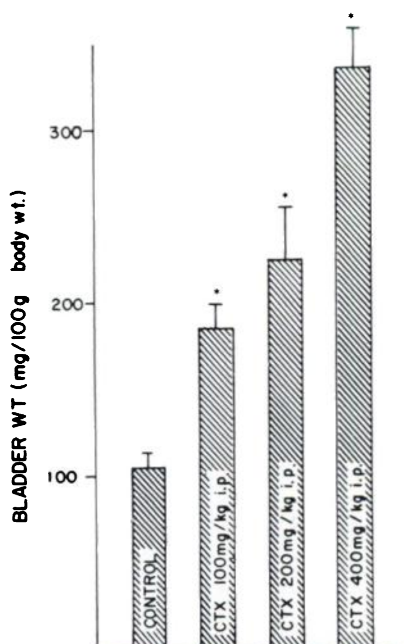

Chart 1. Effect of i.p. administered CTX on murine urinary bladder wet weight. Mice were given various doses of CTX i.p., and 48 hr later bladder weights were measured. Bladder weights were normalized to body weight (mg bladder weight per 100 g body weight). Values are means for 10 mice. *Bars*, S.E. *, values significantly different from control ($p < 0.05$).

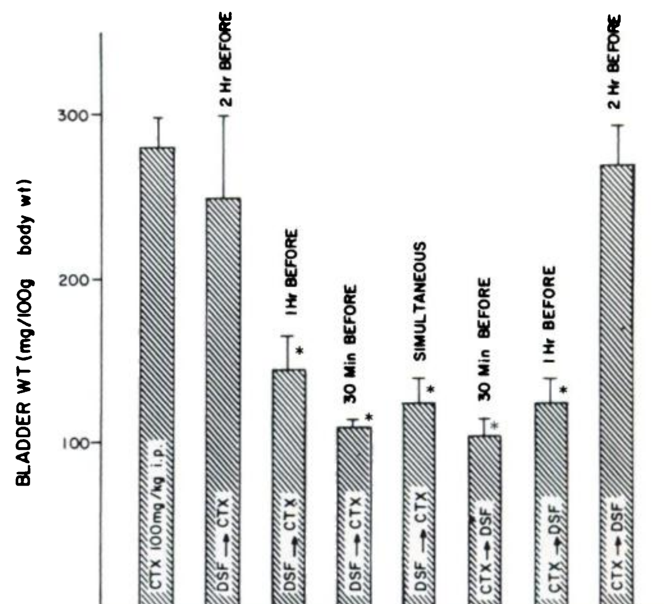

Chart 3. Effect of treatment schedule on the prevention of CTX-induced urinary bladder weight gain by DSF. Mice were given CTX (100 mg/kg i.p.) and DSF (125 mg/kg p.o.) at selected times with respect to CTX. Bladder weights were measured 48 hr after CTX administration. Values are means for 10 mice. *Bars*, S.E. *, values significantly different from CTX alone ($p < 0.05$).

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Since DSF is hydrolyzed rapidly to the sulfhydryl-containing metabolic DDTC *in vivo* (11) and since several other sulfhydryl compounds are reported to be effective uroprotectants (22, 28), it was of interest to determine whether DDTC could also serve as a uroprotectant. To test this, DDTC (125 mg/kg) was administered p.o. at selected times with respect to CTX. Although DDTC did indeed ameliorate the bladder toxicity of CTX, the treatment schedule for DDTC protection was quite different from that observed for DSF (Chart 4). DDTC was most effective when given 2 to 4 hr after CTX but was totally ineffective if administered prior to or simultaneously with CTX.

DSF has been reported to inhibit a number of enzyme systems, including certain enzymes of the mixed-function oxidase system (24), aldehyde oxidase (27), and aldehyde dehydrogenase (8). Since the mixed-function oxidase system is required for metabolic activation of CTX, it is possible that uroprotective potential of DSF resulted from an inhibition of CTX activation. If this was the sole mechanism, one would expect DSF to also eliminate or at least significantly diminish the oncolytic activity of CTX. To test this, L1210-bearing mice were given DSF or DDTC p.o., using the dosing schedule that most effectively prevented CTX-induced bladder damage. As shown in Chart 5, DSF (200 mg/kg) had no intrinsic cytotoxicity, while CTX (150 mg/kg) treatment resulted in a 200% ILS with one long-term survivor (alive 60 days postinoculation). In contrast, DSF administered 30 min prior to CTX significantly increased the percentage of long-term survivors from 5% of mice treated with CTX alone to 70% of mice treated with this drug combination. DDTC (200 mg/kg), on the other hand, had no effect on the oncolytic activity of CTX when administered 4 hr after CTX (data not shown).

One of the more prominent cytotoxic effects of CTX is leukocytopenia (4). To determine whether DSF exacerbated

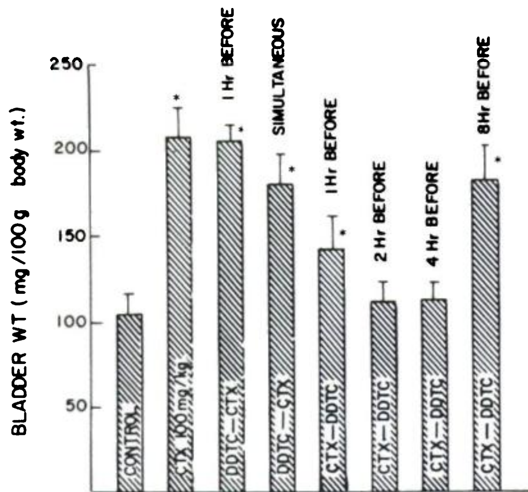

Chart 4. Effect of treatment schedule on the prevention of CTX-induced urinary bladder weight gain by DDTC. Mice were given CTX (100 mg/kg i.p.) and DDTC (125 mg/kg p.o.) at selected times with respect to CTX. Bladder wet weights were measured 48 hr after CTX administration. Values are means for 10 mice. *Bars*, S.E. *, values significantly different from controls ($p < 0.05$).

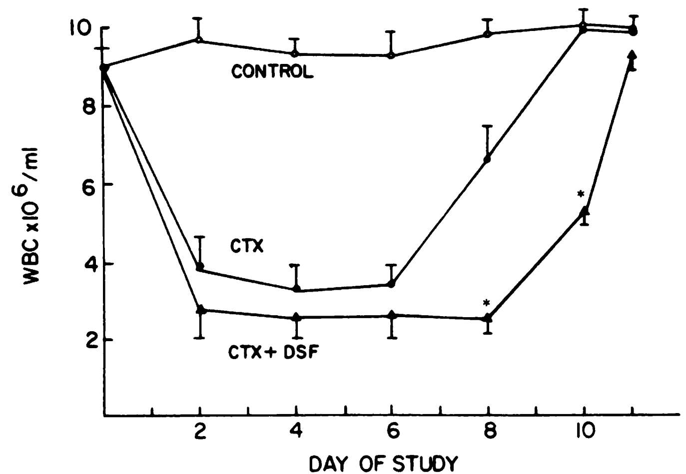

Chart 6. Effect of DSF on CTX-induced leukocytopenia. Mice were given DSF (200 mg/kg p.o.) 30 min before CTX (150 mg/kg i.p.). Mice were bled from the retroorbital sinus at selected times after CTX treatment, and peripheral WBC was measured. Values are means for 6 mice. *Bars*, S.E. *, values for CTX plus DSF values are significantly different from CTX alone ($p < 0.05$).

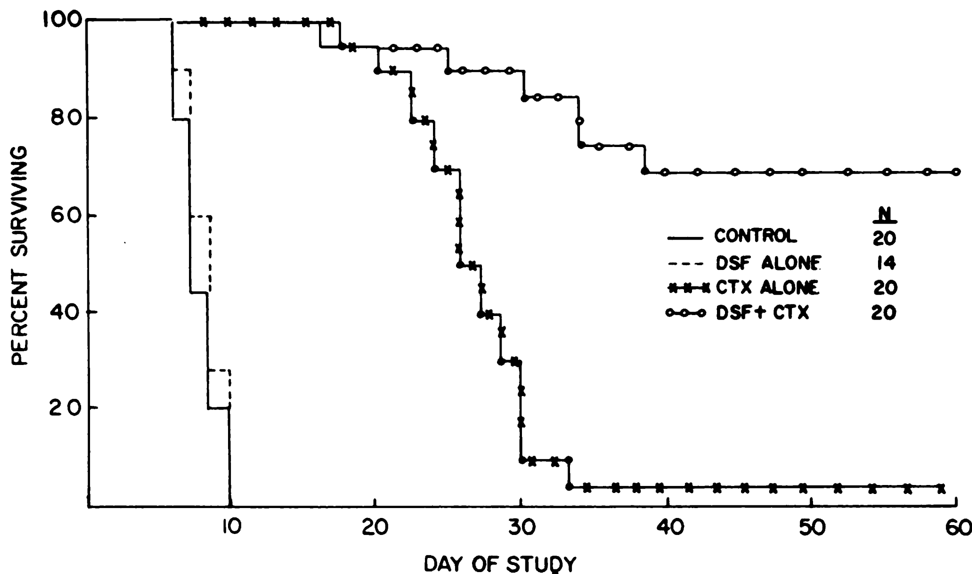

Chart 5. Effect of DSF on the antitumor activity of CTX against L1210 murine leukemia *in vivo*. BD2F₁ mice were inoculated i.p. with 10^6 L1210 cells and later 24 hr treated with 0.9% NaCl solution (control), CTX (150 mg/kg i.p.), DSF (200 mg/kg p.o.), or DSF 30 min before CTX. Animals were observed daily for deaths.

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this effect of CTX treatment, mice were given CTX (150 mg/kg) alone, DSF (200 mg/kg) alone, or DSF 30 min prior to CTX. Mice were then bled from the retroorbital plexus at various times following treatment, and the number of leukocytes was measured. CTX administration resulted in a marked leukocytopenia within 48 hr of treatment, which was maintained until Day 5 (Chart 6). Although DSF had no endogenous leukocytotoxicity, it did increase the duration of CTX-induced leukocytopenia.

DISCUSSION

Urotoxic complications, especially hemorrhagic cystitis (9, 31), are often a serious limiting factor in the therapeutic use of CTX. A number of procedures have been used successfully to treat this untoward effect of CTX, including surgical intervention (14) or direct instillation of formalin (19), phenol (20), or silver nitrate (23). Although controllable and usually reversible, the development of hemorrhagic cystitis necessitates the cessation of CTX administration. Certainly, a much more desirable approach would be to utilize a uroprotective agent capable of preventing hemorrhagic cystitis, thereby allowing the continuation of CTX treatment.

To date, 2 such compounds, NAC (28) and mesnum (22), have had proven uroprotective activity when administered parenterally in combination with CTX. Unfortunately, NAC also produced a concomitant decrease in CTX antitumor efficacy (5). More recently, we have reported that disulfiram prevented CTX-induced cystitis in mice (17, 18). Our current studies have confirmed these initial observations and have extended them by demonstrating that DDTC, a sulfhydryl-containing metabolite of DSF, is also uroprotective and that DSF, but not DDTC, augments the cytotoxicity of CTX.

DSF has been recognized widely as a major adjunct in the treatment of alcoholism (19). Until recently, however, the tissue distribution and metabolism of DSF were only poorly understood. Faiman *et al.* (10, 11) have shown that DSF is metabolized rapidly *in vivo* to DDTC, DDTC methyl ester, inorganic sulfate, and DDTC glucuronide.

Acrolein has been demonstrated to be the causative agent in CTX-induced cystitis (3, 6) and mesnum prevented this toxicity by forming an adduct with acrolein. It is not surprising, therefore, that DDTC is an effective uroprotectant, since it too would appear to be quite capable of adduct formation with acrolein. Indeed, DDTC is likely to be the active moiety when DSF is administered to mice. This supposition is substantiated by the scheduling requirement for uroprotection by either agent. DSF was effective only when administered within 1 hr of CTX, whereas DDTC was effective only when administered 2 to 4 hr after CTX. Under both circumstances, such treatment would result in maximum urinary concentrations of DDTC 3 to 5 hr after the administration of CTX, a time when CTX metabolites would be expected to be at maximal concentrations in mice (26, 30). Interestingly, DDTC has been reported to significantly reduce the renal toxicity of *cis*-diamminedichloroplatinum(II) when administered 2 to 4 hr after the platinum compound (1, 13).

DSF and DDTC have been shown to inhibit a wide range of enzymes, including the mixed-function oxidase system, aldehyde oxidase, and aldehyde dehydrogenase (8, 24, 27). In each case, the mechanism of inhibition has been ascribed to DSF or DDTC binding to sulfhydryl groups on these enzymes (25). Since each of these enzymes plays a significant role in the ultimate metabolism of CTX, either DSF or DDTC could have a profound effect upon the antitumor efficacy of CTX. That the inhibition of mixed-function oxidase was not pharmacologically important was suggested by the observation that neither DSF nor DDTC diminished the oncolytic activity of CTX. Indeed, DSF augmented slightly the efficacy of CTX against L1210 murine leukemia, which is in agreement with observation made in other laboratories.[4] This observation suggests that the inhibition of aldehyde dehydrogenase, an enzyme responsible for the production of the noncytotoxic metabolites carboxyphosphamide and 4-ketocyclophosphamide, may result in an increased production of phosphoramide mustard, the proposed alkylating moiety of CTX, from aldophosphamide. A possible explanation for the lack of DDTC effect on CTX antitumor activity is the treatment schedule used in these experiments. Studies by Struck *et al.* (26) indicate that significant activation of CTX has taken place within 3 hr following CTX administration. Thus, DDTC which was administered p.o. 4 hr after CTX would be expected to have little demonstrable effect on CTX metabolism.

In summary, DSF or its metabolite DDTC effectively prevented CTX-induced cystitis in mice. The treatment schedule necessary for this uroprotective effect as well as previously reported uroprotection by several other sulfhydryl-containing compounds suggest that DDTC is the active uroprotectant. Finally, although both compounds inhibit certain mixed-function oxidase enzymes in the metabolic system required for the activation of CTX, neither compound diminished the oncolytic activity of CTX. These findings suggest that the uroprotection of either DSF or DDTC resulted from adduct formation with acrolein and not by inhibiting the metabolic activation of CTX.

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