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Preclinical report

Relationship between cytocidal activity and glutathione-S-transferase inhibition using doxorubicin coupled to stereoisomers of glutathione with different substrate specificity

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To determine the cytotoxic mode of action of a glutathione (GSH)–doxorubicin (DXR) conjugate, which exhibited potent cytotoxicity against various multidrug-resistant as well as DXR-sensitive cell lines, the molecular interaction between covalent GSH–DXR conjugates and glutathione-S-transferase (GST), a possible molecular target of the conjugates, was investigated. The following four GSH molecules with stereoisomeric forms were prepared: L-Glu–L-Cys–Gly (LL-GSH), D-Glu–L-Cys–Gly (DL-GSH), L-Glu–D-Cys–Gly (LD-GSH) and D-Glu–D-Cys–Gly (DD-GSH). The enzymic activity of GST against each GSH stereoisomer was 88, 38, 8 and 4 nmol/mg/min, respectively, suggesting that the L-form cysteine residue in the molecule was an important substrate of GST. Addition of DXR conjugated with each isomer (10 μM) to a GSH-containing GST assay mixture inhibited the GST activity to 32% for LL-GSH–DXR, 16% for DL-GSH–DXR and 61% for LD-GSH–DXR as compared with the solvent control. Moreover, IC_{50} values for these conjugates were 30, 20 and 250 nM, respectively. The cytocidal activity of each conjugate corresponded to the substrate specificity of GST activity for the GSH isomer. These conjugates bound to the GST molecule, and the binding ability was 0.746, 0.627 and 0.462 mol/mol of GST for LL-GSH–DXR, DL-GSH–DXR and LD-GSH–DXR, respectively. These findings suggested that GSH–DXR interacted with the substrate-binding site of the GST molecule and inhibition of GST activity exhibited potent cytotoxicity. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, cytotoxicity, glutathione–doxorubicin conjugate, glutathione-S-transferase, stereoisomer of glutathione.

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Introduction

The multidrug resistant (MDR) phenotype is a major problem in cancer chemotherapy.[1,2] It has been reported that drug resistance is reversed by a variety of substances, such as an inhibitor of the P-glycoprotein (P-gp) efflux pump and the anti-P-gp antibody for MDR cells,[3–7] and an inhibitor of glutathione-S-transferase (GST) or of glutathione (GSH) synthetase in the GSH/GST detoxification system.

We have reported that doxorubicin (DXR) conjugated with bovine serum albumin (BSA) (BSA–DXR) increased cytotoxicity against several MDR cell lines.[8–10] Further study demonstrated that exhibition of cytotoxicity was based on some degenerative adducts from BSA–DXR, such as small peptide–DXR conjugates, but not the intact BSA–DXR conjugate.[11] There is future potential for clinical use of this novel drug. Therefore, we selected a thiol-containing tripeptide, GSH, as the partner for conventional conjugation with DXR. The GSH coupled to DXR (GSH–DXR) showed excellent cytotoxicity against rat hepatoma AH66 cells.[12] GSH–DXR also demonstrated inhibition of GST enzymic activity, but DXR alone did not, indicating that inhibition of the enzyme makes an important contribution to the expression of potent cytotoxicity of GSH–DXR against AH66 cells.[13] In the present study, we investigated the effect of DXR coupled with a GSH stereoisomer differing in GST substrate specificity[14] on the inhibition of GST activity and the enhancement of cytotoxicity via GST inhibition.

Materials and methods

Materials

DXR was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2-5-diphenyl tetrazorium bromide (MTT), 1-chloro-2,4-dinitrobenzene (CDNB), RNase A, proteinase K and ethidium bromide were obtained from Sigma (St Louis, MO). All other chemicals were of analytical grade.

Cell lines

The rat ascites hepatoma cell line AH66 were cultured with RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (growth medium) under conventional conditions.[9–12]

Synthesis of stereoisomers of GSH

Stereoisomers of GSH, γ-L-Glu–L-Cys–Gly (LL-GSH), γ-D-Glu–L-Cys–Gly (DL-GSH), γ-L-Glu–D-Cys–Gly (LD-GSH) and γ-D-Glu–D-Cys–Gly (DD-GSH), were chemically synthesized by a solid-phase method with a PE Applied Biosystems (Foster City, CA) model 431A peptide synthesizer as described previously.[14]

Conjugate of DXR with GSH isomers

An aliquot of each GSH isomer (1 mg) neutralized with NaOH and 0.5 mg of DXR in 0.5 ml of 0.15 M NaCl containing 0.1% glutaraldehyde were incubated at room temperature for 30 min.[12,13] After incubation, the mixture was applied to Dowex 50W × 8 (Nacalai tesque, Kyoto, Japan; H^+ form, 5 × 15 mm), and the conjugate of DXR with each isomer was eluted with 0.15 M NaCl. The eluate was neutralized immediately with NaOH. Peptide–glutaraldehyde conjugate without coupling to DXR was prepared as follows: 1 mg of each peptide was incubated with 0.1% glutaraldehyde under the same conditions use for the conjugation of DXR with peptides; the preparation was then separated by a Dowex 50W × 8 column. All drugs were filtrated with a 0.45 μM syringe filter (Corning Coster, Tokyo, Japan). The concentration of DXR was measured by absorbance at 495 nm and the concentration of the conjugate expressed as that of DXR.

Cytotoxicity of DXR conjugate

To assess the growth inhibitory effect of the test materials, viable AH66 cells (2×10^4) were cultured continuously for 72 h in a 48-well culture plate (Corning Coster) with 0.5 ml of growth medium containing graded equivalent concentrations of DXR. After incubation, viable cells were determined with the colorimetric assay using MTT as described previously[15] and the results were expressed by the following equation: survival rate (%) = 100 × (absorbance at 570 nm of the drug-exposed cells)/(absorbance at 570 nm of the non-treated control cells).

Assay of GST activity

The scraped and washed cells were sonicated in 10 mM sodium phosphate buffer (pH 7.4) and the resultant suspension was used as the enzymatic source. GST activity was measured at 340 nm ($\varepsilon = 9600$) in 1 mM CDNB, 1 mM GSH and 0.1 M sodium phosphate buffer (pH 6.5) at 37°C for 10 min in the presence or absence of test drugs.[16]

Preparation of cell extract

After treatment of AH66 cells with GSH–DXR, harvested cells were washed with ice-cold 0.15 M NaCl and lysed with ice-cold 0.5% Triton X-100 containing 10 mM Tris–HCl (pH 8.0) and 10 mM EDTA. The cell lysate was spun down at 10 000 *g* for 10 min, and the supernatant was used for assays of DNA fragmentation and caspase-3 activity.

DNA fragmentation assay

After treatment of the cells (2×10^6) with GSH–DXR, the cell extract containing fragmented DNA was incubated with 0.5 mg/ml RNase A at 37°C for 60 min, then with 0.5 mg/ml proteinase K at 37°C for 60 min. After incubation, fragmented DNA which was precipitated by isopropanol was dissolved with 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5% glycerol and 0.05% bromophenol blue. The DNA fragments, separated by 2% agarose gel electrophoresis, were stained with ethidium bromide and photographed on a UV transilluminator.

Assay of caspase-3 activity

Reaction mixtures, which contained 100 μM of DEVD-MCA, the appropriate protein concentration of cell extract, 50 mM HEPES–NaOH (pH 7.5), 10% glycerol and 2 mM dithiothreitol with or without 0.1 μM DEVD-CHO, were monitored for AMC liberation at 37°C for 15 min in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.[17] The caspase-3 proteolytic activity was expressed as the difference between nmol AMC liberated in the presence and absence of the inhibitor/min/mg protein.

Binding of isomeric GSH–DXR to GST

Preparation of a GST-coupled column was conducted as follows. GST that had been purified from rat liver by GSH–agarose column chromatography was coupled to a NHS-activated Sepharose column. After washing and blocking with monoethanolamine, each isomeric GSH–DXR was applied to the column and unbound drug was eluted with PBS. The concentration of DXR in the eluate was monitored at 495 nm, and the amount of conjugate bound to GST was expressed in terms of the difference between the amount of the applied conjugate and that of the eluted conjugate. Next, 2 ml of 10 mM GSH was applied to the column and the eluate was also measured at 495 nm. The concentration of DXR in the eluate was expressed in terms of substitution of conjugate for GSH.

Protein determination

The protein concentration was assayed by a BioRad (Tokyo, Japan) protein assay kit using BSA as the standard.

Results

GST activity for stereoisomers of GSH

Substrate specificity of the four stereoisomers of GSH was examined using CDNB as the second substrate. The GST activities for LL-GSH, DL-GSH, LD-GSH and DD-GSH were 88.3, 38.1, 8.4 and 4.4 nmol/mg/min, respectively (Table 1). The existence of the D-form amino acid residue lowered the substrate specificity of GST activity and GSH containing the D-cysteine residue did not act as a substrate.

Inhibition of GST activity by isomeric GSH–DXR

The activity of GST was also determined in the presence of each conjugate. As shown in Table 2, the GST activity in the presence of LL-GSH–DXR, DL-GSH–DXR, or LD-GSH–DXR at the concentration of 10 μM was inhibited to 32, 15 and 61%, respectively. These conjugates showed dose-dependent inhibition (data not shown).

Cytotoxicity of DXR conjugated with GSH isomers

The following conjugates of DXR with three GSH stereoisomers were prepared: LL-GSH–DXR, DL-GSH–DXR and LD-GSH–DXR. The DD-GSH could not be conjugated with DXR due to interference with the stereostructure. Treatment of AH66 cells for 72 h with each conjugate resulted in IC_{50} values for LL-GSH–DXR, DL-GSH–DXR, and LD-GSH–DXR of 30, 20 and 250 nM, respectively (Figure 1). The cytocidal activity of these conjugates corresponded to the substrate specificity of GST activity for the GSH isomers described above.

Induction of apoptosis by isomeric GSH–DXR

After the AH66 cells were cultured with each conjugate at the respective concentrations of 62.5, 125, 250, 500 and 1000 nM for 24 h, the induction of apoptosis was determined by caspase-3 activation and DNA fragmentation. In non-treated control cells, the caspase-3 activity was 20 pmol/mg/min. Treatment of AH66 cells with LL-GSH–DXR and DL-GSH–DXR showed a dose-dependent and marked increase in caspase-3 activity and DNA fragmentation compared with the control cells (Figure 2a and b). By contrast, LD-GSH–DXR,

Table 1. Difference in substrate specificity of GST toward stereoisomers of GSH

	LL-GSH	DL-GSH	LD-GSH	DD-GSH
GST activity (nmol/mg/min)	88.3±4.3	38.1±2.7	8.4±1.5	4.4±0.6

GST activity was measured at 340 nm in 1 mM CDNB, 1mM stereoisomers of GSH and 0.1 M sodium phosphate buffer (pH 6.5) at 37°C for 10 min with the cell suspension as the enzymatic source.

Table 2. Inhibitory effect of isomeric GSH–DXR on GST activity

	None	LL-GSH-DXR	DL-GSH-DXR	LD-GSH-DXR
GST activity (% of control activity)	83.1 (100)	26.6±4.3 (32)	12.5±2.7 (15)	50.7±1.5 (61)

GST activity (nmol/mg/min) was measured in 1 mM CDNB and 1 mM GSH at 37°C for 10 min with the cell suspensions as the enzymatic source in the presence or absence of 10 μM isomeric GSH–DXR. Each activity was expressed as percent of control activity. Results are means±SD (three independent experiments).

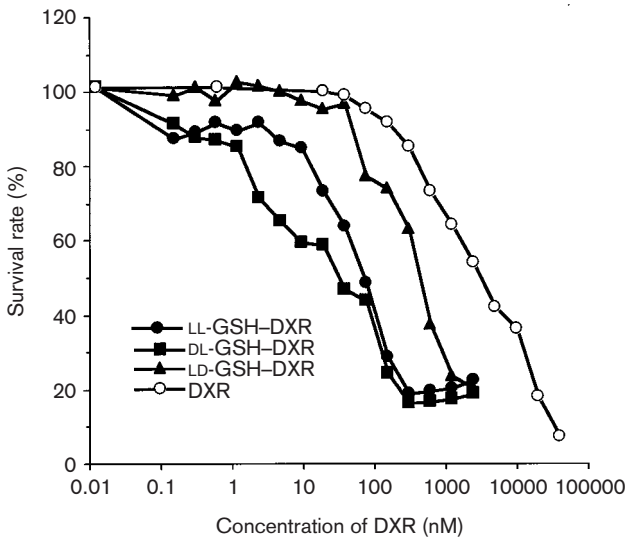


Figure 1. Cytotoxicity of isomeric GSH–DXR against AH66 cells. The concentration of the conjugate expressed as that of DXR. AH66 cells were cultured continuously for 72 h in a 48-well plate with 0.5 ml of growth medium containing graded equivalent concentrations of DXR. After incubation, viable cells were determined with the colorimetric assay using MTT.

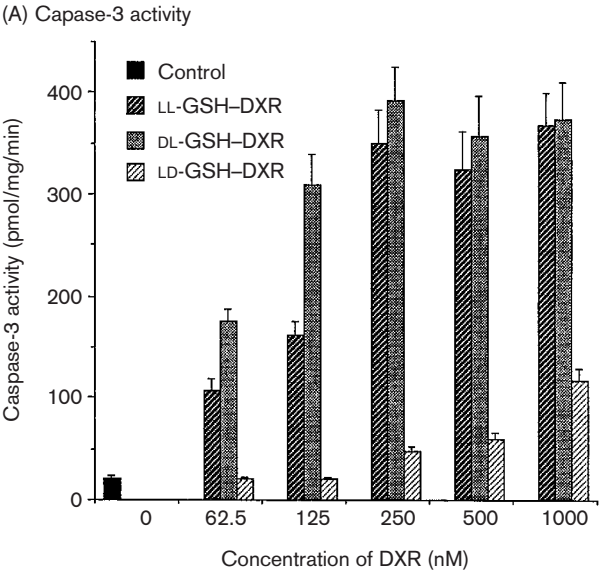
which is composed of the D-cysteine GSH residue, showed a moderate increase in caspase-3 activity up to 120 pmol/mg/min and detectable DNA fragmentation at an extremely high concentration of the conjugate.

Binding of conjugates to GST

To examine competitive binding inhibition of the conjugate with GSH, each conjugate was applied to a GST-coupled column and the amount of bound drug was estimated. LL-GSH–DXR, DL-GSH–DXR and LD-GSH–DXR were bound to the column at the ratios of 0.75, 0.63 and 0.46 mol per 1 mol of GST molecule, respectively (Figure 3). In order to confirm the substrate specificity of GST for GSH stereoisomers, the binding capacity of each isomeric GSH to GST was assayed in the same manner. LL-GSH, DL-GSH and LD-GSH were bound at the ratios of 1.05, 0.43 and 0.09 mol per 1 mol of GST molecule, respectively. These affinities corresponded to the substrate specificity for GSH isomers. Each conjugate, which bound to the GST molecule, was almost replaced to 10 mM GSH.

Discussion

We have synthesized a series of GSH stereoisomers of GSH, which is a substrate for compounds such as GST



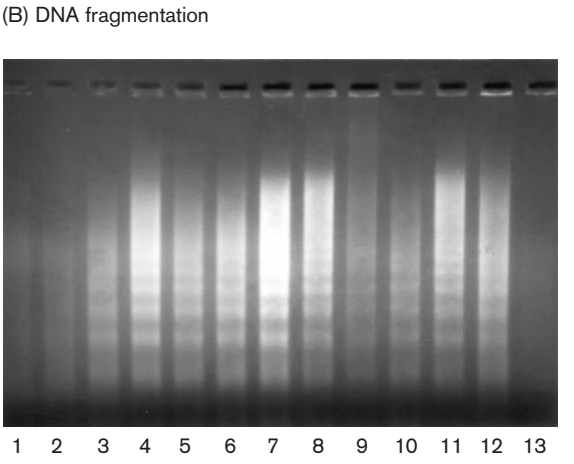


Figure 2. Induction of apoptosis in AH66 cells treated with isomeric GSH–DXR. (A) Caspase-3 activity, (B) DNA fragmentation. Lanes 1, 2, 3 and 4: 125, 250, 500 and 1000 nM LD-GSH–DXR, respectively. Lanes 5, 6, 7 and 8: 62.5, 125, 250 and 500 nM DL-GSH–DXR, respectively. Lanes 9, 10, 11 and 12: 62.5, 125, 250 and 500 nM LL-GSH–DXR, respectively. Lane 13: no treatment. Results are means ± SD (three independent experiments).

and γ-glutamyl transpeptidase. In a previous report, it was shown that LL-GSH and DL-GSH served as substrates, though the DD and LD isomers were inert as substrates in the reaction of GST.[14] In this report, it was confirmed that the L-cysteinyl residue in the substrate was important for the expression of GST activity and that the D configuration of the γ-glutamyl moiety was not as important in binding of the active center of GST. This result is in agreement with the findings of experiments using the GSH analogs, α-L-

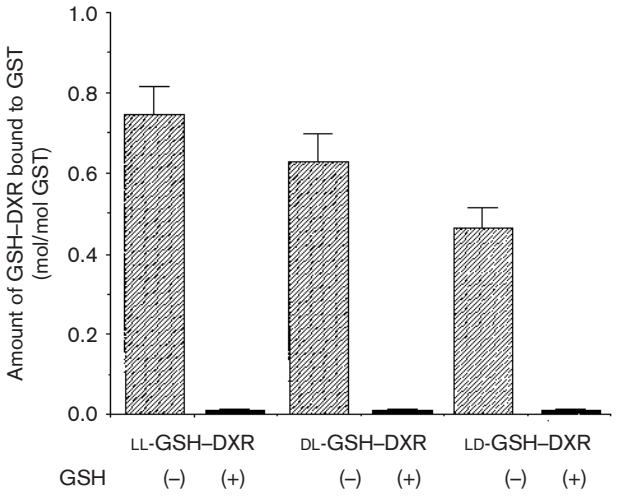


Figure 3. Binding of isomeric GSH–DXR to a purified GST. Binding of GSH–DXR to a GST–Sepharose column was measured. The concentration of DXR in the eluate by 10 mM GSH was expressed as the substitution of GSH–DXR for GSH. Results are means ± SD (three independent experiments).

Glu-L-Cys-Gly, α-D-Glu-L-Cys-Gly and γ-D-Glu-L-Cys-Gly, as substrates in the reaction of GST isozyme 3-3 from rat liver.[18]

Since the previous studies revealed that GSH–DXR exhibited superior cytotoxicity against both DXR-sensitive and -resistant cells relative to DXR,[12] and that the conjugate, but not DXR, inhibited GST activity strongly,[13] it was presumed that the expression of potent cytotoxicity of GSH–DXR was dependent on the inhibition of GST enzyme activity and the conjugate bound to the active center of the GST molecule. Therefore, we examined the molecular interaction between GST and the conjugates of DXR with GSH stereoisomers, which differed in the GST substrate specificity described above.[14] The treatment of AH66 cells with LL-GSH–DXR and DL-GSH–DXR exhibited potent cytotoxicity compared to that obtained with DXR, although treatment with LD-GSH–DXR did not lead to the enhancement of cytotoxicity. However, DD-GSH could not be conjugated with DXR due to the difference in their stereostructures. It was suggested that the DD configuration of both the γ-glutamyl and cysteinyl moieties interfered with the conjugation of DXR. The notion of possible interference of the conjugation of DXR with DD-GSH is supported by reports[14,18,19] that DD-GSH did not serve as a substrate of γ-glutamyl transpeptidase and GST and DD-GSSG (oxidized glutathione) did not act as a substrate of glutathione reductase. Although the cytotoxicity of DD-GSH–DXR could not be obtained because the conjugate was not prepared, it was suggested that the L-cysteine residue of GSH was important for the expression of potent cytotoxicity of GSH–DXR and the cytotoxic efficacy of the conjugates corresponded to the substrate specificity of GST for GSH stereoisomers. Therefore, it was predicted that the conjugates interacted with the active center of the GST molecule and inhibited the enzyme activity. Aliquots of 10 μM of LL-GSH–DXR and DL-GSH–DXR inhibited the activity to 32 and 15% of that in the absence of the conjugate. In contrast, the activity of GST in the presence of 10 μM LD-GSH–DXR was inhibited to only 61% of the control level. This inhibitory effect of each conjugate on GST activity correlated well with the cytocidal activity. The inhibitory effect of the conjugated DXR with GSH isomers on the GST activity was demonstrated dose dependently. This result suggested that the conjugate bound to the active center of the GST molecule via the L-cysteine residue, resulting in the inhibition of enzyme activity. In order to confirm this possibility, the binding capacity of each conjugate to the GST molecule using a GST-coupled column was examined. LL-GSH–DXR, DL-GSH–DXR and LD-GSH–DXR bound to the column at the ratios of 0.75, 0.63 and 0.46 mol per 1 mol GST molecule, respectively. The affinity of LL-GSH–DXR and DL-GSH–DXR to GST was higher than that of LD-GSH–DXR, and corresponded to the potency of GST inhibition by these conjugates (68, 85 and 39% inhibition, respectively) rather than to the substrate specificity for GSH isomers. Since each conjugate that bound to the GST molecule was almost replaced to 10 mM GSH in a reversible manner, but not to the isomers composed of the D-cysteine residue (data not shown), it was suggested that the conjugate showed competitive inhibition against GST activity. This result was supported by a previous report that GSH–DXR showed competitive inhibition by analysis of a Lineweaver–Burk plot.[13] Therefore, the interaction of the conjugates with the active center of the GST molecule via the L-cysteine residue of GSH must inhibit the enzyme activity and exhibit potent cytotoxicity.

We have reported that the treatment of AH66 cells with GSH–DXR induced potent apoptosis.[20] Although these conjugates also induced apoptosis (caspase-3 activation and DNA fragmentation in various degrees), DNA fragmentation induced by LD-GSH–DXR was slight as compared with that by LL-GSH–DXR or DL-GSH–DXR. Replacement of the L-cysteinyl residue for the D counterpart decreased both the expression of cytotoxicity and induction of apoptosis by the conjugate, thereby suggesting that GST inhibition by GSH–DXR induced the apoptosis.

Using GSH stereoisomers, which showed different affinities for the active center of the GST molecule, it was demonstrated that the conjugate of DXR with GSH exhibited potent cytotoxicity via GST inhibition.

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