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Characterization of Novel Anthracycline Prodrugs Activated by Human β-glucuronidase for Use in Antibody-Directed Enzyme Prodrug Therapy

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ABSTRACT. Antibody-directed enzyme prodrug therapy (ADEPT) aims at the specific activation of a prodrug by an enzyme-immuoconjugate localized in tumor tissue. The use of an enzyme of human origin is preferable in ADEPT because it might not be immunogenic when administered to patients. In the case of human β-glucuronidase, prodrugs should be designed that are rapidly and completely activated at a neutral pH. Four new daunorubicin glucuronides were synthesized by coupling a glucuronide group to daunorubicin *via* an aliphatic (GA1 and GB1) or an aromatic (GA3, GB6) carbamate spacer, to be released by electron shift (A-type) or by ring closure (B-type). These prodrugs were characterized *in vitro* for their usefulness in ADEPT and were compared with the previously described prodrugs epirubicin-glucuronide and doxorubicin-nitrophenyl-glucuronide. The four new prodrugs were stable in serum, hydrophilic when compared to the lipophilic daunorubicin, and at least 20-fold less toxic than the parent compound. The hydrolysis rate at clinically relevant enzyme and prodrug concentrations (1 μg/mL human β-glucuronidase, 100 μM prodrug) at pH 6.8 were similar for GA3 ($T_{1/2}$ 160 min) and higher for GB6 ($T_{1/2}$ 40 min) when compared to that of doxorubicin-nitrophenyl-glucuronide ($T_{1/2}$ 170 min). Epirubicin-glucuronide, GA1, and GB1 showed a low hydrolysis rate ($T_{1/2}$ > 400 min). GA1 and GA3, but not GB1 or GB6, were activated to the parent compound. Complete activation was confirmed in OVCAR-3 cells pretreated with a specific antibody-human β-glucuronidase conjugate, where GA3 had similar antiproliferative effects to those of daunorubicin. BIOCHEM PHARMACOL 52;3:455–463, 1996.

KEY WORDS. anthracycline-glucuronide prodrugs; daunorubicin; human β-glucuronidase; ADEPT

The efficacy of chemotherapy for cancer is limited by the occurrence of side effects, because drugs do not distinguish neoplastic cells from normal cells. New methods to increase the selectivity of anticancer agents are under study, including the use of monoclonal antibodies to target cytotoxic treatment to tumor cells. Currently, ADEPT†, in which an antibody targets an enzyme to the tumor site, has been developed [1]. After the antibody-enzyme conjugate has localized into the tumor, a relatively nontoxic prodrug can be converted by the enzyme into an active drug. The generated drug can penetrate into all tumor cells, including those that do not express the relevant antigen.

We have chosen human β-glucuronidase as an enzyme, because it might not be immunogenic upon administration to patients. Human β-glucuronidase is mainly found in the lysosomes and microsomes of normal tissues, and plasma levels of β-glucuronidase are very low [2, 3]. Further, we selected anthracyclines to be used as glucuronidated prodrugs. These drugs are known to be active in a number of tumor types and can enter rapidly into tumor cells.

Four new prodrugs of daunorubicin were synthesized and investigated for their usefulness in ADEPT. The daunorubicin-spacer-glucuronides were designed to be chemically stable in serum, have a low cellular uptake and low antiproliferative effects, and be efficiently activated to the parent drug upon hydrolysis by human β-glucuronidase at a near-neutral pH. The daunorubicin-spacer-glucuronides were synthesized by attaching a spacer molecule to the glucuronide *via* a carbamate linkage [4]. The carbamate spacers between the lipophilic anthracycline and the hydrophilic glucuronide moieties were designed to be released spontaneously from the drug after removal of the glucuronide moiety by human β-glucuronidase-mediated hydrolysis.

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†*Abbreviations:* ADEPT, antibody-directed enzyme prodrug therapy; DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle's medium; Dox-sp-glu, doxorubicin-nitrophenyl-glucuronide; Epi-glu, epirubicin-glucuronide; FCS, fetal calf serum; HSA, human serum albumin; SRB, Sulphorhodamine B.

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Two different types of spacers were used: A-type, which release the spacer by a shift in electrons after hydrolysis; and B-type, in which the spacers were designed to be self-immolative by ring closure after removal of glucuronic acid. For both types of spacers, we designed aliphatic spacers (GA1 and GB1) and aromatic spacers (GA3 and GB6) to determine differences in the hydrolysis rate (Fig. 1).

The experiments presented focus on the comparison of the four daunorubicin-spacer-glucuronides with two anthracycline prodrugs that have been previously reported [5, 6]. Enzymatic activation was carried out by recombinant human β-glucuronidase produced by a mouse cell line transfected with the human β-glucuronidase gene. Attention was paid to differences between the various prodrugs with respect to K_m and V_{max} and to the hydrolysis half-life times at clinically relevant enzyme and prodrug concentrations. The *in vitro* antiproliferative effects of the different (pro)drugs in combination with a specific antibody-human β-glucuronidase conjugate were compared to determine the most effective prodrug with ADEPT.

MATERIALS AND METHODS

Cell Lines

The human ovarian cancer cell line OVCAR-3 [7] was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan, UT, U.S.A.), 50 IU/mL penicillin and 50 μg/mL streptomycin (ICN, Costa Mesa, CA, U.S.A.) in a humidified atmosphere containing 5% CO_2 at 37°C.

The mouse L-MPR 13-2-1 MTX 3.2 cell line (kindly provided by Prof. W. S. Sly, St. Louis University, St. Louis, MO, U.S.A.) [8] contains the genes for human β-glucuronidase (EC 3.2.21) and for dihydrofolate reductase (EC 1.5.1.3, DHFR) integrated in its genome. The human β-glucuronidase gene is co-amplified with DHFR and overexpressed in the presence of methotrexate. The L-MPR cells were grown in the same culture medium as the OVCAR-3 cell line, except for the 5% heat-inactivated FCS dialyzed against PBS to reduce the folate contents. Methotrexate (Lederle, Etten-Leur, The Netherlands) was added to the medium in a final concentration of 3.2 μM.

Drugs and Prodrugs

Epirubicin (Farmitalia Carlo Erba, Nivelles, Belgium), doxorubicin (Farmitalia), and daunorubicin (Société Parisienne d'expansion Chimique, Paris, France) were purchased as powders. Epi-glu was isolated from the urine of patients treated with epirubicin (75 to 120 mg/m^2 IV) as described by Haisma *et al.* [5]. In short, the urine was filtered through paper and the pH adjusted to 2.5 with 12 M phosphoric acid. Methanol was added to a final concentration of 20% and the metabolite Epi-glu purified on a silica-C18 column (15 cm × 1.6 cm I.D., 0.03 μm; Serva, Heidelberg, Germany). Dox-sp-glu [6] was a gift from Dr. K. Bosslet, Behringwerke, Marburg, Germany. The synthesis of the four daunorubicin-spacer-glucuronides has been partially described [4]. The structural formulas are depicted in Fig. 1. The structures of the prodrugs were confirmed through elemental analysis, ^{1}H-NMR (400 mHz, $CDCl_3$) and mass spectroscopy. Stock solutions of drugs and prodrugs were prepared in sterile water and stored at −20°C.

β-Glucuronidase

Human β-glucuronidase was isolated from the culture medium of L-MPR cells. The medium was harvested every 4 to 7 days and azide was added to a final concentration of 0.02% (w/v) to prevent bacterial growth. The medium was stored at 4°C until use. For isolation of the enzyme, the medium was acidified to pH 4.5 with acetic acid, centrifuged for 60 min at 80,000 *g* and filtered through a 0.2 μm filter (Nalge, Hereford, England). The enzyme was purified by 3 steps of ion-exchange chromatography. The first and third steps were anion-exchange chromatography (HiTrapSP; Pharmacia, Uppsala, Sweden) and the second step was cation-exchange chromatography (HiTrapQ; Pharmacia). Purity of the enzyme was verified by SDS-PAGE.

The specific activity of human β-glucuronidase was determined by measuring the hydrolysis of *p*-nitrophenyl-β-D-glucuronide to *p*-nitrophenol and glucuronic acid. Dilutions of the enzyme with known protein concentrations were made in 0.1% (w/v) BSA in 50 mM acetic acid pH 4.2. To 1 part of enzyme, 10 parts of *p*-nitrophenyl-β-D-glucuronide (10 mM) in either 0.1% (w/v) BSA/PBS at neutral pH or in 0.1% (w/v) BSA in 50 mM acetic acid pH 4.2 were added. After a 30-min incubation at 37°C, 15 parts of 1 M glycine/1 M NaOH pH 10.6 were added to stop the reaction and the extinction was read at a wavelength of 405 nm with an ELISA plate reader (Labsystems, Helsinki, Finland). The purified human β-glucuronidase had a molecular weight of 280 kDa and an optimal activity at pH 4.2 (30 U/mg at 37°C). The activity at pH 6.8 was 3 U/mg at 37°C.

For experiments where complete activation of prodrugs was required, an excess of β-glucuronidase from *E. coli* was used (Boehringer Mannheim, Mannheim, Germany). The enzyme was diluted in 0.1% (w/v) BSA/PBS. The activity of the enzyme is optimal at neutral pH (72.4 U/mg at 37°C).

Characteristics of (Pro)drugs

The stability of prodrugs was determined in 0.1% (w/v) BSA/PBS at pH 7.4, as well as in human serum after an incubation period of 24 hr at 37°C. The samples were analyzed by HPLC on a C18 reversed phase column (Chromsep 2 × 100 mm × 4.6 mm, I.D., 3 μm; Chrompack, Middelburg, The Netherlands). Samples were prepared for HPLC analysis by dilution of 10 μL in 140 μL ice-cold methanol.

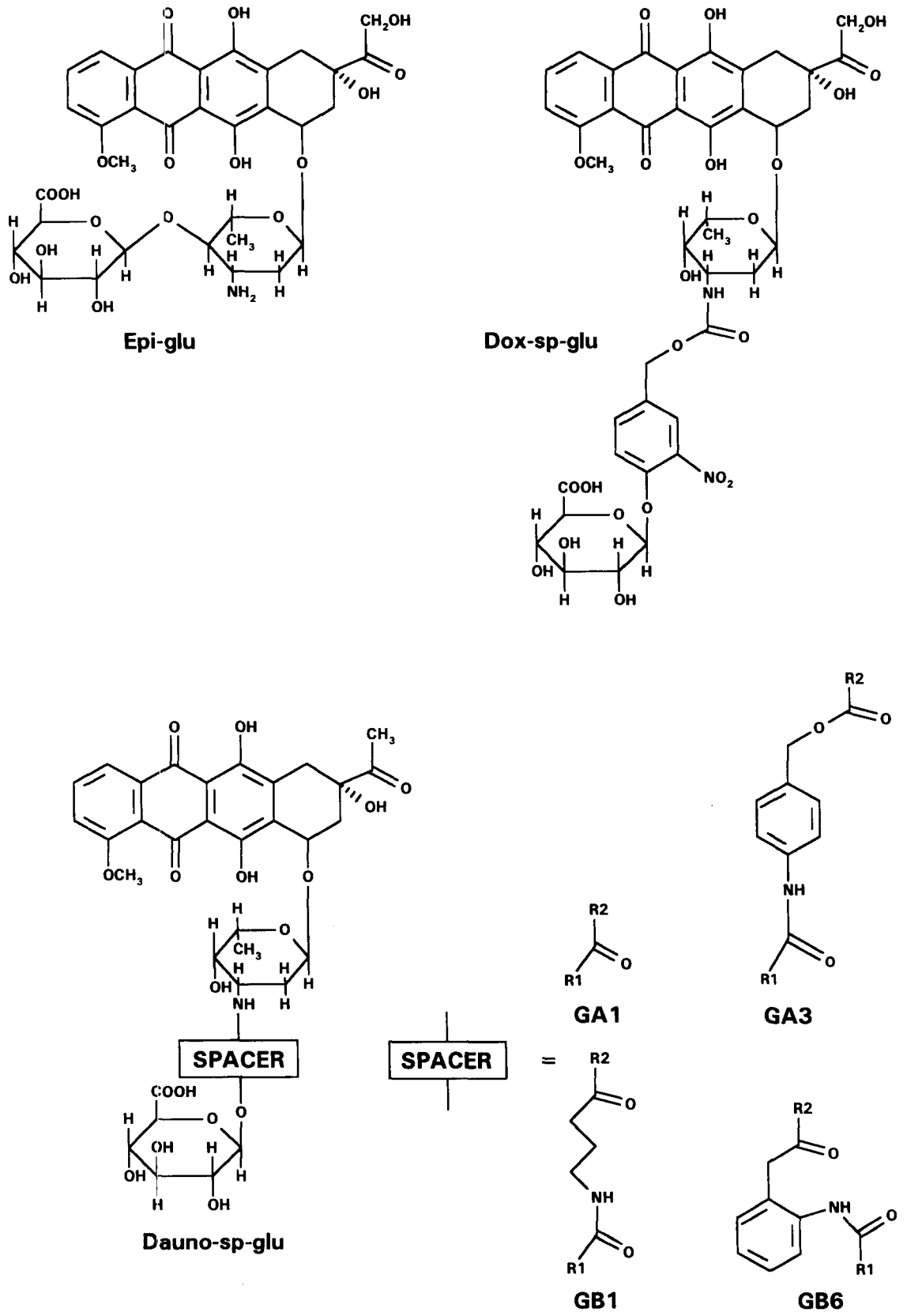

FIG. 1. Structural formulas of the prodrugs epirubicin-glucuronide (Epi-glu), doxorubicin-nitrophenyl-glucuronide (Dox-sp-glu), and the four daunorubicin-spacer-glucuronides (GA1, GA3, GB1, and GB6). For spacers: R1 = glucuronide moiety and R2 = anthracycline moiety.

After 10 min at 0°C, the samples were centrifuged for 4 min at 16,000 *g*. Thereafter, 100 μL was diluted with 25 μL of 12 mM H_3PO_4 and 50 μL was loaded on the column. The drug or prodrug was eluted from the column with 15 mM phosphate, 2 mM triethylamine, and 33% (v/v) acetonitrile at pH 3.5 and analyzed with a fluorescence detector (Jasco 821-FP; Separations, H.I. Ambacht, The Netherlands) using an excitation wavelength of 480 nm and an emission wavelength of 580 nm. Calibration of the system was performed as described [9]. Recoveries of the different anthracyclines ranged from 67.5 to 100%, with detection limits ranging from 4 to 10 pM.

The hydrolysis of the prodrugs by human recombinant β-glucuronidase was determined in PBS at pH 6.8, reflecting the tumor interstitial pH [10]. A range of prodrug concentrations (10^{-5} to 5×10^{-3} M) was tested against two different human β-glucuronidase concentrations (1 μg/mL and 10 μg/mL) to determine the hydrolysis expressed as K_m and V_{max} values. The prodrug and the enzyme were diluted in 0.1% (w/v) BSA/PBS at pH 6.8. After incubation for 30 min at 37°C, the reaction was stopped by adding methanol (see above). Samples were stored at −20°C until HPLC analysis on a C18 reversed-phase column. K_m and V_{max} values were calculated from direct linear plots as described by Eisenthal and Cornish-Bowden [11]. K_m values $>5 \times 10^{-3}$ M could not be determined accurately because 5×10^{-3} M was the highest concentration of prodrug tested.

To further characterize the enzymatic conversion of the prodrugs by human β-glucuronidase, the half-lives of the hydrolysis of prodrugs were determined at clinically relevant concentrations of the enzyme (1 μg/mL) and the prodrugs (100 μM).

The hydrophilicity of drugs and prodrugs was analyzed and given as octanol/PBS partition coefficients. Drugs or prodrugs were dissolved in water to a concentration of 1 mM. An aliquot of 5 μL was added to 500 μL octanol and, then, 500 μL PBS at pH 7.4 was added. The mixture was incubated for 4 hr at 37°C, with rigorous shaking once every hour. Aliquots of 100 μL of both phases were taken and diluted in 2 mL of methanol. The fluorescence was read (excitation wavelength of 480 nm and emission wavelength of 560 nm; Perkin Elmer 3000, Norwalk, CT, U.S.A.), and the octanol/PBS partition coefficient was calculated after subtraction of the background of the octanol and PBS controls.

The protein binding of the prodrugs was determined by the use of a Dianorm dialysis apparatus (Diachema, Zurich, Switzerland). Two 500 μL chambers were separated by a 5000 Da cutoff dialysis membrane. One chamber was filled with prodrug or drug diluted in 4% HSA in PBS at pH 7.4. The second chamber was filled with PBS. After closing the chambers, the different samples were incubated for 4 hr at 37°C under rotation. Samples from both chambers were diluted 20 times in 7.5% (v/v) acetonitrile in PBS at a pH adjusted to 3.5 with 1 M phosphoric acid, and the fluorescence was read in a fluorescence detector as described above. The concentration of the anthracyclines was determined by comparison with standards for each (pro)drug in the same diluent (range 0.01 to 10 μM). Because drug concentrations in both chambers were measured, loss to dialysis membranes and chamber walls was not considered to cause major errors [12].

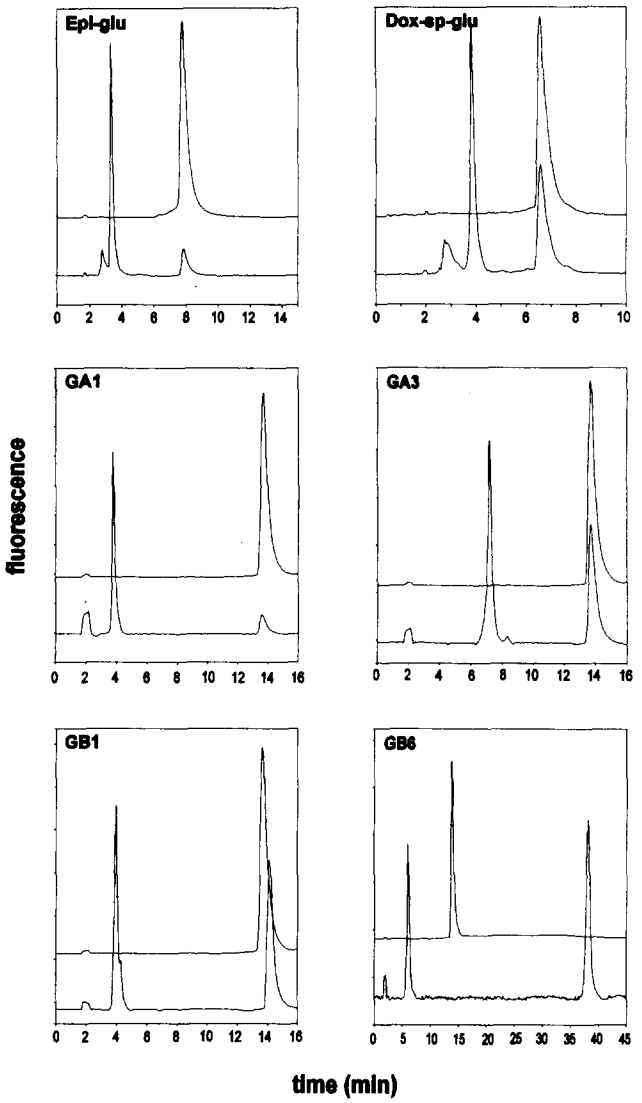

FIG. 2. Chromatograms of epirubicin, doxorubicin, and daunorubicin (100 μM, upper line) and epirubicin-glucuronide (Epi-glu), doxorubicin-nitrophenyl-glucuronide (Dox-sp-glu) or the newly synthesized prodrugs of daunorubicin (100 μM, lower line) after hydrolysis by human β-glucuronidase at 1 μg/mL (Dox-sp-glu, GA3, and GB6) or 10 μg/mL (Epi-glu, GA1, and GB1) for 30 min at pH 6.8 and 37°C. The chromatographic conditions are given in Materials and Methods.

The uptake of drugs and prodrugs was measured in OVCAR-3 cells. Cells were harvested and resuspended in DMEM without Phenol Red at pH 7.4 to a concentration of 10^7 cells/mL. An aliquot of 100 μL of drug or prodrug at 20 μM in medium supplemented with 0.1% (w/v) BSA at pH 7.4 was added to 100 μL of cells. After a 30-min incu-

TABLE 1. Hydrolysis characteristics of prodrugs in the presence of human β-glucuronidase at pH 6.8 and 37°C

	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (mol/mol·s)	k_{cat}/K_m (1/s·M)
Epi-glu	>5000*	0.7†	3.3†	<650
Dox-sp-glu	880	19.1	89.1	101,280
GA1	>5000*	0.5†	2.3†	<470
GA3	2070	35.5	165.7	80,030
GB1	>5000*	1.8†	8.4†	<1680
GB6	840	19.2	89.6	106,650

k_m and V_{max} were calculated from direct linear plots as described in Materials and Methods. Data represent the mean of 3 independent experiments, performed in duplicate. Standard deviations were less than 30%. *K_m values >5 × 10^{-3} M could not be determined accurately because this was the highest concentration of prodrug tested. †at 1 × 10^{-3} M prodrug.

bation at 37°C, the cells were washed 3 times with ice-cold PBS. The cells were dissolved in 500 μL 7.5% (v/v) acetonitrile in PBS adjusted to pH 3.5 with H_3PO_4. Undissolved cell particles were removed by centrifugation (3 min at 16,000 *g*). The samples were analyzed by measuring the fluorescence in a fluorescence detector as described above. The concentration of the anthracyclines was determined by comparison with standards for each (pro)drug added to the cells in the same diluent (range 0.01 to 10 μM) and corrected for recovery of the (pro)drugs from the cells.

In Vitro Antiproliferative Effects

The antiproliferative effects of the drugs, prodrugs, and prodrugs after enzymatic hydrolysis were compared by measuring the growth of OVCAR-3 cells with the protein dye stain Sulphorhodamine B (SRB; Sigma, St. Louis MO, U.S.A.). In short, cells were harvested with 0.25% trypsin and 0.2% EDTA in PBS to obtain a single cell suspension and seeded in 96-well tissue culture plates (2 × 10^6 cells/mL, 10 μL/well, 3 wells per concentration). Drug or prodrug was added (10 μL/well) at different concentrations, with a range of 3 or more logs. In separate wells, an excess of β-glucuronidase was present to determine the antiproliferative effects of the prodrug hydrolyzed by the enzyme. After 24 hr of incubation, 200 μL of culture medium (supplemented DMEM) was added and the cells were allowed to proliferate for another 72 hr. Cells were fixed with 25% trichloroacetic acid for 1 hr at 4°C and washed with water. The cells were stained with 0.4% SRB in 1% (v/v) acetic acid for 15 min at room temperature, washed with 1% (v/v) acetic acid and air dried. Bound SRB was solubilized with 10 mM unbuffered Tris and the extinction was read at 492 nm. The extinction was linear from 1000 up to 200,000 cells/well. Separate wells were fixed 24 hr after seeding to subtract background staining. The antiproliferative effects were determined and expressed as IC_{50} values, the (pro)drug concentration that give 50% growth inhibition compared to control cell growth.

Statistical Methods

The *t*-test with a confidence level of 95% was used to determine whether or not the properties of the prodrugs differed significantly from those of the parent drugs (Table 2).

RESULTS

Stability and Hydrolysis of Prodrugs

Chemical stability was >95% for all prodrugs after a 24-hr incubation period in PBS/BSA as well as in human serum, both tested at 37°C. No parent drug was detected after HPLC analysis. Thus, the prodrugs were not expected to show spontaneous conversion in the *in vivo* situation.

The prodrugs were incubated with recombinant human β-glucuronidase to determine their hydrolysis rates. HPLC analysis showed that Epi-glu, Dox-sp-glu, GA1, and GA3 were hydrolyzed to their parent drugs. Sample chromatograms of the hydrolysis of the newly synthesized prodrugs are shown in Fig. 2. A new product was formed after hydrolysis of GB1 and GB6. Apparently, after release of the glucuronic acid and CO_2, there was no release of the spacer from the anthracycline molecule. Prolonged incubations of up to 48 hr with enzyme showed that the prodrugs Epi-glu, Dox-sp-glu, GA1, and GA3 were completely hydrolyzed to their parent drugs, whereas GB1 and GB6 were completely hydrolyzed to spacer-drug molecules.

The V_{max} and the K_m for the different prodrugs were measured with human β-glucuronidase (Table 1). The prodrugs containing aliphatic spacers, Epi-glu, GA1, and GB1 showed relatively high K_m values. Dox-sp-glu, GA3, and GB6, which contain an aromatic spacer, showed lower values that should be more favourable for enzymatic conversion. The V_{max} values of Epi-glu and GA1 were very low, less than 2 μmol/min/mg at 1 mM prodrug concentration. GA3 showed the highest V_{max} of 35.5 μmol/min/mg. Dox-sp-glu and GB6 showed intermediate V_{max} values. Figure 3 depicts the hydrolysis of prodrugs at 100 μM incubated with human β-glucuronidase at 1 μg/mL as concentrations that are clinically relevant. The prodrugs with low K_{cat}/K_m values, Epi-glu, GA1, and GB1, were slowly hydrolyzed

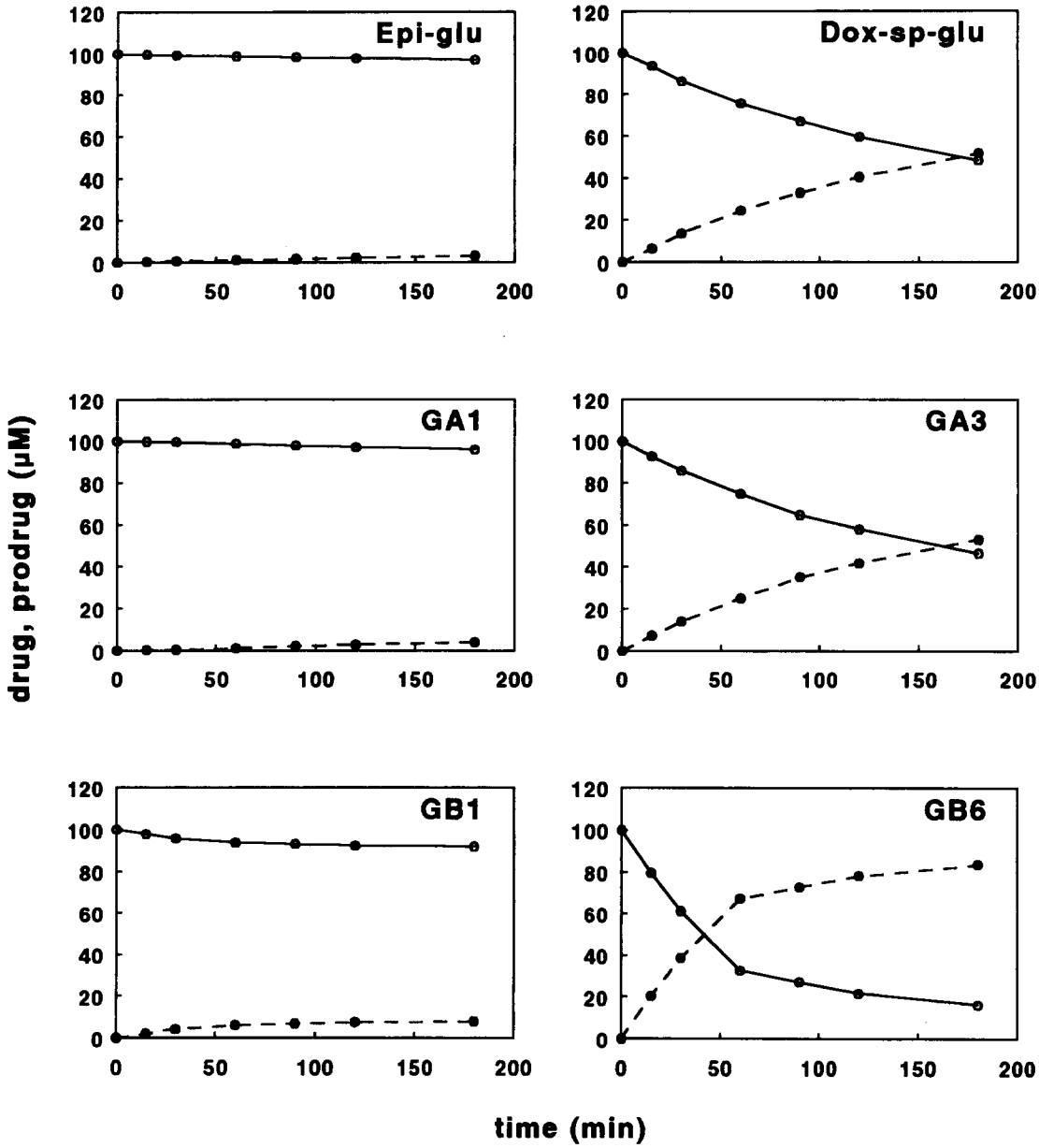

FIG. 3. Hydrolysis of prodrugs (○) to drug or drug-spacer (●) by human β-glucuronidase. Prodrugs were incubated at a concentration of 100 μM with 1 μg/mL enzyme in 0.1% (w/v) BSA/PBS at pH 6.8 and 37°C. Drug and prodrug concentrations were measured by HPLC as described in Materials and Methods.

with a $T_{1/2}$ of 3400, 2300, and 425 min, respectively. For Dox-sp-glu ($T_{1/2}$ 170 min), GA3 ($T_{1/2}$ 160 min) and, especially, for GB6 ($T_{1/2}$ 40 min), short half-lives were measured for their enzymatic conversion, which corresponds to their higher K_{cat}/K_m values.

Hydrophilicity, Protein Binding, and Cellular Uptake of (Pro)drugs.

The octanol/PBS partition coefficient gives a measure of the hydrophilicity of the (pro)drugs. In contrast to the drugs epirubicin, doxorubicin, and daunorubicin, more hydrophilic prodrugs are less able to pass the cell membrane. The tested prodrugs invariably showed statistically significant lower values, thereby indicating their hydrophilic character (Table 2).

The protein binding of the prodrugs was in the same range, but slightly lower when compared to that of the parent drugs (Table 2). No high values were found, which would indicate free prodrug to be available for enzymatic activation at the tumor site.

The cellular uptake of the parent drugs was high in OVCAR-3 cells, whereas no or little uptake could be detected for all prodrugs (Table 2). Upon microscopic inspection, the slightly higher uptake in the case of Epi-glu appeared to be located mainly at the outside of the cell membrane. GA1, GA3, or GB6, incubated with an excess of β-glucuronidase, showed increased cellular uptake com-

TABLE 2. Hydrophilicity, protein binding, cellular uptake, and antiproliferative effects of drugs and prodrugs

	Octanol/ PBS†	Protein binding‡	Cellular uptake§	IC_{50}‖	Prodrug/ drug¶
Epirubicin	8.50 (0.55)	68 (7.6)	340 (28)	0.6 (0.2)	
Epi-glu	0.17 (0.03)*	58 (5.8)	150 (25)*	50.0 (10.0)*	83
Doxorubicin	2.50 (0.16)	70 (6.0)	337 (43)	0.6 (0.2)	
Dox-sp-glu	0.18 (0.02)*	57 (5.7)*	28 (4)*	9.1 (1.7)*	15
Daunorubicin	12.55 (0.99)	64 (5.9)	349 (25)	1.2 (0.5)	
GA1	0.01 (0.001)*	45 (5.7)*	15 *	40.0 (10.0)*	33
GA3	0.02 (0.001)*	67 (3.0)	31 (12)*	21.6 (4.7)*	18
GB1	0.01 (0.001)*	42 (0.2)*	15 *	>100 *	>100
GB6	0.08 (0.01)*	72 (4.5)*	32 (6)*	>20 *	>20
GA1 + GUS#			384 (21)	1.1 (0.4)	0.9
GA3 +GUS#			343 (11)	1.3 (0.5)	1.1
GB1 + GUS#			121 (8)*	>100 *	>100
GB6 + GUS#			518 (8)*	>20 *	>20

Data represent the mean (SD) of 3 independent experiments. *indicates a significant difference when compared to the parent drug. †hydrophilicity expressed as the octanol/PBS ratio. ‡percentage of total (pro)drug bound to 4% HSA in PBS, pH 7.4. §pmol/10^6 OVCAR-3 cells (detection limit 15 pmol/10^6 cells). ‖IC_{50} is the pro(drug) concentration (μM) that gives 50% cell growth inhibition in OVCAR-3 cells compared to untreated cells. ¶ratio of the IC_{50} values of prodrug and corresponding parent drug. #excess of β-glucuronidase.

pared to the respective prodrugs, but this was not observed for GB1. This finding corresponds with the HPLC data, which indicated that GA1 and GA3 were activated to daunorubicin. GB1 and GB6 could not be activated to the parent drug, and the resulting spacer-drug molecule showed a lower (GB1) or higher (GB6) cellular uptake compared to daunorubicin.

In Vitro Antiproliferative Effects

For the drugs epirubicin, doxorubicin, and daunorubicin, IC_{50} values for OVCAR-3 cells were 0.6, 0.6, and 1.2 μM, respectively. As expected, the prodrugs were less capable of inhibiting cell growth (Table 2). The ratio between the antiproliferative effects of the prodrug and the parent drug varied from 15 in the case of Dox-sp-glu to >100 for GB1. When prodrugs were incubated with an excess of β-glucuronidase, the antiproliferative effects of GA1 and GA3 returned to a value similar to that of daunorubicin. This indicated that the prodrugs could be activated completely to the parent drug. HPLC analysis showed that GB1 and GB6 were not hydrolyzed to an active drug by β-glucuronidase. This was confirmed by the persistence of high IC_{50} values.

The antiproliferative effects of GA1 and GA3 were also measured with OVCAR-3 cells pretreated with saturating amounts of a conjugate between monoclonal antibody 323/A3 and human β-glucuronidase [5]. As illustrated in Fig. 4, GA3 was completely activated to the parent drug by the conjugate. The slow hydrolysis rate of GA1 did not result in antiproliferative effects in OVCAR-3 cells.

DISCUSSION

Anthracyclines are effective antitumor agents and can rapidly diffuse through cell membranes into cells of both normal and malignant tissues. Anthracycline prodrugs were synthesized for use in ADEPT on the assumption that increased hydrophilicity would preclude cellular uptake and reduce cytotoxicity. This was accomplished by linking a glucuronic acid group to the drug moiety, directly for Epi-glu or *via* different spacers for Dox-sp-glu, GA1, GA3, GB1, and GB6 (Fig. 1). *In vitro*, the synthesized daunorubicin-spacer-glucuronides were at least 20 times less toxic than daunorubicin. Another requirement to be dealt with for this type of prodrug was the specific activation by human β-glucuronidase to the parent drug at neutral pH. Indeed, Epi-glu, Dox-sp-glu, GA1, and GA3 could be activated to epirubicin, doxorubicin, and daunorubicin, respectively. The spacers were designed to give different activation rates of the prodrugs by human β-glucuronidase. The prodrugs with aliphatic spacers were activated slowly, whereas the prodrugs containing aromatic spacers had high hydrolysis rates.

Several enzymes are being investigated for use in tumor site-specific prodrug activation. We, and others, have chosen enzymes of human origin to prevent immunogenicity, such as that observed following administration of bacterial enzymes to patients [13]. Alkaline phosphatase was not suitable because of its abundant presence in the blood, resulting in untimely activation of the prodrug with systemic administration [14]. Our enzyme of choice, lysosomal human β-glucuronidase, is present in only minimal concentrations in the circulation. A disadvantage of human β-glucuronidase is the low pH optimum, pH 4.2. The enzyme has an approximately 10-fold lower hydrolysis rate at the pH found in the interstitium of tumor tissue, which is acidic to near-neutral [10].

We evaluated the activation of prodrug to drug at clinically achievable concentrations of enzyme and prodrug at near neutral pH. Human β-glucuronidase was used at 1 μg/mL, achieved by a tumor uptake of 0.001% of a 100-mg

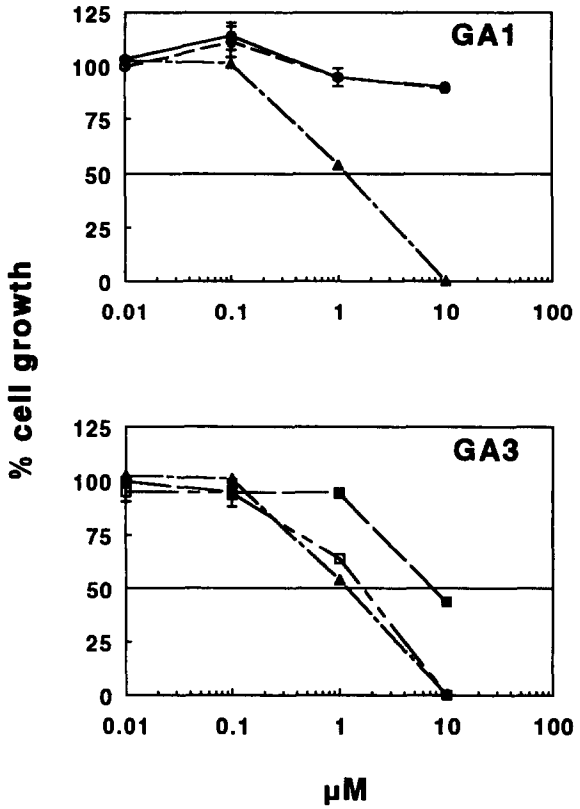

FIG. 4. Growth inhibition of OVCAR-3 cells by daunorubicin (▲), GA1 (●), GA3 (■), and by GA1 (○) and GA3 (□) in the presence of a specific antibody-human β-glucuronidase conjugate. Cells were exposed to the drugs for 24 hr in triplicate wells in 96-well microtiter plates. The cell numbers were determined after an additional 72-hr incubation. Results are expressed as the percentage of growth inhibition relative to control cell growth, determined by staining with Sulphorhodamine B. Standard errors are indicated.

patient dose. This is well within the range of the levels reached by radiolabelled monoclonal antibodies [1]. The prodrugs were used at a concentration of 100 μM, which is approximately 10 times higher than the plasma concentrations of anthracyclines found in patients [15], and should be tolerated when prodrugs are 10-fold less toxic than the parent drugs. The prodrugs GA1 and GB1 with aliphatic spacer-moieties were slowly hydrolyzed by human β-glucuronidase. This may be due to the proximity of the bulky aglycon, which could sterically hinder access to the enzyme. Short half-lives and high k_{cat}/K_m ratios were found for Dox-sp-glu, GA3, and GB6, which contain an aromatic spacer-moiety. The greater distance between the glucuronic acid and the aglycon or the superior leaving group could be the reason for the observed higher hydrolysis rates.

GA1 and GA3 were hydrolyzed to daunorubicin with release of the spacers by a shift in electrons. GB1 and GB6, which were designed with a self-immolating spacer, were not hydrolysed to daunorubicin. Instead, after release of glucuronic acid, we observed that part of the spacer remained attached to the drug. Despite the very rapid hydrolysis of GB6 by human β-glucuronidase ($T_{1/2}$ 40 min), the remaining spacer-drug molecule was nontoxic and, thus, not suitable for ADEPT.

Epi-glu and Dox-sp-glu were described earlier as potential candidates for use in ADEPT [5, 6]. Epi-glu was rapidly hydrolyzed by bacterial β-glucuronidase, but we found a very low hydrolysis rate for human β-glucuronidase ($T_{1/2}$ 3400 min) at clinically relevant concentrations of enzyme and prodrug at neutral pH, pH 6.8. The hydrolysis of Dox-sp-glu by the human enzyme was considerably faster ($T_{1/2}$ 170 min). It is noteworthy that the group of Bosslet [6] found activation of Dox-sp-glu at pH 7.2 by the human β-glucuronidase moiety of their fusion protein, where the fusion protein consisted of a human β-glucuronidase and a humanized monoclonal antibody. However, after hydrolysis of Dox-sp-glu, an intermediate was detected before the parent drug was generated. This intermediate drug-spacer molecule may have a possible disadvantage, as ultimate cellular drug uptake may be hampered by a slow diffusion of the spacer-drug molecule through the cell membrane. In addition, the intermediate may diffuse to the circulation and, then, enter normal tissues after spontaneous conversion to doxorubicin. Two of our anthracycline glucuronides, GA3 ($T_{1/2}$ 160 min) and GB6 ($T_{1/2}$ 40 min), showed similar or higher activation rates when compared to Dox-sp-glu. Unfortunately, GB6 was not converted to the parent compound. Upon hydrolysis, GA3 did not show an intermediate drug-spacer molecule and was immediately converted to daunorubicin.

As discussed by Yuan *et al.* [16], the rate of activation of the prodrugs is a very important variable in ADEPT. When the hydrolysis rate is high, almost all of the prodrug will be converted into drug in both tumor and plasma, because there will always be some circulating conjugate. In contrast, a very low conversion rate will result in a high tumour: plasma ratio for the drug, but the actual drug levels in the tumor may be too low to be toxic to the cells. *In vitro*, we, indeed, found that GA3, with its high hydrolysis rate, but not GA1, which is hydrolysed very slowly, could be activated to toxic concentrations of daunorubicin by a conjugate specifically bound to OVCAR-3 tumour cells. In our future *in vivo* studies we will seek to determine the usefulness of GA3, and derivatives thereof, for treatment at the time monoclonal antibody-human β-glucuronidase conjugates have localized into human tumour xenografts.

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