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Short Communication

INHIBITION OF GLUTATHIONE-RELATED ENZYMES AND CYTOTOXICITY OF ETHACRYNIC ACID AND CYCLOSPORINE

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Abstract—Glutathione (GSH) is an endogenous thiol that detoxifies active oxygen and reactive species formed during intermediary metabolism and drug detoxification. Compounds with a range of potential toxicities were tested for their abilities to affect GSH reductase and GSH *S*-transferase activities, which are each components of the two principal detoxification pathways in which GSH participates. A high performance liquid chromatographic method for determining oxidized and reduced GSH was modified to assay GSH reductase activity. With this method it was possible to demonstrate that ethacrynic acid, which inhibits GSH *S*-transferase, also inhibits the activity of GSH reductase. Inhibition of GSH reductase by ethacrynic acid was similar to that seen with carmustine (BCNU). GSH reductase activity was not affected by cis- or transplatin, buthionine sulfoximine, other loop diuretics, cyclosporine A or aminoglycosides. Cyclosporine inhibited GSH *S*-transferase at 50 μM and higher concentrations. These results support a role for GSH-mediated detoxification mechanisms in ethacrynic acid- and cyclosporine-associated cytotoxicity, which may mediate their toxicities and their potential as adjunctive agents in antineoplastic therapy. A better understanding of the mechanism of their toxicity can greatly extend the clinical usefulness of these agents, as this toxicity is the basis of both their therapeutic and antitherapeutic actions.

Key words: cancer; chemotherapy; antineoplastic agent; glutathione *S*-transferase; glutathione reductase; diuretic

Certain therapeutic agents, including aminoglycoside antibiotics, diuretics, and antineoplastic agents such as cisplatin, share significant toxicity for two particular organs—the inner ear and the kidney [1]. Other exclusively nephrotoxic agents, such as cyclosporine, may share similar properties but are excluded from the inner ear space. A common property of this oto- and/or nephrotoxic potential is its profound enhancement by the concurrent administration of ethacrynic acid. Such a combination can result in irreversible deafness and kidney damage after a single combined administration of quite modest doses of these drugs [2–6]. Two of these same drugs, ethacrynic acid and cyclosporine, have been investigated as adjunctive agents in cancer chemotherapy. They are used to reverse or overcome multidrug resistance by potentiating the cytotoxicity of other antineoplastic agents. A better understanding of the mechanism of their toxicity can greatly extend the clinical usefulness of these agents, as this toxicity is the basis of both their therapeutic and antitherapeutic actions. The organ-specific toxicity and its potentiation by these different drugs in combination suggest a common mechanism or shared biochemical pathway. However, no mechanism has been advanced that can account for the potent synergy and the organ specificity that have been observed.

Ethacrynic acid and related "loop" diuretics are known to be potent inhibitors of GSH§-*S*-transferases, and have been reported to bind irreversibly to these enzymes [6–8]. We have proposed that inhibition or inactivation of GSH-mediated drug detoxification mechanisms may constitute the final common pathway by which these otherwise structurally and functionally dissimilar drugs may potentiate each others' toxicity [9–11]. This same mechanism may also underlie the therapeutic efficacy of these drugs as adjunctive antineoplastic agents. This cytotoxicity may occur through depletion of GSH from body tissues as a consequence of the detoxification of metabolically generated free radicals and reactive metabolites, or by the inhibition of enzymes involved in GSH use. This concept is supported by the recent work of Huang and Schacht [12], who report that such a reactive metabolite may be formed from aminoglycosides, and by Zenner *et al.* [13], who have demonstrated glutathione inhibition of gentamicin ototoxicity. A further linkage between oto- and nephrotoxicity may be the rate of GSH turnover, which has already been implicated as a factor in nephrotoxicity [14]. We report further data on the interactions of ethacrynic acid and cyclosporine with GSH-mediated detoxification mechanisms that may mediate their cytotoxicity and their potential as adjunctive antineoplastic agents.

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§ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; BSO, buthionine sulfoximine; BCNU, carmustine (1,3-bischloroethyl-nitrosourea); and EC, electrochemical detection.

Materials and Methods

Drugs and Chemicals. All drugs used were purchased from the Sigma Chemical Co., except for carmustine (BCNU; a gift of Bristol Laboratories), sodium ethacrynate (a gift of Merck, Sharpe & Dohme), cyclosporine A (a gift of Sandoz Pharmaceuticals), piretanide (a gift of Hoechst) and bumetanide (a gift of Hoffmann-LaRoche). BSO is a compound specifically designed to reduce GSH synthesis and lower GSH levels in tissue by the inhibition of γ-glutamylcysteine synthetase [15], without activity on other drug-metabolizing pathways [14] or inherent toxicity [15].

Glutathione measurements. Both GSSG and GSH levels were determined using HPLC with EC, as modified [10, 11] from the method of Allison and Shoup [16]. Sensitivity of this method is 35 nM for GSH and 60 nM for GSSG which is several orders of magnitude below the levels found in most tissues [17]. Representative chromatograms demonstrating the HPLC separation and identification of GSH and GSSG in standards and tissue extracts can be found in an earlier publication [10].

In vitro *enzyme assays.* GSH *S*-transferase activity was assayed by the method of Habig *et al.* [18], with 0.75 μM CDNB (Sigma) as substrate. Rat kidney GSH *S*-transferase (Sigma) was used. GSH reductase activity was determined by measuring the production of GSH from GSSG with the HPLC–EC method described above. A solution of 0.1 mM NADPH, 1.0 mM GSSG, 0.5 mM EDTA and 0.0005 U/mL of GSH reductase (bovine, Sigma) was incubated at 30° for 30 min. The final volume was 1.0 mL. At the end of the incubation period, 500 μL of the incubation solution was added to 500 μL of HPLC mobile phase and filtered through a 0.45 μM pore size Nylon-66 filter. The filtrate was then injected directly into the HPLC and assayed as described above. The reaction was linear with respect to GSSG concentration up to 2.0 mM. This assay was not affected by BSO, a γ-glutamylcysteinyl-synthetase inhibitor, indicating that the assay measures GSH production via GSH reductase activity rather than *de novo* synthesis. Results of enzyme inhibition studies shown in Tables 1 and 2 are the means of five different determinations done in triplicate, for each drug and concentration. Results are compared to the control value, using the Student's *t*-test.

Results

Glutathione reductase activity was measured *in vitro*, using the HPLC–EC method to follow the production of GSH from GSSG (Table 1). The antineoplastic agent BCNU demonstrated its known ability to inhibit GSH reductase activity in our assay. Ethacrynic acid was also found to be an effective inhibitor of GSH reductase, with activity very similar to that of BCNU. Reductase activity was not affected by cisplatin, transplatin, BSO, other loop diuretics, cyclosporine A, or aminoglycoside antibiotics. GSH *S*-transferase activity (Table 2) was likewise not affected by BSO, BCNU, or aminoglycoside antibiotics, but was inhibited strongly by ethacrynic acid and at higher concentrations by bumetanide. Ethacrynic acid appeared to have about ten times greater potency in inhibiting GSH *S*-transferase relative to GSH reductase activity. Cyclosporine inhibited GSH *S*-transferase effectively at 50 μM and higher concentrations (Table 2). No effect was seen on GSH reductase activity at 1.0 mM cyclosporine, which was sufficient to inhibit GSH *S*-transferase completely under these experimental conditions.

Discussion

This work supports the contention that a common metabolic pathway that involves GSH may be the mechanism underlying the cytotoxicity of ethacrynic acid and cyclosporine. This cytotoxicity is the basis for the potential of these drugs as antineoplastic agents. We further propose that xenobiotic metabolism and detoxification may constitute the final common pathway that is shared by the synergistic oto- and/or nephrotoxic agents. Glutathione participates in the metabolism and detoxification of electrophilic drugs and metabolites and active oxygen species via reactions catalyzed by GSH-*S*-transferases, transpeptidases, transhydrogenases, and peroxidases and reductases [15, 17, 18]. Perturbation of the GSH system by drug actions on different sites in the GSH pathways may be the basis of the toxic interactions of these agents.

Cytotoxic interactions may occur because of depletion of GSH from body tissues as a consequence of drug metabolism, by the inhibition of one or more of these enzymes involved in GSH utilization, and/or via the generation of toxic thiols via β-lyase action on GSH-conjugates [19]. Manipulation of endogenous GSH levels and the activities of GSH-related enzymes may modulate the toxicities of many of these drugs, which otherwise limit their clinical usefulness, and these manipulations may underlie the therapeutic efficacy of ethacrynic acid and cyclosporine in antineoplastic therapy.

The adjunctive use of ethacrynic acid to overcome drug resistance in cancer chemotherapy has received much interest recently [20–24]. A phase I clinical trial has demonstrated that tolerable doses of ethacrynic acid may be used to inhibit GSH *S*-transferase activity in cells of cancer patients undergoing chemotherapy with alkylating agents such as thiotepa [23]. However, chronic exposure of tumor cells to ethacrynic acid may cause them to become resistant to this adjunctive agent because of induction of GSH *S*-transferase, which can occur at the transcriptional level [24]. The present results suggest that ethacrynic acid may act clinically by inhibiting GSH reductase, as well as by inhibiting GSH *S*-transferase [6, 7] and depleting tissue GSH [9, 10]. These multiple actions may underlie its therapeutic potential as an antineoplastic agent.

Cyclosporine is another agent with nephrotoxic potential that is now being considered as an adjunctive agent in antineoplastic therapy [25–28]. There are conflicting reports on the effect of cyclosporine on GSH levels in different organs and tissues [11, 29]. This laboratory has shown earlier that cyclosporine administration results in a more than 6-fold increase in kidney [GSH], while other tissues tested are unaffected [11]. The present findings suggest that this GSH increase may be a homeostatic mechanism of the affected tissues in attempting to compensate for the cyclosporine-induced inhibition of GSH *S*-transferase. This enzyme inhibition may underlie the cytotoxicity of cyclosporine.

Another aspect of this mechanism with important clinical implications is the profound potentiation of oto- and nephrotoxicity by ethacrynic acid. The inhibition of GSH *S*-transferases by ethacrynic acid is well-known [6, 7]. In the present study ethacrynic acid was found to inhibit not only GSH *S*-transferase but also GSH reductase, as has been reported in canine erythrocytes [30]. In earlier studies [9], we found that depletion of tissue GSH by BSO results in greatly enhanced toxicity of ethacrynic acid and aminoglycoside antibiotics. This is consistent with the very steep dose–response relationship for cytotoxic potentiation by ethacrynic acid [4]. It suggests that a minimum level of inhibition of the GSH-related enzymes by ethacrynic acid is required for the expression of cytotoxicity [9, 10].

A linkage between oto- and nephrotoxicity may be GSH turnover, which has already been implicated in the kidney toxicity of other drugs. A rapid turnover makes the tissue very susceptible to metabolic damage by facile depletion of GSH [14]. Toxicological and/or therapeutic enhancement seen after depletion of GSH is consistent with many reports in the literature [9, 10, 14, 31–37]. The lack of potentiation of ethacrynic acid ototoxicity in an acute study of guinea pigs depleted of GSH by BSO pretreatment has also been reported [38]. However, these investigators were using a near maximal dose of ethacrynic acid [4] that may have precluded the expression of any greater toxicity consistent

Table 1. Effects of potential ototoxic agents on glutathione reductase activity

Drug	Concentration (mM)	Glutathione reductase activity (nmol formed/mg/min)	% Inhibition (vs control)
Control		16.3 ± 0.7	
Cisplatin	0.1	16.1 ± 0.8	0
Transplatin	0.1	15.8 ± 1.9	3
BSO	1.0	17.2 ± 1.3	0
BCNU	0.1	11.4 ± 0.8	30
	1.0	0 ± 0.2*	100
Ethacrynic acid	0.01	14.3 ± 1.1	12
	0.05	13.4 ± 1.3	18
	0.1	9.5 ± 0.4*	42
	0.5	1.1 ± 0.3*	93
	1.0	0 ± 0.1*	100
Furosemide	1.0	16.5 ± 1.7	0
Bumetanide	1.0	16.0 ± 0.8	1
Piretanide	1.0	15.9 ± 1.1	2
Streptomycin	1.0	16.6 ± 2.3	0
Neomycin	1.0	16.7 ± 1.6	0
Kanamycin	4.1	15.9 ± 0.9	2
Cyclosporine A	1.0	16.4 ± 1.2	0

Results are means ± SEM of five different determinations done in triplicate, for each drug and concentration.

* $P < 0.001$, relative to control (Student's *t*-test).

Table 2. Effects of potential ototoxic agents on glutathione *S*-transferase activity

Drug	Concentration (mM)	Glutathione reductase activity (nmol formed/mg/min)	% Inhibition (vs control)
Control		92.9 ± 4.1	
Cisplatin	0.1	84.5 ± 6.3	8
Transplatin	0.1	82.3 ± 6.4	11
Ethacrynic acid	0.05	6.2 ± 0.3*	93
Furosemide	0.05	80.9 ± 10.3	13
Bumetanide	0.25	42.3 ± 2.8*	54
Piretanide	0.1	85.2 ± 2.3	8
Cyclosporine A	0.01	92.0 ± 2.5	1
	0.05	42.5 ± 3.0*	54
	0.2	25.3 ± 0.6*	73
	0.5	9.6 ± 0.4*	90

Results are means ± SEM of five different determinations done in triplicate, for each drug and concentration.

* $P < 0.001$, relative to control (Student's *t*-test).

with potentiation. Recent studies of ototoxicity have identified a close link between GSH and toxic mechanisms [9, 10, 13].

Cisplatin has also been reported to inhibit GSH peroxidase [39], and the inhibition of GSH peroxidase in kidney mitochondria has been related to the development of nephrotoxicity [40]. It is not known if inhibition of GSH peroxidase, which has been localized in the cochlea [41], is related to ototoxicity. Both cis- and transplatin (100 μM) modestly inhibited GSH *S*-transferase (8 and 11%, respectively) (Table 2). The combined effects of these actions of cisplatin may be a component of its toxicity. In unpublished studies, we have seen acute toxicity and mortality with cisplatin after BSO that speaks strongly for a potentiative mechanism that can be prevented or ameliorated by GSH.

At present, both ethacrynic acid and cyclosporine are being considered for use as adjunctive agents in cancer chemotherapy, based on their ability to enhance the cytotoxicity of other antineoplastic agents. These quite dissimilar agents may share a common mechanism that underlies their potential efficacy. Understanding this mechanism may result in safer and more effective use of these agents.

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