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# Comparison of Two Anthracycline-Based Prodrugs for Activation by a Monoclonal Antibody–β-Glucuronidase Conjugate in the Specific Treatment of Cancer

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## ABSTRACT

Antibody-directed enzyme prodrug therapy (ADEPT) may improve the therapeutic index of cytostatic agents. We compared two prodrugs, epirubicin-glucuronide (Epi-glu) and doxorubicin-spacer-glucuronide (Dox-sp-glu), for their cytotoxicity on activation by a monoclonal antibody–enzyme conjugate bound to tumor cells. The results showed that the prodrugs were 10 (Dox-sp-glu) and 100 (Epi-glu) times less toxic than the parent drugs against OVCAR-3 cells. This difference was a result of the hydrophilic property of the prodrugs resulting in a reduced cellular uptake. The enzyme-catalyzed hydrolysis of Dox-sp-glu by *E. coli*-derived β-glucuronidase (GUS) ($K_m$ 500 μ*M*, $V_{max}$ 21,000 μmol/min/g) was much more efficient than that of Epi-glu ($K_m$ 10 μ*M*, $V_{max}$ 40 μmol/min/g). Incubation of OVCAR-3 cells with an enzyme-immunoconjugate prepared from monoclonal antibody 323/A3 and *E. coli*-derived GUS before treatment with prodrugs completely restored the cytotoxicity of the prodrugs to the level of the parent drugs.

**Index Entries:** ADEPT; prodrug; anthracyclines; monoclonal antibody; β-glucuronidase.

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## INTRODUCTION

Antibody-directed enzyme prodrug therapy (ADEPT) aims at the specific treatment of cancer. Tumor-selective monoclonal antibodies (MAbs) are used to carry enzymes to tumor cells. The enzyme converts a relatively nontoxic prodrug, which is administered after the conjugate has localized in tumor lesions, into an active cytotoxic agent.

Previous work in our laboratory has shown that a conjugate between *E. coli*-derived β-glucuronidase (GUS) and MAb 323/A3 induces selective cytotoxicity on hydrolysis of the prodrug epirubicin-glucuronide (Epi-glu) by the enzyme (*1*). The major problem that appeared in this study was the slow conversion of prodrug into drug by the enzyme. In addition, for future clinical studies we anticipate the replacement of *E. coli*-derived enzyme by GUS of human origin. Then, at neutral pH, one may expect an even lower conversion rate. Both aspects necessitated that we select a better prodrug with a favorable hydrolysis rate.

In our next experiments we compared a new prodrug, doxorubicin-spacer-glucuronide (Dox-sp-glu), with Epi-glu for its stability, enzymatic hydrolysis rate, and cytotoxicity. We could demonstrate that Dox-sp-glu could be converted into Dox by a 323/A3–GUS conjugate bound to the tumor cells. The activation of Dox-sp-glu occurred at a more efficient hydrolysis rate than that of Epi-glu.

## METHODS

### Antibody, Enzyme, and Conjugate

Purified murine MAb 323/A3 (2) was kindly provided (S. O. Warnaar, Centocor Europe, Leiden, The Netherlands). The antibody is directed against a membrane glycoprotein that is highly expressed in a variety of carcinomas.

GUS from *E. coli* K12 was purchased from Boehringer (Mannheim, Germany). The enzyme activity was measured with *p*-nitrophenyl-β-D-glucuronide (1 m*M* in 0.1*M* sodium acetate pH 4.2). Samples were incubated with substrate for 30 min at 37°C. The absorbance at 405 nm was read after raising the pH to 10.6 with 1*M* glycine/NaOH.

MAb 323/A3 and GUS were conjugated using heterobifunctional cross-linkers. SATA-derivatized (Pierce, Oud Beierland, The Netherlands) GUS was reacted with SMCC-treated (Pierce) 323/A3. The conjugate was purified on a Mono Q column (Pharmacia, Woerden, The Netherlands). The immunoreactivity was estimated by measuring the amount of enzyme-immunoconjugate bound to glutaraldehyde-fixed OVCAR-3 cells.

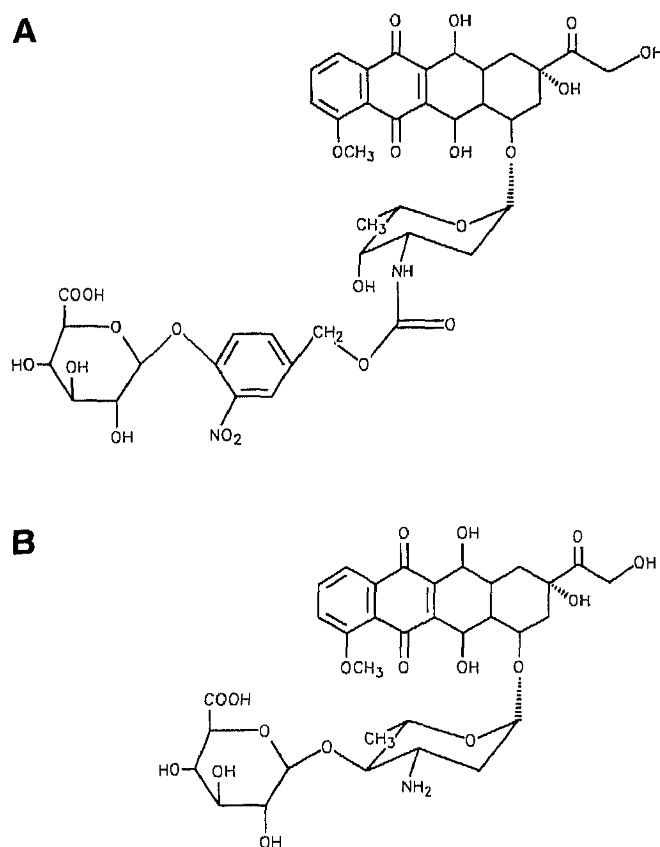


Fig. 1. Molecular structures of (**A**) Dox-spacer-glu and (**B**) Epi-glu.

## Prodrugs

Epi-glu (Fig. 1) was isolated from urine of patients treated with epirubicin by reversed-phase high-pressure liquid chromatography (HPLC). Dox-sp-glu (3; Fig. 1) was a gift from K. Bosslet (Behringwerke, Marburg, Germany). Purity of Epi-glu or Dox-sp-glu and the formation of Epi or Dox after hydrolysis by GUS were measured by reversed-phase HPLC and fluorescence detection. Each run included standards of the prodrugs.

The stability of Epi-glu and Dox-sp-glu was studied under various conditions in vitro. Prodrug (10 $\mu M$ final concentration) was added to human plasma, serum, or tissue culture medium (DMEM, with 10% FCS). At different time intervals (0, 4, and 24 h) samples were taken and analyzed by HPLC for the presence of drug and derivatives.

To further characterize the prodrugs, we determined the physicochemical parameters. The apparent partition coefficient was measured by

dissolving the drugs in octanol at a concentration of 10 $\mu M$. An equal volume of phosphate-buffered saline (PBS) pH 7.4 was added and, after vigorous mixing for 3 h at 37°C, the concentration of the drugs in the two solutions was measured by fluorometry. The protein binding was determined using equilibrium dialysis (4). Drugs at 10 $\mu M$ were dissolved in PBS pH 7.4 containing 4% human serum albumin and dialyzed against PBS for 4 h at 37°C. The concentrations of the drugs were determined by fluorometry.

The enzymatic parameters $V_{max}$ and $K_m$ were determined for Epi-glu and Dox-sp-glu, using a 30-min incubation at 37°C in PBS containing 0.1% bovine serum albumin (BSA). Hydrolysis of the prodrugs was measured with reversed-phase HPLC.

### In Vitro Cytotoxicity

The human ovarian cancer cell line NIH:OVCAR-3 (5) was used to determine the cytotoxicity and cellular uptake of the prodrugs. Cells were cultured as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 m$M$ L-glutamine, 50 IU $mL^{-1}$ penicillin, and 50 $\mu g$ $mL^{-1}$ streptomycin. The cellular uptake of the prodrugs at 10 m$M$ concentration was measured by fluorometry after incubating OVCAR-3 cells for 30 min at 37°C. For cytotoxicity assays cells were plated at a concentration of $10^6$ $mL^{-1}$ and exposed to varying concentrations of prodrugs in MEM. After 24 h fresh DMEM was added and the incubation continued for another 72 h. Cell growth was determined by measurement of the absorbance at 540 nm of trichloroacetic acid-fixed and sulforhodamine-B-stained cells. The effect of the conjugate on the cytotoxicity was measured by pretreating the cells with conjugate at 10 $\mu$g/mL (1).

## RESULTS

### Antibody, Enzyme, and Conjugate

GUS was covalently linked to 323/A3 through a stable thioether bond. The conjugate was purified by ion-exchange chromatography and retained > 90% GUS activity. The immunoreactive fraction measured on OVCAR-3 cells was at least 50%.

### Characterization of Prodrugs

The stability of the prodrugs was measured in tissue culture medium, plasma, and serum. Epi-glu and Dox-sp-glu showed no evidence of formation of the parent drugs after incubation for 24 h at 37°C (data not shown). The apparent partition coefficient between octanol and PBS was different for the drugs and prodrugs. The polar glucuronic acid-derivatives of the

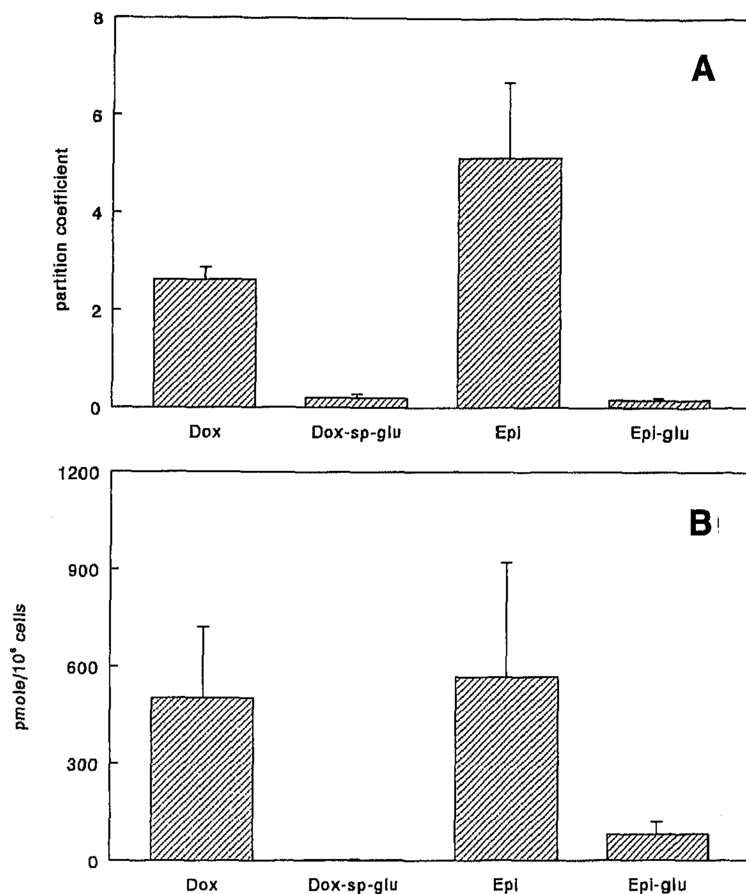


Fig. 2. Partition coefficients in octanol/PBS (**A**) and cellular accumulation in OVCAR-3 cells (**B**) of Epi-glu and Dox-spacer-glu.

Table 1
Enzymatic Hydrolysis of Prodrugs[a]

| Enzyme | $V_{max}$, μmol/min/g | $K_m$, μM |
|---|---|---|
| Epi-glu | 40 | 10 |
| Dox-spacer-glu | 21,000 | 500 |

[a] Values of $K_m$ and $V_{max}$ were derived from Eady-Hofstee plots.

drugs were much more water soluble than the parent drugs, with almost all of the prodrugs remaining in the water phase (Fig. 2A). The protein binding of the prodrugs was similar, approx 60%. The enzymatic parameters $V_{max}$ and $k_m$ were determined with *E. coli*-derived GUS. Dox-sp-glu was more efficiently converted into the parent drug than Epi-glu (Table 1).

### Cytotoxicity of Prodrugs

First, we determined whether the increased hydrophylicity of the prodrugs resulted in a reduced cellular accumulation. Both prodrugs showed a dramatic reduction in uptake measured with OVCAR-3 cells as compared to the uptake of the parent drugs. The cellular accumulation of Dox-sp-glu was slightly less than that of Epi-glu (Fig. 2B). Examination of the cells by fluorescence microscopy revealed no nuclear uptake of the prodrugs, but only a faint membrane staining. This was in contrast to the cells exposed to the drugs, which showed a predominant staining of the nucleus.

Then, the cytotoxicity of the prodrugs was determined with OVCAR-3 cells, using the sulforhodamine-B protein dye assay. When cells were exposed to prodrug for 24 h, Epi-glu was approx 100-fold less toxic than Epi, whereas Dox-sp-glu was approx 10 times less toxic than Dox. Pretreatment of OVCAR-3 cells with saturating concentrations (10 $\mu$g/mL) of enzyme-immunoconjugate completely reversed the cytotoxicity of both prodrugs (Fig. 3).

## DISCUSSION

The prodrugs Epi-glu and Dox-sp-glu were found to be 100- and 10-fold less toxic, respectively, than their parent drugs as caused by a decreased cellular accumulation rate. The prodrugs could be efficiently hydrolyzed into the parent drugs by an enzyme-immunoconjugate bound to tumor cells. The enzymatic hydrolysis of Dox-spacer-glu was much more efficient than that of Epi-glu.

Epirubicin is a stereoisomer of doxorubicin. In vitro, both drugs are equally toxic to OVCAR-3 cells. The prodrug Epi-glu was approx 10-fold less toxic than Dox-sp-glu, despite an apparent lower cellular accumulation rate of Dox-sp-glu. Because both prodrugs are stable in serum, plasma, and medium, the relatively high toxicity of Dox-sp-glu could be caused by a small amount of GUS released from the OVCAR-3 cells into the medium. Dox-sp-glu was shown to be more susceptible to enzyme activation. The presence of a phenol spacer in Dox-sp-glu may be the reason that this prodrug is more efficiently hydrolyzed by GUS when compared to Epi-glu.

Preliminary clinical experience has confirmed the feasibility of ADEPT. Bagshawe et al. (6) studied the bacterial enzyme carboxypeptidase G2 conjugated to a murine anticarcinoembryonic antigen antibody in combination with a mustard glutamate prodrug in patients with advanced colorectal cancer. Minor responses were noted in all four patients treated, but all developed antibodies to mouse IgG and carboxypeptidase G2. This study indicates that nonimmunogenic enzymes as well as human(ized) antibodies are to be preferred.

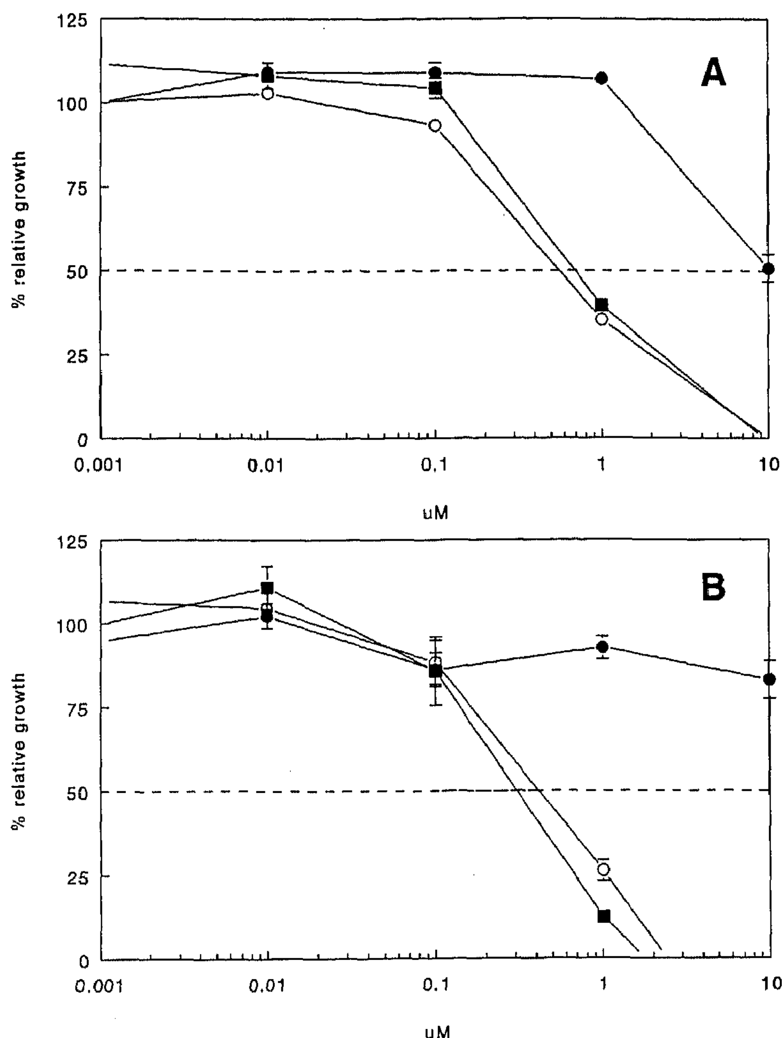


Fig. 3. Growth inhibition of OVCAR-3 cells. (**A**) Dox (○), Dox-spacer-glu (●), and Dox-spacer-glu after preincubation of cells with 323/A3-GUS conjugate (■). (**B**) Epi (○), Epi-glu (●), and Epi-glu after preincubation of cells with 323/A3-GUS enzyme–immunoconjugate (■).

In our study, we presently consider human GUS as a possible enzyme for prodrug activation. Human GUS has a much lower enzyme activity at neutral pH than *E. coli*-derived GUS. Therefore, the more efficient enzymatic hydrolysis of Dox-sp-glu makes this prodrug of greater value for use with human GUS. In addition, the recently developed fusion protein techniques (7) should improve the production of predefined humanized antibody–enzyme molecules.

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