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L-Cysteine prodrug protects against cyclophosphamide urotoxicity without compromising therapeutic activity

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Summary. 2(*R,S*)-D-*ribo*-(1′,2′,3′,4′-Tetrahydroxybutyl)-thiazolidine-4(*R*)-carboxylic acid (RibCys) is a prodrug of L-cysteine that releases the sulfhydryl amino acid after nonenzymatic ring opening and hydrolysis. The L-cysteine then elevates glutathione (GSH) levels by stimulating its biosynthesis. RibCys was investigated for its ability to protect CDF_1 mice from the potent urotoxicity of cyclophosphamide (CTX) without compromising the therapeutic utility of the drug. RibCys induced a significant reduction in weight loss of the animals and in bladder inflammation at 48 h after CTX administration; however, bladder tissue remained inflamed as compared with that in controls. Bladder histology also showed some pathological changes in the presence of RibCys. In contrast, all parameters of toxicity (body weight loss, bladder inflammation, and pathological abnormalities) had been virtually reversed by day 21 after administration. In tests against L1210 leukemia, RibCys did not interfere with CTX anticancer activity. From these preliminary studies, RibCys appears to be a likely candidate for protecting against long-term CTX toxicity, perhaps reversing the original damage caused by a very high dose, without compromising the therapeutic utility of the alkylating agent.

Introduction

While the development of novel cytotoxic agents continues, the use of currently available anticancer drugs to their fullest potential remains a distinct challenge. A persistent problem stems from the drugs' lack of specificity to target tissue, which generates sometimes intolerable and always undesirable toxic side effects. One approach to combatting this dilemma involves the coadministration of a protective compound that reduces the nonspecific toxicity of the chemotherapeutic agent but does not detract from its cancer-fighting ability.

In this context, we are investigating the protective ability of a prodrug of L-cysteine against the potent urotoxicity of cyclophosphamide (CTX). The prodrug of interest, 2(*R,S*)-D-*ribo*-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine-4(*R*)-carboxylic acid (RibCys; Fig. 1), functions by liberating L-cysteine, which is capable of elevating cellular levels of the endogenous protective agent glutathione (GSH) [26]. GSH has long been recognized as a component of critical detoxification pathways; therefore, modulation of GSH metabolism is a reasonable target for the development of protective drugs.

CTX is used against some sarcomas [14], lymphoma [27], and carcinoma of the lung [19] and breast [7]. It is also given prior to organ transplantation because of its immunosuppressive activity [36]. Unfortunately, CTX exhibits dose-dependent/dose-limiting toxicity to the bone marrow and bladder, which reduces its overall effectiveness [1]. This drug must be metabolically activated by components of the microsomal mixed-function oxidase system to generate cytotoxic species. Of these, acrolein [24] and chloroacetaldehyde [25] are considered to be the prime candidates responsible for the potent urotoxicity of CTX. The phosphoramide mustard also liberated during CTX metabolism is not thought to contribute to the bladder damage, but rather is responsible for the antitumor activity of CTX [28].

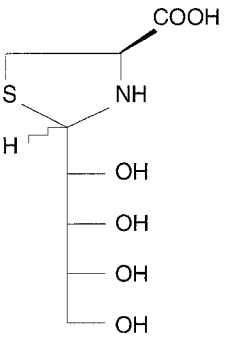

Fig. 1. Structure of RibCys

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GSH is known to be important in the detoxication of oxazaphosphorine metabolites [2, 12, 15]; therefore, much work has centered on relieving the toxicity of CTX by using thiol-containing compounds that may achieve "sulfhydryl" rescue. We set out to determine whether a thiazolidine prodrug of L-cysteine, such as RibCys, could function as a protective agent against CTX urotoxicity without compromising its useful therapeutic activity.

Materials and methods

Drugs. CTX, ribose, and L-cysteine were purchased from Sigma Chemical Company (St. Louis, Mo.) and were used without further purification. RibCys was synthesized and characterized as described previously [26].

Animals. Male CDF_1 mice weighing 13–15 g were obtained from Charles River Laboratories (Wilmington, Mass.) and were used when they had reached the 20-g range. Animals were housed over cedar shavings and were maintained on a 12-h light/dark cycle in a humidity- and temperature-controlled facility. Animals were fasted for 10 h prior to studies except when indicated; water was available ad libitum. Food was resupplied after injections except in the GSH elevation study, in which the animals remained unfed for the duration of the experiment. All animal studies were performed in compliance with guidelines established in "Guide for the Care and Use of Laboratory Animals", published by the United States Department of Health and Human Services (NIH publication 86-23, revised 1985). Animals were housed in facilities accredited by the American Association of Laboratory Animals Care (AAALAC), and the research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. Treatment groups ranged from 5 to 12 mice. Intraperitoneal injections were accomplished with the animals held loosely; no anesthetic was used during the injections. Statistical significance between data groups was determined using the Student's *t*-test.

Acute toxicity. High-dose CTX (300 mg/kg, i.p.) was given to mice, followed 30 min later by saline or RibCys (8 mmol/kg, i.p.). Another set of animals received only the RibCys injection. The mice were weighed daily and were killed after 48 h. The bladders were blotted dry, examined visually, and weighed. Macroscopic assessment of tissue damage was made using the following rating scale [3]: 0, no swelling or abnormal coloration; 1, inflammation corresponding to an increase of <30% in bladder weight, with slight coloration; 2, moderate inflammation corresponding to an increase of between 30% and 60% in bladder weight, with increased coloration; and 3, severe inflammation corresponding to an increase of >60% in bladder weight, with dark coloration. The scores for test groups were recorded as the weighted average for all animals in a given group.

The bladders were then fixed in buffered formalin solution. Histological sections were prepared and were stained with hematoxylin and eosin. Pathological changes in the organ were rated by one author (R. T. Z), who was unaware of sample identification. The grading system was based on the following scale: 0, normal; 1+, mild edema of the lamina propria; 2+, marked edema of the lamina propria associated with polymorphonuclear leukocyte infiltration; and 3+, marked edema of the lamina propria accompanied by hemorrhage and severe disruption of the mucosa. Again, the scores were recorded as the weighted average of all animals in an experimental group. Organ weight was expressed in milligrams of bladder weight per 100 g body weight.

Chronic toxicity. The protocol for the acute toxicity study was followed, except that the animals were observed for 21 days. Mortality was noted, and bladders were removed immediately on the death of the animal or after it had been killed on day 21.

Antitumor activity. Animals were inoculated with 10^6 L1210 leukemia cells in the lower right quadrant of the peritoneum. At 1 day after tumor cell implantation, separate treatment groups received either (1) CTX (150 mg/kg, i.p.) plus saline, (2) CTX (150 mg/kg, i.p.) followed by RibCys (8 mmol/kg, i.p.) 30 min later, (3) saline plus RibCys (8 mmol/kg, i.p.), or (4) vehicle only. Drugs were injected into the upper left quadrant of the peritoneum. Animals were observed daily for 60 days. Antitumor activity was determined by calculations of %T/C [34], which is defined as the median survival (MS) of dying mice in the treatment group (T) divided by that of the control group (C), multiplied by 100.

GSH measurements. Total glutathione (hereafter referred to as GSH) was measured in bladder tissue at 1, 2, 4, 8, and 16 h after RibCys administration (8 mmol/kg, i.p.) or in fed or fasted untreated controls using the Tietze method [33], with the following modifications. The entire bladder was weighed and homogenized in 5 ml 5% sulfosalicylic acid (w/v). After centrifugation at 3,000 *g* for 10 min, the supernatants were diluted 10-fold in 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and were kept on ice. A 300-µl aliquot of this solution was combined in a 1.5 ml cuvette with 450 µl buffer, 100 µl glutathione reductase (5 units/ml), and 50 µl 5,5′-dithio-*bis*(2-nitrobenzate) (DTNB; 4 mg/ml). After 1 min incubation, 100 µl reduced nicotinamide adenine dinucleotide phosphate (NADPH; 2 mg/ml) was added, and the absorbance at 412 nm was monitored for 2 min. The change in absorbance per minute was determined, and this value was converted to micromoles of GSH per gram of tissue by comparison with known standards. All measurements were made between 7 and 9 a.m. to avoid problems with the known diurnal variation in GSH levels.

Results

Effect of RibCys on acute toxicity

As shown in Table 1, the administration of a high dose of CTX (300 mg/kg) caused a decrease in body weight coupled with a significant increase in bladder weight. Macroscopic evaluation of the bladder showed dramatic inflammation and hemorrhage in the organ. Profound pathological changes in the bladder tissue were also visible by light microscopic examination of tissue slides. RibCys (8 mmol/kg), given 30 min later, reversed the weight loss of the animals. It also significantly reduced the bladder inflammation but did not bring bladder weights back to levels recorded for untreated animals. Visual evaluation revealed a decrease in tissue changes following the administration of RibCys but, again, did not show complete reversal of these changes. Histological sections examined at this point showed that damage had been done to the bladder tissue in some animals even in the presence of RibCys. RibCys itself exhibited no toxicity to the bladder.

Effect of RibCys on chronic toxicity

During the 21-day experiment, CTX continued to exhibit its powerful toxicity in terms of both bladder inflammation and weight loss of the animals (Table 2). Pronounced pathological abnormalities were also clearly revealed in the bladder tissue by light microscopy and visual examination. RibCys showed dramatic protective abilities at this time point, resulting in the virtually complete reversal of all parameters of toxicity measured (body weight loss, bladder weight gain, and macroscopic and microscopic rating of bladder tissue).

Table 1. Effect of RibCys on the toxicity of CTX at 48 h

Treatment[a]		Body wt. change	Relative bladder wt[b]	Composite macroscopic rating[c]	Composite microscopic rating[c]
CTX	RibCys				
–	–	+6.2%	82.9 ± 1.9	0 ±0	0 ±0
+	–	–7.8%	203.4 ± 5.6*	3 ±0	1.9 ± 0.3
+	+	+2.4%	129.8 ± 7.3**	1.7 ± 0.3	1.2 ± 0.4
–	+	+7.3%	84.8 ± 2.7	0 ±0	0 ±0

Data represent mean values ± SE
[a] CTX (300 mg/kg, i.p.) was followed 30 min later by RibCys (8 mmol/kg, i.p.). Saline was substituted for drugs where indicated
[b] Expressed as mg/100 g body wt.
[c] Indicates the weighted average of all animals in a treatment group; see Materials and methods for rating definition
* Statistically significant difference from untreated control ($P < 0.05$); ** Statistically significant difference from both untreated controls and CTX-treated animals ($P < 0.05$)

Table 2. Effect of RibCys on the toxicity of CTX at 21 days

Treatment[a]		Survival	Body wt. change	Relative bladder wt[b]	Composite macroscopic rating[c]	Composite microscopic rating[c]
CTX	RibCys					
–	–	100%	+28%	74.3 ± 2.7	0 ±0	0 ±0
+	–	17%	–23.9%	148.3 ± 6.2	3 ±0	3 ±0
+	+	83%	+24.8%	89.1 ± 5.2*	0.3 ± 0.2	0.5 ± 0.5

Data represent mean values ± SE
[a, b, c] See footnotes to Table 1
* Statistically significant difference from CTX-treated animals ($P < 0.05$)

Effect of RibCys on antitumor activity of CTX

The interference by RibCys with CTX antitumor activity was examined against L1210 leukemia cells transplanted into mice. As seen in Table 3, animals given a 150 mg/kg dose of CTX displayed prolonged survival as compared with untreated controls. The administration of RibCys (8 mmol/kg) 30 min later failed to interfere with the therapeutic efficacy of CTX. RibCys itself showed no antitumor activity.

The life span of animals that had been inoculated with L1210 leukemia cells was longer than that generally found in the literature. Usually, untreated mice succumb to the lethal effects of this tumor in about 11–14 days. The longer lifetimes may be attributed to the characteristics of the tumor cells, to the method of implantation, or to physical parameters of the strain or batch of animals used in this study. However, the critical differences between experimental and control treatment groups remain.

Effect of RibCys on bladder GSH

The ability of RibCys to elevate GSH levels in the bladder was studied at various times after RibCys administration and in fed and fasted controls. Fasting produced a decrease of about 40% in GSH levels, as expected. The time course for GSH elevation in the bladder is shown in Table 4. GSH levels had significantly increased to >2 times the baseline values by 4 h after RibCys administration and remained at this elevated level through the 16-h time point.

Table 3. Interference by RibCys with the antitumor activity of CTX against L1210 leukemia

Treatment[a]		MS[b]	%T/C[c]	Survivors for >60 days (n)
CTX	RibCys			
–	–	24.2	–	0/5
+	–	38.6	159	2/5
+	+	41.8	173	0/6
–	+	21	87	0/5

[a] CTX (150 mg/kg, i.p.) was given 1 day after tumor cell implantation; RibCys (8 mmol/kg, i.p.) followed 30 min later when indicated
[b] MS was determined only using dying animals
[c] See Materials and methods for % T/C calculation

Table 4. Effect of RibCys on bladder GSH levels

Time (h) after RibCys[a]	GSH (μmol/g tissue)
0 (fed controls)	1.71 ± 0.11*
0 (fasted controls)	1.03 ± 0.23
1	1.89 ± 0.05*,**
2	1.89 ± 0.06*,**
4	2.19 ± 0.08*,**
8	2.13 ± 0.09*,**
16	2.12 ± 0.08*,**

Data represent mean values ± SE
[a] RibCys injected i.p. at 8 mmol/kg
* Significantly higher than in fasted controls ($P < 0.05$); ** Significantly higher than in fasted controls ($P < 0.01$)

Discussion

Two categories of sulfhydryl protective agents are recognized as being active against CTX toxicity [4, 8, 9, 15, 28, 31, 32, 34, 35]. The first category, which is composed of agents that most likely interact directly with CTX's activated intermediates, is best represented by sodium 2-mercaptoethane sulfonate (mesna) [5]. Mesna exhibits powerful protective capabilities that are dependent on the dose of oxazaphosphorine and on the route and timing of administration. Mesna's toxicity is considered to be low; however, it is known to cause central nervous system disturbances [21] along with severe hypersensitivity in some individuals [18]. It also exhibits limited cell permeability [6].

The second category consists of agents that serve as precursors to GSH and may protect against CTX's urotoxicity by stimulating the biosynthesis of this critical cellular thiol and serve to amplify the existing detoxification pathway. *N*-Acetylcysteine (NAC) and cysteine itself represent this class of agents. Although mesna has enjoyed more clinical use, the pharmacokinetics of the cysteines are actually superior to those of mesna [35]. Again, the toxicity of NAC and cysteine is considered to be low but cannot be ignored [10, 11, 16, 17, 20, 22, 23, 29, 30].

Both mesna and cysteine suffer from a similar chemical drawback to their use in high concentrations. The free thiol groups of these agents are oxidized to the inactive disulfide form in a concentration-dependent fashion. The active free sulfhydryl groups are re-released only following enzymatic reduction of the disulfide in the renal tubules, for example. Depending on the kinetics of acetyl cleavage of NAC in a given system, initially high levels of free cysteine can be expected from this agent as well, which would be subject to the undesired oxidative deactivation.

RibCys, a prodrug of cysteine, is expected to reproduce most closely the effects of free cysteine. The amino acid is released from the thiazolidine by nonenzymatic ring opening and hydrolysis, a mechanism of action that is unique as compared with that of any other type of sulfhydryl protective agent. This equilibrium-controlled dissociation offers a slow-release form of cysteine, which reduces the problems associated with high levels of the free amino acid (toxicity, mutagenicity, oxidation of free thiol) but exhibits cysteine's superior pharmacokinetic profile and capitalizes on its natural transport phenomena.

In perhaps the first study on bladder GSH carried out in this context, RibCys showed its ability to elevate GSH levels by >2-fold that found in controls. Dramatic elevation was not expected because GSH biosynthesis is stringently controlled by feedback inhibition. The value of these GSH elevation studies lies not in the absolute increase in GSH achieved, but rather in the ability of RibCys to maintain GSH levels during continued fasting of the animals. This study argues favorably for the hypothesis that RibCys can serve as a slow-release, depot form of L-cysteine.

RibCys was shown to have a positive effect on the microscopic characteristics of the bladder. Usually, only a macroscopic evaluation of the tissue is made. One study by Hacker et al. [13], however, revealed that tetraethylthiuram disulfide (disulfiram) cotreatment yielded no microscopic evidence of bladder damage at 48 h. The dose of CTX used in that study was 100 mg/kg, which produces bladder toxicity, albeit at a drastically reduced level as compared with the 300 mg/kg used in the current studies.

In conclusion, RibCys has shown the ability to protect against the potent urotoxicity of high-dose CTX after 21 days without adversely affecting its antitumor activity. Numerous studies concerning the dose-response and the time and route of administration will assist in the evaluation of RibCys as a sulfhydryl rescue agent for CTX.

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