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Cytosolic β-glycosidases for activation of glycoside prodrugs of daunorubicin

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Abstract

Human cytosolic β-glycosidase is a small monomeric enzyme that is active under physiological conditions, which might be ideal for enzyme–prodrug therapy. We have previously reported the synthesis of a galactoside (DNR-GlA3) and a glucoside (DNR-GsA3) prodrug of daunorubicin. In the present study, we established that cellular uptake of DNR-GlA3 and DNR-GsA3 was low in contrast to that of daunorubicin. Recombinant human β-glycosidase converted both prodrugs to daunorubicin as shown by liquid chromatography. The kinetics of the conversion of DNR-GlA3 and DNR-GsA3 by human β-glycosidase, however, was unfavorable as the K_m values were, respectively, 3- and 6-fold higher than those of another mammalian β-glycosidase of bovine origin. The V_{max} values were, respectively, 3.3 and 8.5 nmol/hr/mg as compared to 158.3 and 147.8 nmol/hr/mg of the bovine enzyme. Treatment of OVCAR-3 cells with human β-glycosidase (0.5 U/mL) and 0.5 μM DNR-GlA3 or DNR-GsA3 resulted in, respectively, 86 and 81% cell growth inhibition, while the prodrugs alone inhibited growth to only 19 and 1%. Treatment of cells with the bovine enzyme and the prodrugs inhibited cell growth more efficiently. We conclude that the endogenous intracellular β-glycosidase is not available for extracellular prodrug activation. Thus, the incorporation of the enzyme in enzyme–prodrug therapy might be an elegant approach to achieve tumor-specific prodrug conversion. The efficiency of glycoside prodrug conversion might be improved by design of a prodrug that is more readily activated by human β-glycosidase or by evolution of the enzyme into a mutant form that displays high activity towards these prodrugs.

Keywords: Anthracyclines; Cancer chemotherapy; Cytosolic β-glycosidase; Galactoside; Glucoside; Prodrug

1. Introduction

The selective activation of a relatively non-toxic prodrug by an enzyme present in the tumor tissue should enhance the drug concentration in the tumor as compared to the active drug given by a systemic route. The activating enzyme should be localized exclusively in the tumor or have a relatively high ratio in the tumor vs. normal tissues and blood. The latter can be achieved by targeting the enzyme to the tumor using an antibody directed against a tumor antigen (ADEPT) [1]. Alternatively, the gene encoding the enzyme can be used to transduce tumor cells (GDEPT) [2,3].

The enzyme in use for selective prodrug activation should ideally have the following properties. First, it should not be available for activation of the prodrug in normal tissue. Second, the activating enzyme should be able to effect high catalytic activity under physiological conditions. Third, the enzyme should preferably be active as a small monomer, because a large molecule is hampered in its diffusion through the tumor. Finally, for use in ADEPT, the enzyme should be easily produced in large quantities. Therefore, the enzyme should preferably not

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Abbreviations: ADEPT, antibody-directed enzyme–prodrug therapy; GDEPT, gene-directed enzyme–prodrug therapy; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; 4-MuGal, 4-methylumbelliferyl-β-D-galactopyranoside; 4-MuGlc, 4-methylumbelliferyl-β-D-glucopyranoside; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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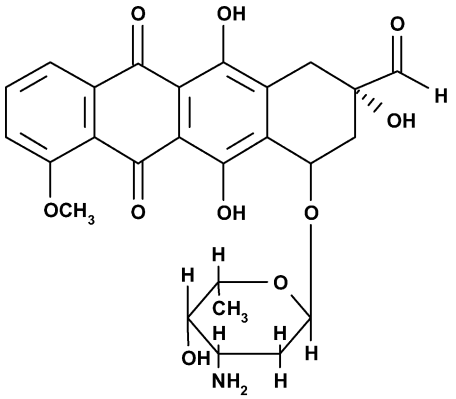
need posttranslational modifications, such as *N*-glycosylation, because this would confine production of the protein to a mammalian system instead of the more cost-effective bacterial or yeast systems.

To meet the requirement that the prodrug-converting enzyme should not be available for activation of the prodrug in normal tissues, enzymes of non-mammalian origin, for example obtained from bacteria or viruses, such as carboxypeptidase G2 [4], cytosine deaminase [5] or thymidine kinase [6] could be used. The main disadvantage of these enzymes, however, is their potential to elicit an immune response in humans [7]. A challenging alternative option is the use of human enzymes of which the endogenous counterpart is not accessible to the prodrug, like human lysosomal β-glucuronidase. The intracellular enzyme, which has been successfully used in ADEPT [8] and GDEPT [9–11], is not available for activation of the highly hydrophilic glucuronide prodrugs due to their low cellular uptake [12]. Human β-glucuronidase, however, is not the most optimal enzyme for enzyme prodrug therapy. It is active at a low pH, and displays only 10% activity at a neutral pH. In addition, it is active as a tetramer of about 300 kDa and it needs to be glycosylated to exert its activity.

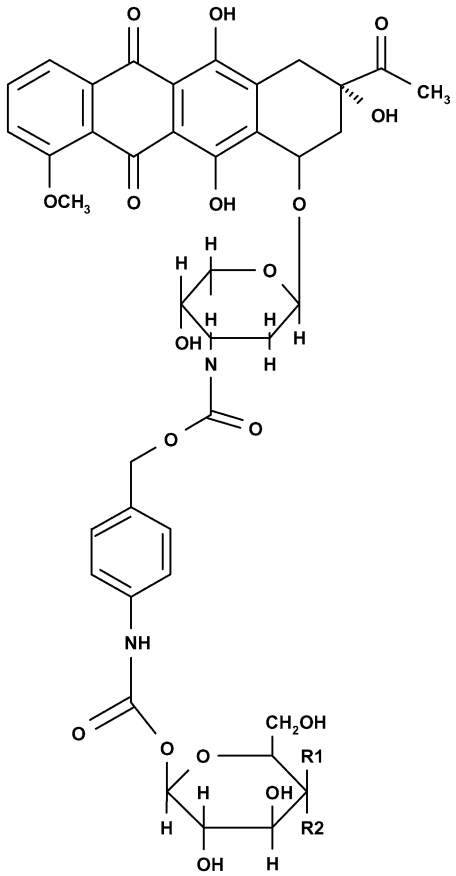
We hypothesized that human cytosolic β-glycosidase could be an ideal enzyme for induction of cell death by conversion of a glycoside prodrug into a toxic drug. First, the endogenous human enzyme is present in the cytosol of liver, spleen, intestinal cells and lymphocytes and is probably not accessible for a glycoside prodrug in the circulation. A glycoside prodrug will likely remain extracellularly, just as glucuronide prodrugs [12]. Second, human cytosolic β-glycosidase efficiently hydrolyzes the galactoside and glucoside substrates, 4-MuGal and 4-MuGlc, under physiological pH and temperature. Finally, it is a small monomeric protein of 55 kDa that does not need glycosylation for activity [13].

Human cytosolic β-glycosidase is expected to activate prodrugs possessing a β-galactoside or β-glucoside promoiety. The β-glycosidase from the plant sweet almond has already been used *in vitro* for tumor-specific activation of the naturally occurring cyanogenic glucoside, amygdalin [14]. Galactoside and glucoside prodrugs of daunorubicin, designated DNR-GlA3 and DNR-GsA3, respectively, have been synthesized for application in selective chemotherapy (Fig. 1). These prodrugs have been shown to be approximately 20 times less toxic than the active drug daunorubicin and they can be activated by bovine liver β-galactosidase, which we will further refer to as bovine β-glycosidase as it displays both β-D-galactosidase and β-D-glucosidase activity [15]. Similar galactoside prodrugs of daunorubicin have been shown to be activated by *Escherichia coli* β-galactosidase [16].

In the current study, we investigated the usefulness of human β-glycosidase for selective activation of DNR-GsA3 and DNR-GlA3 and compared its efficiency with that of bovine β-glycosidase.



Daunorubicin



DNR-GlA3: R1 = H, R2 = OH
DNR-GsA3: R1 = OH, R2 = H

Fig. 1. Molecular structures of daunorubicin and its galactoside and glucoside prodrugs, *N*-[4-daunorubicin-*N*-carbonyl(oxymethyl)phenyl] *O*-β-galactosyl carbamate (DNR-GlA3) and *N*-[4-daunorubicin-*N*-carbonyl(oxymethyl)phenyl] *O*-β-glucosyl carbamate (DNR-GsA3), respectively.

2. Materials and methods

2.1. Cell culture

The COS-7 and CHO cell lines were obtained from the American Type Culture Collection and were grown in DMEM (Life Technologies) supplemented with 5%

heat-inactivated fetal calf serum (Life Technologies), 50 IU/mL penicillin and 50 μg/mL streptomycin (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°. The human ovarian cancer cell line OVCAR-3 [17] was grown under similar conditions, but supplemented with 10% heat-inactivated fetal calf serum.

2.2. Characterization of glucoside and galactoside prodrugs of daunorubicin

Stock solutions of galactoside and glucoside prodrug of daunorubicin, DNR-GlA3 (11.7 mM) and DNR-GsA3 (15.6 mM), respectively, were prepared in DMSO. The purity and stability of DNR-GlA3 and DNR-GsA3 (10 μM) were determined in PBS with 0.1% BSA at pH 7.4 at 37°. Samples (20 μL) were taken at different time-points. Sample preparation for HPLC analysis was performed as described in the next section.

The uptake of daunorubicin (Société Parisienne d'Expansion Chimique), DNR-GlA3 and DNR-GsA3 was measured in OVCAR-3 cells. The prodrugs DNR-GlA3 or DNR-GsA3 were synthesized as described [15]. Cells were harvested and resuspended in MEM without Phenol Red supplemented with 0.1% BSA to a concentration of 2.7×10^7 cells/mL. An aliquot of 75 μL of drug or prodrug at 40 μM in MEM/0.1% BSA was added to 75 μL of cells. After a 30-min incubation at 37°, supernatants were collected for analysis and the cells were washed three times with ice-cold PBS. The cells were dissolved in 150 μL PBS, freeze–thawed three times and sonicated for 5 min. Undissolved cell particles were removed by centrifugation at 16,000 *g* for 4 min. The samples were analyzed in a spectrophotometer (UltrospecIII, Pharmacia) at 495 nm. The amount of (pro)drug in the cells and supernatants was expressed as a percentage of the amount of (pro)drug in cells after a 0-min incubation. The experiment was performed four times in duplicate samples.

The lipophilicity of DNR-GlA3 and DNR-GsA3 was analyzed by determination of the octanol/PBS partition coefficient. Daunorubicin was used as control drug. The (pro)drugs were dissolved in 1 mL octanol to a concentration of 10 μM and, then, 1 mL PBS was added. The mixture was incubated for 3 hr at 37° under continuous rigorous shaking (300 rpm). The phases were separated and the ratio of (pro)drug in octanol and PBS was determined by measuring the absorbance of both phases at 495 nm (UltrospecIII, Pharmacia). The octanol/PBS partition coefficient was calculated after subtraction of the background from octanol and PBS. The experiment was carried out three times.

2.3. HPLC

Samples (10 μL) were treated by the addition of 90 μL acetonitrile in order to precipitate proteins. After incubation at −20° for 10 min, samples were centrifuged at 14,000 *g* for 10 min. The supernatant (80 μL) was collected and 80 μL phosphate buffer (0.5 mM triethylamine/20 mM sodium phosphate) was added. Samples were stored at −20° until HPLC analysis.

Samples (50 μL) were loaded on a HPLC C18 reversed-phase column (Chromsep, 2 mm × 100 mm × 4.6 mm, 3 μm particle size; Chrompack). The drug or prodrugs were eluted from the column with 0.5 mM triethylamine/20 mM sodium phosphate and 50% acetonitrile and detected with a fluorescence detector (Jasco, Model 821-FP; Separations) using an excitation wavelength of 480 nm and an emission wavelength of 590 nm. The elution peaks in the chromatograms were integrated and calculated using the Gyncosoft program (Gynkotek, Version 5.3E).

2.4. Expression and purification of cytosolic β-glycosidase

For transient expression of human cytosolic β-glycosidase, the mammalian expression vector containing the cDNA encoding the enzyme with myc- and 6His-tag [18] was used to transfect COS-7 cells. The expression vector is further referred to as pGlyc. The cells were transfected with pGlyc using Lipofectamine Plus reagent, according to the protocol provided by the manufacturer (Life Technologies). Cells were grown for 48 hr before harvesting. The cells were taken up in PBS and lysed by freeze–thawing in liquid nitrogen for three times. The expressed enzyme was purified by a nickel-nitrilotriacetate spin column procedure (Qiagen) according to the manufacturer's instructions for purification of proteins containing a 6His-tag under native conditions. Purity was checked by SDS–PAGE and Coomassie Blue staining. For calculation of the specific activity of purified human cytosolic β-glycosidase, the enzyme activity was determined as described below and the protein concentration was measured using the BCA protein assay kit (Pierce) according to the protocol.

2.5. Enzyme activity assay

The β-D-galactosidase activity from the β-glycosidase enzyme was measured using 4-MuGal (Fluka) as substrate. The reactions contained 1.0 mM 4-MuGal in PBS with 0.1% BSA and up to 10 μL purified β-glycosidase or cell lysate or supernatant of transfected cells, in a final volume of 100 μL at 37°. The reaction was stopped after 1 hr incubation, by adding half the reaction volume to 1 mL 0.1 M glycine/NaOH buffer, pH 10.6. Substrate conversion to 4-methylumbelliferone was measured in a fluorescence spectrometer (Perkin-Elmer) at an excitation wavelength of 360 nm, and an emission wavelength of 470 nm.

One unit of β-glycosidase was defined as the amount of enzyme required for a similar extent of conversion of the substrate 4-MuGal as observed from one unit of bovine liver β-galactosidase (0.17 U/mg protein; Sigma Chemical Co.), further referred to as bovine β-glycosidase, after incubation for 1 hr at 37°.

2.6. Prodrug–enzyme kinetics

DNR-GlA3 and DNR-GsA3 (concentration range from 0.0025 to 1 mM) were incubated with purified cytosolic β-glycosidase or an equal amount of β-D-galactosidase activity of bovine β-glycosidase (0.25 U/mL) for determination of the K_m and V_{max} values. Samples (10 μL) were taken after 15 hr for the human enzyme and after 8 min for the bovine enzyme and prepared for HPLC analysis as described. The experiment was repeated two to four times for the different enzyme–prodrug combinations.

2.7. In vitro antiproliferative effects

OVCAR-3 cells were seeded in a 96-wells microtiter plate at 5000 cells/well in triplicate. After 24 hr, DNR-GlA3 or DNR-GsA3 was added in a final concentration of 0.5 μM in the culture medium with or without purified human β-glycosidase (0.05 or 0.5 U/mL). After 72 hr, the wells were incubated with the cell proliferation reagent WST-1 (Roche), a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases [19], for 1 hr at 37°. The absorbance was measured at a wavelength of 450 nm. The antiproliferative effects in treated cells were expressed as a percentage of control cell growth.

3. Results

3.1. Characterization of glucoside and galactoside prodrugs of daunorubicin

The stability was >95% for both prodrugs after a 24-hr incubation period in PBS/0.1% BSA as well as in human serum, both tested at 37°. In order to demonstrate that the glycoside prodrugs of daunorubicin remain extracellularly, we determined the uptake of DNR-GlA3 and DNR-GsA3 in OVCAR-3 cells. The cellular uptake of DNR-GlA3 and DNR-GsA3 was low as compared to that of daunorubicin (Fig. 2). While most of the DNR-GlA3 and DNR-GsA3 could be measured in the supernatant (76.3 and 75.5%, respectively), most of the daunorubicin could be detected in the cellular fraction (66.9%).

The lipophilicity of the prodrugs and the parent drug was measured by the octanol/PBS partition coefficient. The values (±SD) for the partition coefficient of daunorubicin and the prodrugs, DNR-GlA3 and DNR-GsA3, were 14.3 (±0.1), 18.9 (±5.73), and 14.7 (±1.65), respectively, and they were not significantly different (ANOVA, $P > 0.05$).

3.2. Activation of glucoside and galactoside prodrugs of daunorubicin by human β-glycosidase

Recombinant human liver cytosolic β-glycosidase has β-D-galactosidase and β-D-glucosidase activity [18]. Therefore, this enzyme is expected to activate the daunorubicin prodrugs DNR-GlA3 and DNR-GsA3 that contain a β-D-galactoside and a β-D-glucoside promoiety, respectively (Fig. 1) [15]. Human cytosolic β-glycosidase purified from COS-7 cells transfected with pGlyc [18] had a specific activity of 1.45×10^6 nmol/hr/mg for the substrate 4-MuGal. For comparison, an approximately three times lower specific activity (4.25×10^5 nmol/hr/mg) has been reported for human cytosolic β-glycosidase purified from human liver [13]. To determine the conversion of DNR-GlA3 and DNR-GsA3 into daunorubicin by human β-glycosidase, the prodrugs were incubated with 0.8 U/mL of the purified enzyme and daunorubicin production was measured by HPLC (Fig. 3). Approximately 75% of DNR-GlA3 and 90% of DNR-GsA3 was hydrolyzed to daunorubicin. Using similar experimental conditions, we have demonstrated that bovine β-glycosidase could completely convert DNR-GlA3 and DNR-GsA3 into daunorubicin at a

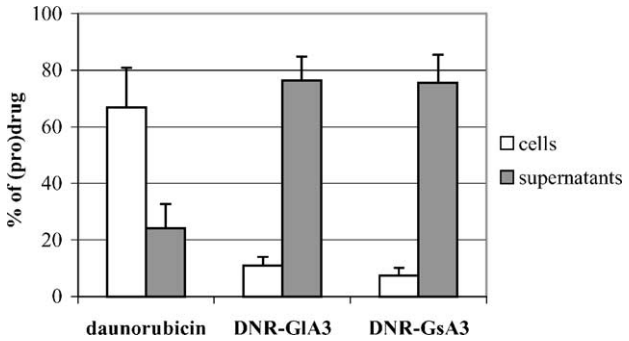


Fig. 2. Uptake of daunorubicin, DNR-GlA3 and DNR-GsA3 in OVCAR-3 cells. OVCAR-3 cells (2×10^6) were incubated with daunorubicin, DNR-GlA3 and DNR-GsA3 (20 μM) at 37° for 30 min. The amount of (pro)drug in the cells and the supernatants was determined by a spectrophotometer at 495 nm and expressed as a percentage of the amount of (pro)drug at the start of the experiment. The experiment was performed four times in duplicate samples. Bars: ±SEM.

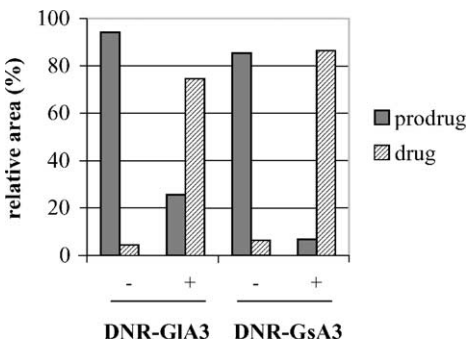


Fig. 3. HPLC analysis of the hydrolysis of DNR-GlA3 or DNR-GsA3 to daunorubicin by human β-glycosidase. DNR-GlA3 and DNR-GsA3 (10 μM) were incubated with purified human cytosolic β-glycosidase (0.8 U/mL) (+) or no enzyme (−). The amount of β-D-galactosidase activity of the enzyme used in the reactions was determined by 4-MuGal conversion at neutral pH and 37°. Samples were analyzed by HPLC after overnight incubation at 37° and a neutral pH. The peak areas of DNR-GlA3, DNR-GsA3, and daunorubicin were expressed as a percentage of the total peak area in a sample. Data are shown from one experiment.

Table 1
Enzyme kinetics of human and bovine β-glycosidase for DNR-GlA3 and DNR-GsA3 prodrugs

Enzyme	K_m (mM) ± SEM		V_{max} (nmol/hr/mg) ± SEM	
	DNR-GlA3	DNR-GsA3	DNR-GlA3	DNR-GsA3
Human β-glycosidase	0.16 ± 0.02	0.24 ± 0.03	3.3 ± 0.2	8.5 ± 0.9
Bovine β-glycosidase	0.05 ± 0.02	0.04 ± 0.01	158.3 ± 23.9	147.8 ± 15.3

Equal amounts of β-D-galactosidase activity (0.25 U/mL) of purified human and bovine β-glycosidase were incubated with a range of concentrations of DNR-GlA3 and DNR-GsA3. Kinetic constants were derived from linear Lineweaver–Burk plots.

much lower concentration [15]. Thus, the human enzyme was less efficient in the activation of the DNR-GlA3 and DNR-GsA3 prodrugs than the bovine species.

To directly compare the kinetics of prodrug hydrolysis by both enzymes, we determined their K_m and V_{max} values for DNR-GlA3 and DNR-GsA3 (Table 1). A low K_m and a high V_{max} would be an indication for relatively efficient conversion reactions. The K_m values of human β-glycosidase for the prodrugs were higher than those of the bovine enzyme, i.e. approximately 3- and 6-fold for DNR-GlA3 and DNR-GsA3, respectively. Furthermore, the V_{max} values of the human enzyme were considerably lower than those of the bovine enzyme, i.e. approximately 50- and 20-fold for DNR-GlA3 and DNR-GsA3, respectively.

3.3. Cytotoxicity of human cytosolic β-glycosidase with glucoside and galactoside prodrugs of daunorubicin

OVCAR-3 cells were incubated with human β-glycosidase (0.5 U/mL) and DNR-GlA3 or DNR-GsA3 prodrugs at a concentration of 0.5 μM. At this concentration, daunorubicin gives almost 100% growth inhibition (Fig. 4). OVCAR-3 cell growth was inhibited by approximately 19 or 1% after treatment, respectively, with DNR-GlA3 or DNR-GsA3 alone. The incubation of cells with either prodrug in combination with human β-glycosidase resulted in, respectively, 86 and 81% growth inhibition. Human β-glycosidase alone had no effect on cell growth.

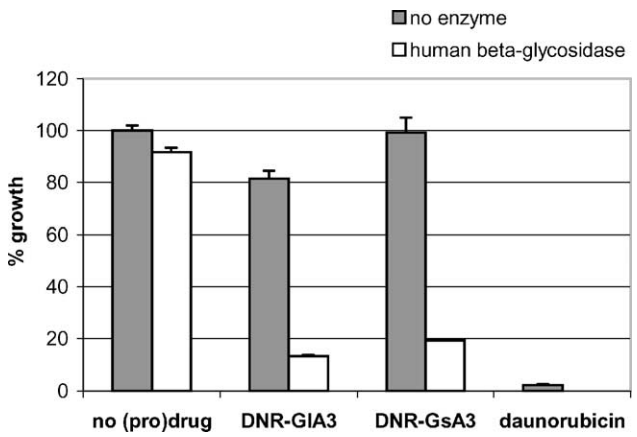


Fig. 4. Antiproliferative effects of daunorubicin or DNR-GlA3 or DNR-GsA3 without or with human cytosolic β-glycosidase on OVCAR-3 cells. Cells were plated at 5000 cells/well. After 24 hr, prodrug was added in a final concentration of 0.5 μM in the culture medium with or without purified human β-glycosidase (0.5 U/mL). As a control, treatment with 0.5 μM daunorubicin was included. After 72 hr, cell growth was measured with the cell proliferation reagent, WST-1, and the growth inhibitory effect was determined. Growth of treated cells was expressed as a percentage of control cell growth. Data are shown from one experiment carried out in triplicate wells. Bars: ±SD.

Treatment of cells with the bovine enzyme and the glycoside prodrugs resulted in almost 100% cell growth inhibition at a 10 times lower concentration of activity of 0.05 U/mL due to more efficient prodrug conversion (data not shown). At this enzyme concentration, the combination of human β-glycosidase and the DNR-GlA3 or DNR-GsA3 prodrugs resulted in a very limited growth inhibition of approximately 10% more than that after treatment with DNR-GlA3 or DNR-GsA3 alone (data not shown).

4. Discussion

We investigated the suitability of a neutral β-glycosidase, namely, human cytosolic β-glycosidase, in combination with the previously synthesized glycoside prodrugs of daunorubicin, DNR-GlA3 and DNR-GsA3 [15], for enzyme–prodrug therapy. In this study, we established that DNR-GlA3 and DNR-GsA3 were stable in human serum. Thus, these glycoside prodrugs are not expected to show spontaneous conversion in the *in vivo* situation. Furthermore, DNR-GlA3 and DNR-GsA3 remained extracellularly (Fig. 2). In other words, the endogenous intracellular β-glycosidase is not available for prodrug activation. The glycoside prodrugs can only be activated by exogenous enzyme that is present outside the tumor cells conferring specificity for this enzyme–prodrug system. In contrast, the parent drug, daunorubicin, liberated from the prodrugs, was readily taken up by the cells strongly limiting its leakage into the circulation. The reason for the difference in cellular uptake is not clear. It could not be attributed to a difference in lipophilicity, because the partition coefficients in octanol/PBS were similar. It might be explained by the difference in intracellular binding between daunorubicin and the larger glycoside prodrugs of daunorubicin. The high binding of daunorubicin is associated with a high apparent intracellular volume for this drug [20].

The purified human β-glycosidase could hydrolyze the prodrugs DNR-GlA3 and DNR-GsA3 to daunorubicin (Fig. 3). Another neutral β-glycosidase, bovine β-glycosidase, was more efficient in the conversion of the

prodrugs, as judged from the K_m and V_{max} values, while the same amount of β-D-galactosidase activity was used as measured by 4-MuGal conversion (Table 1). These observations indicated that the generation of daunorubicin from DNR-GlA3 or DNR-GsA3 by human cytosolic β-glycosidase was less efficient. It seems that this human enzyme has different substrate specificity than the bovine enzyme. In other words, DNR-GlA3 and DNR-GsA3 are more suitable substrates for bovine liver β-glycosidase than for human cytosolic β-glycosidase.

Human ovarian cancer cell growth was inhibited by exposure to human β-glycosidase and DNR-GlA3 and DNR-GsA3 (Fig. 4). Treatment of cells with the bovine enzyme, however, resulted in almost complete cell growth inhibition at a 10 times lower concentration of enzyme activity (0.05 U/mL). At the same enzyme concentration, cell growth after treatment with the human enzyme in combination with the prodrugs was inhibited only approximately 10% more than after treatment with the prodrugs alone. These results were in agreement with the relatively low efficiency of hydrolysis of DNR-GlA3 and DNR-GsA3 by the human enzyme as compared to that by the bovine enzyme.

A considerable bystander effect *in vivo* has been demonstrated as a result of extracellular prodrug conversion by tumor-selective expression of a cell-surface tethered or secreted enzyme in suicide gene therapy [10,11,21]. For the possible use of human cytosolic β-glycosidase for activation of an extracellular glycoside prodrug in suicide gene therapy, we made a construct, psGlyc, in which the gene encoding human cytosolic β-glycosidase is preceded by a signal sequence for secretion. COS-7 and CHO cells transfected with psGlyc showed expression of active human cytosolic β-glycosidase, but no secretion of the active enzyme as judged by Western blot analysis and an enzyme activity assay (data not shown). The same expression vector containing the sequence encoding the IgGκ leader peptide has been successfully employed to obtain a secreted version of other proteins that are normally not secreted [22]. Therefore, the reason for the lack of secretion of human β-glycosidase can probably not be explained by the way the construct was made, but should be sought into the sequence encoding the enzyme.

In conclusion, cytosolic β-glycosidases have favorable characteristics for enzyme–prodrug therapy. Human cytosolic β-glycosidase has the advantage that repeated administration is possible in contrast to the enzyme of bovine origin. Whether or not the efficacy of DNR-GlA3 and DNR-GsA3 conversion in combination with this human enzyme is sufficient to support its incorporation in targeted enzyme–prodrug therapy remains to be shown in a more complex pharmacokinetic model. It might be possible to improve the efficacy of prodrug conversion by designing a prodrug that is more readily activated by human cytosolic β-glycosidase. For example, the use of elongated spacers in anticancer prodrugs has been reported to result in enhanced drug release, possibly because of reduced steric hindrance [23]. Another possibility might be the mutagenesis of the gene encoding human cytosolic β-glycosidase to obtain a form of the enzyme that displays higher activity towards DNR-GlA3 and DNR-GsA3 (for review see [24]). For site-directed mutagenesis, knowledge of the sequence encoding bovine β-glycosidase would be useful as this enzyme efficiently activates DNR-GlA3 and DNR-GsA3. For use in GDEPT, mutagenesis might also be considered to obtain a version of human β-glycosidase that is secreted by human malignant cells.

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