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Drug conjugate of doxorubicin with glutathione is a potent reverser of multidrug resistance in rat hepatoma cells

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A recent study has suggested that degraded adducts smaller than 2 kDa in molecular weight of bovine serum albumin (BSA)-conjugated doxorubicin (DXR) (BSA–DXR) might exhibit cytotoxicity against multidrug resistant (MDR) cells. To investigate this notion further, intracellular accumulation and cytotoxicity of DXR coupled to several small peptides, such as glycylglycine (diGly), glycylglycylglycine (triGly), reduced glutathione (GSH) and oxidized glutathione (GSSG), were investigated using DXR-sensitive (AH66P) and DXR-resistant (AH66DR) rat hepatoma cell lines. Against both AH66P and AH66DR cells, diGly-conjugated DXR (diGly–DXR) and triGly-conjugated DXR (triGly–DXR) demonstrated the same cytotoxic activity as DXR, and the accumulation of both conjugates in the two cell lines was almost similar to that of DXR. After treatment of AH66DR cells with 5 μM verapamil [an inhibitor of P-glycoprotein (Pgp)], the intracellular levels of diGly–DXR and triGly–DXR were markedly increased and consequent cytotoxicity was improved. On the other hand, GSH-conjugated DXR (GSH–DXR) showed 9- and 7.5-fold more cytotoxic activity than BSA–DXR against AH66P and AH66DR cells, respectively. GSH–DXR accumulated rapidly in AH66DR cells, probably by the same mechanism as in AH66P cells, because the treatment of AH66DR cells with verapamil did not cause a significant increase in the intracellular drug level as compared with that in cells treated without verapamil. The levels of cytotoxicity and accumulation of GSSG–DXR were the same as those of BSA–DXR for both cell lines. These results indicate that GSH–DXR exerts potent cytotoxicity against both cell lines among the peptide–DXR conjugates examined because of the rapid uptake and high accumulation of GSH–DXR similar to that of DXR without efflux.

Key words: Doxorubicin, glutathione, multidrug resistance, P-glycoprotein, rat hepatoma cell.

Introduction

Multidrug resistance (MDR) is a major problem in cancer chemotherapy.[1,2] It has been reported that MDR can be reversed by a variety of substances, such as an inhibitor of the P-glycoprotein (Pgp) efflux pump and anti-Pgp antibody.[3–8] We have reported that bovine serum albumin (BSA)-conjugated doxorubicin (DXR) (BSA–DXR) increased cytotoxicity markedly against several MDR cell lines.[9–11] Our reports demonstrated that BSA–DXR was slowly internalized and not pumped out by Pgp, resulting in increased accumulation and improved cytotoxicity of the drug. Moreover, a recent study revealed that lysosomal breakdown of internalized BSA–DXR followed by liberation of the degraded active adducts of the conjugate that were smaller than 2 kDa in molecular weight must be an essential stage in the development of cytotoxicity against tumor cells with the MDR phenotype.[12] To confirm whether the small adduct exhibited cytotoxicity against MDR cells, the accumulation and cytotoxic activity of newly prepared conjugates of DXR with several small peptides were determined.

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Materials and methods

Materials

DXR was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). BSA, reduced glutathione (GSH), oxidized glutathione (GSSG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazorium bromide (MTT) and verapamil were obtained from Sigma (St Louis, MO). Dowex 50W × 8, glycylglycine (diGly), glycylglycylglycine (triGly), glutaraldehyde and Triton X-100 were purchased from Nakarai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Cell lines

Rat ascites hepatoma cell line, AH66P, and DXR-resistant mutant subline, AH66DR (10 μM DXR resistance), were cultured with RPMI 1640 containing 10% heat-inactivated fetal bovine serum (growth medium) under conventional conditions.[9]

Conjugation of DXR with various peptides

One mg of each peptide (diGly, triGly, GSH or GSSG 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. After incubation, the mixture was applied to Dowex 50W × 8 (H^+ form, 5 × 15 mm) and the conjugate of DXR with each peptide 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 used for the conjugation of DXR with peptides; the preparation was then separated by a Dowex 50W × 8 column. BSA–DXR was prepared as described previously.[9–11] All drugs were filtrated with a 0.45 μm syringe filter (Corning Coster Japan, Tokyo, Japan). The concentration of DXR was measured by absorbance at 495 nm. Each conjugate of DXR with diGly, triGly, GSH or GSSG was expressed as diGly–DXR, triGly–DXR, GSH–DXR or GSSG–DXR respectively.

Growth inhibitory effect of DXR and conjugates

To assess the growth inhibitory effect of the test materials, viable AH66P and AH66DR cells (2×10^4) were cultured continuously for 96 h in a 48-well culture plate (Corning Coster Japan) with 0.5 ml of growth media containing graded equivalent concentrations of DXR in the presence or absence of 5 μM verapamil. After incubation, viable cells were determined with the colorimetric assay using MTT as described previously[13] 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].

Accumulation of the drugs

After incubation of cells (1×10^6 cells/2 ml of growth media) with 5 μM DXR or conjugates for various periods of time in the presence or absence of 5 μM verapamil, the harvested and washed cells were sonicated mildly in ice-cold 10 mM Tris–HCl (pH 7.4). The plasma membrane was removed by centrifugation as described previously[14] and the resultant supernatant was mixed with the same volumes of 2% Triton X-100 in 20 mM sodium phosphate buffer (pH 7.4). DXR in the mixture, which represented intracellular DXR, was measured by fluorospectrometry set at an emission wavelength of 580 nm with an excitation wavelength of 470 nm using authentic DXR as a standard. The results were expressed by the following equation: drug accumulation rate (%) = 100 × [intracellular DXR (nmol) per 1×10^6 cells]/[DXR (nmol) added to media].

Results

Growth inhibitory effect of peptide-conjugated DXR

DiGly–DXR and tri-Gly–DXR exhibited the same cytotoxic activity as DXR against AH66P and AH66DR cells, respectively (Figure 1 and Table 1).

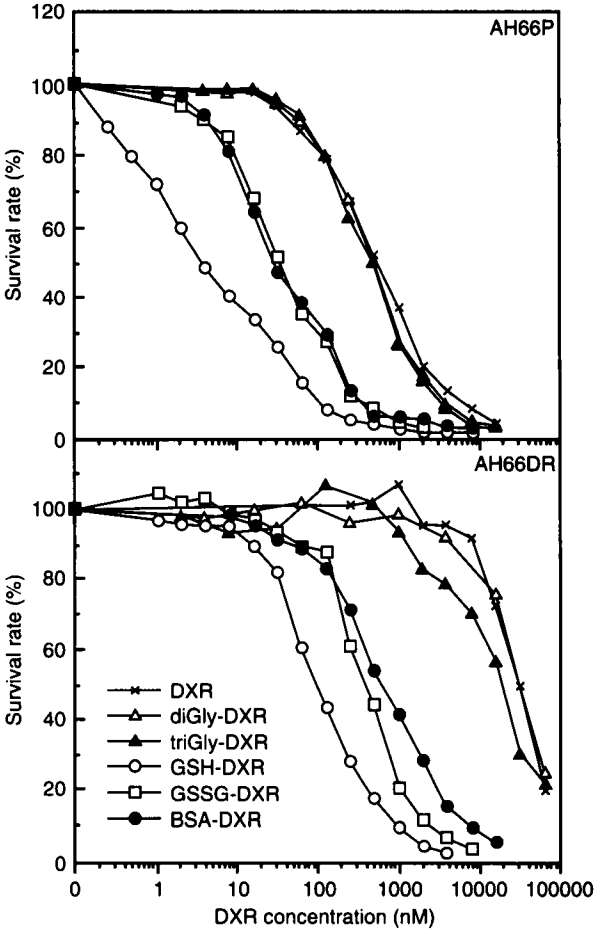

Figure 1. Cytotoxicity of DXR and peptide-conjugated DXR against AH66P and AH66DR cells. Cytotoxicity was expressed as survival rate versus equivalent concentrations of DXR. IC_{50} value for each drug is shown in Table 1.

Table 1. Effect of verapamil (VPL) on IC_{50} values for peptide-conjugated DXR and the drug accumulation rates in AH66P and AH66DR cells.

Drugs	IC_{50} values (nM)			Drug accumulation rates (%)		
	AH66P	AH66DR		AH66P	AH66DR	
	−VPL	−VPL	+VPL	−VPL	−VPL	+VPL
DXR	600 ± 90	32000 ± 15000	900 ± 190	17.1 ± 2.0	2.5 ± 0.8	14.3 ± 2.3
diGly-DXR	500 ± 120	30000 ± 10000	900 ± 120	17.9 ± 3.2	1.5 ± 0.6	15.4 ± 1.6
triGly-DXR	500 ± 70	20000 ± 5000	700 ± 210	16.9 ± 1.9	3.4 ± 1.1	13.9 ± 1.3
GSH-DXR	3.5 ± 1.1	80 ± 16	16 ± 4	15.0 ± 0.9	11.4 ± 1.6	14.0 ± 1.1
GSSG-DXR	30 ± 4.6	400 ± 70	50 ± 10	10.2 ± 2.1	10.3 ± 1.6	12.4 ± 2.2
BSA-DXR	30 ± 4.0	600 ± 90	40 ± 15	11.3 ± 1.8	9.7 ± 0.7	12.1 ± 1.5

Incubation was carried out in the presence or absence of 5 μM VPL. IC_{50} values were expressed as equivalent concentrations of DXR. Results are means ± SD (four or five independent experiments). Drug accumulation rate was expressed as intracellular DXR relative to DXR added to the medium in 24 h of incubation. For details, see Materials and methods.

When both sensitive and resistant cell lines were cultured with GSSG-DXR-containing media, the IC_{50} value for the drug was at the same level as that for BSA-DXR. By contrast, from the IC_{50} levels, it can be deduced that the cytotoxic activity of GSH-DXR was 9- and 7.5-fold greater than that of BSA-DXR against AH66P and AH66DR cells, respectively. None of the peptide-glutaraldehyde conjugates without DXR exhibited any cytotoxic activity against both AH66P and AH66DR cells up to 1 mM of equivalent concentrations of the peptide (data not shown).

Accumulation of peptide-conjugated DXR

In AH66P cells, the uptake and accumulation of diGly-DXR, triGly-DXR or GSH-DXR was similar to those of DXR (Figure 2). In AH66DR cells, conjugation of diGly or triGly to DXR did not increase the intracellular accumulation of the drugs; however, GSH-DXR accumulated rapidly in the cells with an almost similar uptake pattern to DXR accumulation in the AH66P cells (Figure 2). The intracellular accumulation of GSSG-DXR increased gradually up to 24 h in both AH66P and AH66DR cells, and was almost at the same level as that of BSA-DXR (Table 1).

Effect of verapamil

The accumulation of each of DXR, diGly-DXR or triGly-DXR in the AH66DR cells was markedly increased in the presence of verapamil, and the IC_{50} values of these drugs were at the same levels as the drugs used against AH66P cells treated without verapamil. When the AH66DR cells were treated with GSH-DXR, the addition of verapamil did not induce any significant increase in cytotoxicity nor in the intracellular amount of the drug as compared with that in the cells treated without verapamil. By contrast, the treatment of AH66DR cells with verapamil increased the cytotoxicity of BSA-DXR 15-fold as compared with the use of BSA-DXR against the cells treated without verapamil; however, the accumulation rate for the drug was increased only 2.4% in AH66DR cells. Treatment of AH66DR cells with verapamil did not show any cytotoxic activity up to the concentration of 10 μM (data not shown).

Discussion

In our previous report, we demonstrated that BSA-DXR taken up into the cells was degraded in lysosomes and the resultant active adducts exhibited cytotoxicity against MDR cells.[12] As our findings

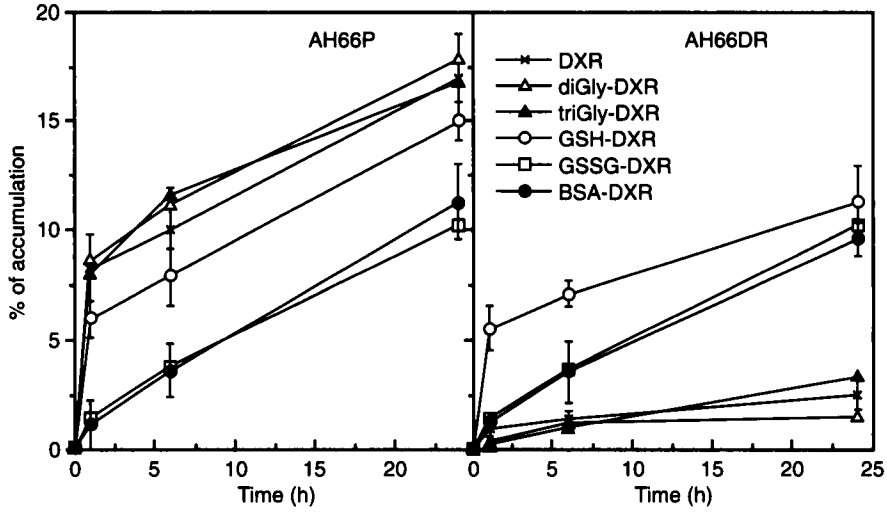

Figure 2. Time-dependent accumulation of DXR and peptide-conjugated DXR in AH66P and AH66DR cells. After incubation of the cells with 5 μM DXR and peptide-conjugated DXR, the internalized conjugate was measured by fluorospectrometry (Ex/Em = 470/580 nm). Drug accumulation rate was expressed as intracellular DXR relative to DXR added to the media. Results are means ± SD (three independent experiments).

strongly indicated that the adduct was a peptide–DXR complex with a molecular weight of less than 2 kDa, several peptides were coupled to DXR and their cytotoxic activities were evaluated using AH66P and AH66DR cells. GSH–DXR exhibited the most potent cytotoxic activity against AH66P as well as AH66DR cells among the peptide–DXR conjugates examined. By contrast, diGly–DXR, triGly–DXR and GSSG–DXR demonstrated only the same cytotoxic activity as DXR and BSA–DXR, respectively, against both cell lines.

To investigate whether or not Pgp caused efflux of these conjugates, the effect of verapamil on the accumulation and cytotoxicity of the drugs was determined using AH66DR cells. Co-treatment of the cells with DXR, diGly–DXR or triGly–DXR and verapamil caused a marked increase in intracellular drug level, leading to potent cytotoxicity against the cells. Consequently, IC_{50} values for DXR, diGly–DXR or triGly–DXR against AH66DR cells reached the same levels as those against the AH66P cells without verapamil treatment. Accordingly, these two peptide-conjugated DXR are most likely effluxed from AH66DR cells by the same mechanism via the Pgp pump as that for reduced retention of the intracellular DXR. By contrast, the addition of verapamil to the AH66DR cells failed to cause any further increase in the cytotoxicity of GSH–DXR compared to the cells cultured without verapamil. This result might be attributable to the fact that GSH–DXR accumulated in AH66DR cells with minimal efflux by Pgp because the treatment of AH66DR cells with verapamil caused only a slight increase in the intracellular GSH–DXR level compared to that in AH66DR cells treated without verapamil. On the other hand, co-treatment of the resistant cells with BSA–DXR and verapamil resulted in an extremely enhanced cytotoxicity (15-fold) as compared with treatment of the cells with only BSA–DXR despite the slight increase (approximately 2.4%) in intracellular drug accumulation. This discrepancy strongly indicated that part of the active adducts degraded from BSA–DXR might be transported out of the cells as increased accumulation of the adduct after treatment with verapamil led to potent cytotoxicity against AH66DR cells. From the results of the present study, we concluded that GSH–DXR accumulated rapidly in both AH66P and AH66DR cells with minimal efflux by Pgp, thereby resulting in potent cytotoxicity against both cell lines. Although the accumulation of GSH–DXR in AH66P cells was at a somewhat lower level than that of DXR, the cytotoxicity of GSH–DXR was 170-fold higher than that of DXR. GSH–DXR may exhibit cytotoxicity through some unknown mechanisms other than intercalation of DXR with DNA, such as antagonistic functions towards GSH and an inhibitory effect on topoisomerase II. Further investigation will be necessary to elucidate the precise role of GSH–DXR in the manifestation of cytotoxicity against tumor cells, including MDR cells.

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