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A methylester of the glucuronide prodrug DOX-GA3 for improvement of tumor-selective chemotherapy

Michelle de Graaf[a,1], Tapio J. Nevalainen[b,1,2], Hans W. Scheeren[b], Herbert M. Pinedo[a], Hidde J. Haisma[c], Epie Boven[a,*]

[a]*Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands*
[b]*Department of Organic Chemistry, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands*
[c]*Department of Therapeutic Gene Modulation, University Center for Pharmacy, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

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Abstract

The glucuronide prodrug of doxorubicin, DOX-GA3, can be selectively activated in tumors by extracellular human β-glucuronidase, resulting in a better therapeutic index than doxorubicin. DOX-GA3, however, is rapidly excreted by the kidney. We hypothesized that slow release of DOX-GA3 from its methylester, DOX-mGA3, by esterase activity in blood would result in improved circulation half-life ($t_{1/2}$) of DOX-GA3. DOX-mGA3 was synthesized more efficiently with an overall yield of 60% as compared to 37% in the case of DOX-GA3. We showed that DOX-mGA3 was enzymatically converted to DOX-GA3 with a $t_{1/2}$ of approximately 0.5 min in mouse plasma to 2.5 h in human plasma, which was in agreement with differences in esterase activity between species. DOX-mGA3, similar to DOX-GA3, was at least 37-fold less potent than the parent drug doxorubicin in growth inhibition of four different human malignant cell lines in vitro. Incubation of OVCAR-3 cells with DOX-mGA3 in combination with an excess of human β-glucuronidase (0.05 U mL^{-1}) resulted in a similar growth inhibition to that of doxorubicin. Intravenous administration of DOX-mGA3 in FMa-bearing mice resulted in an area under the concentration versus time curve (AUC) of DOX-GA3 in tumor and most normal tissues that was 2.5- to 3-fold higher than after the same dose of DOX-GA3 itself. In tumor tissue, this was accompanied by a 2.7-fold increase in the AUC of doxorubicin from DOX-mGA3 than from DOX-GA3. In conclusion, an advantage of DOX-mGA3 over DOX-GA3 is that this prodrug can be produced with a higher yield. Another important advantage is the improved pharmacokinetics of the lipophilic DOX-mGA3 as compared to that of the hydrophilic DOX-GA3. This effect may even be more pronounced in man, because of the lower plasma esterase activity than measured in mice.
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1. Introduction

An important metabolic route in the liver is the generation of drug glucuronides, which are biologically or chemically less reactive and exhibit higher polarity and excretability than the corresponding parent aglycones. In fact, such metabolites could be useful as prodrugs. These naturally occurring glucuronide prodrugs are less toxic than their parent compounds due to increased hydrophilicity, resulting in decreased cellular uptake [1]. They can be selectively activated in the tumor by β-glucuronidase, which is released in necrotic tumor areas. An example of a naturally occurring glucuronide prodrug is the metabolite of aniline mustard. Efficacy of treatment with aniline mustard has directly been related to the level of β-glucuronidase in mouse tumors [2]. A clinical trial of aniline mustard in patients with advanced cancer has shown a relation between β-glucuronidase activity in aspirate and imprint preparations of tumor cells, which was assessed by a cytochemical technique, and patient's response [3]. Success of that trial was limited, since very high tissue levels of β-glucuronidase were required. The need for

Abbreviations: AUC, area under the concentration versus time curve; BNNP, bis(4-nitrophenyl)phosphate; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; HPLC, high-performance liquid chromatography; MTD, maximum tolerated dose; PBS, phosphate-buffered saline; *p*NPA*cp*-nitrophenyl acetate

* Corresponding author. Tel.: +31 20 4444336; fax: +31 20 4444355.
E-mail address: e.boven@vumc.nl (E. Boven).
[1] Both authors have equally contributed to this work.
[2] Present address: Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, Kuopio 70211, Finland.

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these very high levels might be attributed to slow activation of the aniline mustard glucuronide by the enzyme. Synthetic glucuronide prodrugs that are more efficiently hydrolyzed have been developed for enzyme prodrug therapy (for review see [4]).

We have synthesized glucuronide prodrugs of daunorubicin and doxorubicin, designated daunorubicin-GA3 (DNR-GA3) and doxorubicin-GA3 (DOX-GA3), respectively [5], which contain a self-eliminating *p*-aminobenzyloxycarbonyl spacer. Both prodrugs can be completely converted to doxorubicin by human β-glucuronidase in vitro and they were shown to be relatively non-toxic in vivo. The maximum tolerated dose (MTD) of DOX-GA3 in tumor-bearing nude mice, based on the occurrence of 10% weight loss, was approximately 500 mg kg^{-1} i.v., compared to 8 mg kg^{-1} for doxorubicin [6]. Administration of glucuronide prodrugs, including DOX-GA3, to nude mice bearing human ovarian cancer xenografts at half of the MTD has resulted in concentrations of the parent drug in plasma that were significantly lower, while the concentrations of the parent drug in tumor tissue were higher than after administration of the parent drug itself [6–8]. The increased concentration of the parent drug in tumor tissue after prodrug administration is beneficial as it enhances the therapeutic effect [7,9–11]. The increased efficacy was specifically observed in larger tumors containing a higher proportion of necrotic areas.

A disadvantage of hydrophilic glucuronide prodrugs is their rapid elimination by the kidneys. In earlier experiments in tumor-bearing nude mice, DOX-GA3 had completely disappeared from plasma within 4 h after i.v. administration of 250 mg kg^{-1} of the prodrug [6]. The rapid elimination of glucuronide prodrugs poses a major problem for their use in cancer treatment, as this means that very high doses are required. We hypothesized that a methyl ester of DOX-GA3, DOX-mGA3, would be more lipophilic than DOX-GA3 and of better use in enzyme prodrug therapy. The methyl ester of other methylated glucuronide prodrugs has been reported to be hydrolyzed by carboxylesterases [12,13]. When administered in vivo, the methyl group of DOX-mGA3 might be removed by carboxylesterase activity in plasma to yield the original DOX-GA3 prodrug, which could in turn be activated by human β-glucuronidase (Fig. 1). We hypothesized that slow release of DOX-GA3 after administration of DOX-mGA3 might result in improved pharmacokinetics of the glucuronide prodrug in vivo. Therefore, the use of the lipophilic DOX-mGA3, which can be considered a pro-prodrug, might allow lower doses to be administered to achieve the same therapeutic effect.

Another disadvantage of glucuronide prodrugs, especially those based on doxorubicin, is the relatively inefficient synthesis [14–16]. A maximum yield of no more than 37% of DOX-GA3 can be obtained as final product, which can be explained as follows. The precursor of DOX-GA3 having the glucuronic acid group protected as methyl ester and tri-acetate, has to be hydrolyzed under strong basic conditions. In this basic reaction mixture, however, extensive side-product formation takes place due to base lability of doxorubicin. This step accounts for the main reduction in the yield of DOX-GA3. We anticipated that the production of DOX-mGA3 (Fig. 2) should result in a much higher yield. The hydrolysis of only the tri-acetate, and not the methyl group, in the last step of the synthesis could be performed under less basic conditions for a shorter time period and, therefore, would result in less side-product formation.

In the present study, we synthesized DOX-mGA3. We investigated its stability and the antiproliferative effects of DOX-mGA3 in vitro and compared the data with the results obtained with DOX-GA3. We analyzed the conversion of DOX-mGA3 into DOX-GA3 by esterase activity in plasma of mice, rats and humans and subsequent hydrolysis to doxorubicin. We also determined whether DOX-mGA3 could be converted into DOX-GA3 and doxorubicin in vivo.

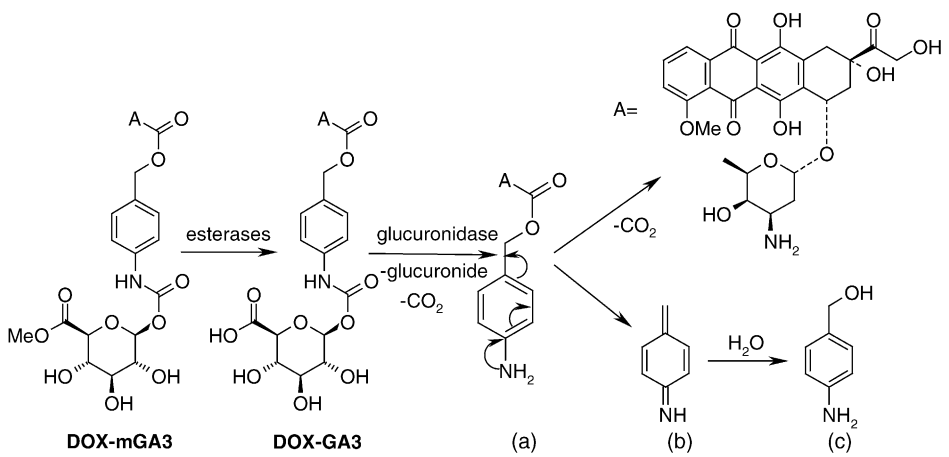
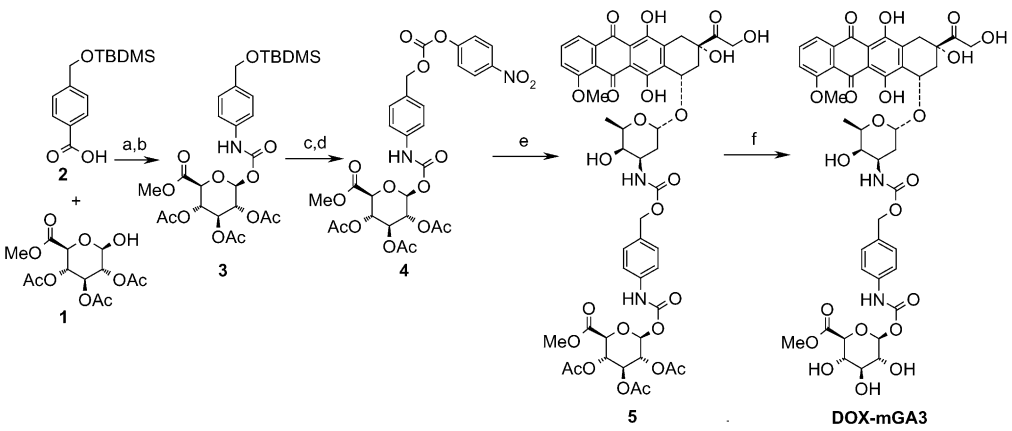


Fig. 1. The methyl glucuronate prodrug will be hydrolyzed first to liberate its glucuronide by esterases. The formed acid should be further cleaved by β-glucuronidase and spontaneous release of CO_2 to the drug-spacer molecule (**a**). The electron-releasing free amino-group will trigger the 1,6-elimination process and second molecule of CO_2 resulting in release of the parent antracycline and iminoquinone methide (**b**) transition state that is rapidly hydrolyzed to the non-toxic 4-aminobenzyl alcohol (**c**).



a) DPPA, Et_3N, at room temperature for 18 hr, b) at 80 °C for 3 hr, c) HoAc/H_2O/THF 1:1:1, at room temperature for 2 hr, d) 4-nitrophenyl chloroformate, pyridine, THF , at room temperature for 3 hr, e) Et_3N, DMF, at room temperature for 16 hr, f) NaOMe, MeOH, at 0°C for 1 hr.

Fig. 2. The synthesis of the methylated glucuronide prodrugs of doxorubicin, DOX-mGA3. For details see Section 2.

2. Materials and methods

2.1. Synthesis of N-[4-(doxorubicin-N-carbonyl-oxymethyl)phenyl] O-(methyl-O-β-glucuronyl) carbamate (DOX-mGA3)

The synthesis of the protected glucuronide prodrug of doxorubicin (Fig. 2) was performed using the same methodology as described by Leenders et al. [16]. Methyl-2,3,4-tri-O-acetyl D-glucuronic acid (**1**) was obtained as described [17]. The β-glucuronyl carbamate spacer moiety was prepared from TBDMS protected 4-hydroxymethyl-benzoic acid (**2**) by the modified Curtius reaction [16]. The resulting protected pro-moiety (**3**) was coupled to the anthracycline after deprotection of the benzyl alcohol functionality and transformation to the active carbonate (**4**) using 4-nitrophenyl chloroformate. The resulting tri-acetylated prodrugs (**5**) were deacetylated as described below to obtain the methyl ester prodrug, DOX-mGA3.

An 0.1 N solution of NaOMe in methanol (1.9 mL, 0.21 mmol) at 0 °C was added to a solution of **5** [16] (225 mg, 0.21 mmol) in anhydrous mixture of methanol (50 mL) and tetrahydrofuran (10 mL), and the deep blue solution was stirred for 1 h under argon. Thereafter, the solution was neutralized by the addition of the H^+ form of Dowex 50 WX2 cation-exchange resin. Filtration followed by evaporation under reduced pressure resulted in a crude residue, which was purified with column chromatography (SiO_2 MeOH/CH_2Cl_2, 1/10), giving DOX-mGA3 as amorphous red solid (117 mg, 0.126 mmol), mp 172–174 °C. The overall yield was 60%. Anal. calc. for $C_{43}H_{46}N_2O_{21}\cdot 1.5H_2O$: C, 54.15; H, 5.18; N, 2.94. Found: C, 53.97; H, 5.48; N, 3.16. NMR [300 MHz, $(CD_3)_2SO$)] δ 1.12 (2H, d, J = 7.0 Hz, 5′-Me), 1.45 (1H, dd, J = 12.7 Hz, J = 3.9 Hz, $2'_{eq}$-H), 1.83 (1H, dt, J = 12.7 Hz, J = 3.5 Hz, $2'_{ax}$-H), 2.09 (1H, dd, J = 14.4 Hz, J = 5.7 Hz, $8'_{ax}$-H), 2.20 (1H, d, J = 3.5 Hz, J = 14.9 Hz, $8'_{eq}$-H), 2.91 (1H, d, J = 18.8 Hz 10_{eq}-H), 2.99 (1H, d, J = 18.8 Hz 10_{ax}-H), 3.14–3.50 (4H, m, 3′-H, Gluc2,3,4,-H), 3.66 (2H, s, CO_2Me) 3.70 (1H, m, 4′-H), 3.88 (1H, d, J = 9.7 Hz, Gluc5-H), 3.97 (3H, s, 4-OMe), 4.12 (1H, m, 5′-H), 4.57 (2H, d, J = 6.1 Hz, 9-CH_2OH), 4.70 (1H, d, J = 5.7 Hz, 4′-OH), 4.85 (1H, d, J = 6.1 Hz, OH), 4.87 (2H, s, ArCH_2-), 4.92 (1H, t, J = 4.4 Hz, 7-H), 5.21 (1H, d, J = 2.6 Hz, 1-H′), 5.33 (1H, d, J = 4.4 Hz, OH), 5.42 (3H, m, Gluc1-H, OH, 9-OH), 6.84 (1H, d, J = 7.9 Hz, 3′-NH-), 7.24 (2H, d, J = 8.4 Hz, Ar3,5-H), 7.41 (2H, d, J = 8.4 Hz, Ar2,6-H), 7.63 (1H, t, J = 4.8 Hz, 3-H), 7.89 (2H, d, J = 4.4 Hz,1,2-H), 9.36 (1H, bs, ArNH-), 9.97 (1H, bs, 11-OH), 10.12 (1H, bs, 6-OH).

2.2. In vitro characteristics of DOX-mGA3

A stock solution of DOX-mGA3 (7.5 mM) was prepared in DMSO. The purity and stability of DOX-mGA3 (10 μM) was determined in phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) at pH 7.4 at 37 °C. Samples (20 μL) were taken at different time points. Analysis with reversed-phase HPLC was performed as described in the next section.

The hydrophilicity of DOX-mGA3 was analyzed by determination of the octanol/PBS partition coefficient. DOX-GA3 and doxorubicin were used as control drugs. Doxorubicin was derived from Pharmacia & Upjohn. The (pro)drugs were dissolved in 1 mL of octanol to a concentration of 10 μM, and then, 1 mL of PBS was added. The mixture was incubated for 3 h at 37 °C under continuous rigorous shaking (300 rpm). Aliquots of 100 μL of both phases were collected and diluted in 1 mL of methanol. The ratio of (pro)drug in octanol and PBS was determined by measuring the fluorescence of both phases (excitation wavelength 480 nm and emission wavelength of 580 nm; Perkin Elmer 3000). The octanol/PBS partition coefficient was calculated after subtraction of the background from octanol and PBS.

The hydrolysis of the methyl group of DOX-mGA3 (10 μM) in human, mouse and rat plasma was analyzed. Samples (20 μL) were taken at different time points and prepared for HPLC analysis. As a control, the hydrolysis of DOX-mGA3 (6 μM) by 3 units of carboxylesterase of porcine liver (Sigma–Aldrich) was checked in 100 μL of PBS/0.1% BSA after incubation at 37 °C for 45 min.

In order to demonstrate that DOX-mGA3 is not a good substrate for human recombinant β-glucuronidase, DOX-mGA3 (100 μM in PBS) was diluted in PBS/0.1% BSA with human β-glucuronidase (10 μg mL^{-1}) to a final concentration of 10 μM. At different time points, samples (20 μL) were taken and prepared for HPLC analysis. For measurement of the half-life of the hydrolysis of DOX-GA3 generated from DOX-mGA3, DOX-mGA3 was diluted in mouse plasma (100 μM) and incubated for 30 min at 37 °C. This mixture was then incubated in PBS/0.1% BSA with human β-glucuronidase (10 μg mL^{-1}) to a final prodrug concentration of 10 μM as described above.

2.3. HPLC analysis

Samples (20 μL) were treated by the addition of 80 μL acetonitrile in order to precipitate proteins. After incubation at −20 °C for 10 min, samples were centrifuged at 16,000 × *g* for 10 min. The supernatant (80 μL) was collected and 80 μL of phosphate buffer (0.5 mM triethylamine/20 mM sodium phosphate) was added.

The HPLC apparatus consisted of an autosampler (Marathon-XT), a pump (Separations, Model 300), and a fluorescence detector (Jasco, Model 821-FP: excitation 480 nm; emission 590 nm). Samples (50 μL) were loaded on a reversed-phase column (Chromsep C_{18}, 2 mm × 100 mm × 4.6 mm, 3 μm particle size). Elution was done with eluens in an isocratic run (0.5 mM triethylamine/20 mM sodium phosphate:acetonitrile = 3:2). Calibration of the system was performed as previously described [18] and the detection limit was 0.1 μM. In each cluster of runs, standards of doxorubicin, DOX-GA3 and DOX-mGA3 served as references at the beginning and at the end of the cluster. Control plasma and tissue samples spiked with known concentrations of the different compounds were included in the biodistribution experiments. No degradation was observed and the recovery of the compounds was taken into consideration in the analysis. The elution peaks in the chromatogram were integrated using the Gyncosoft program (Gynkotek, Version 5.3E).

2.4. Esterase activity assay

Human, mouse or rat plasma was incubated with 200 μL of 100 mM Tris–HCl, pH 8.0, containing 1 mM *p*-nitrophenyl acetate (*p*NPAc, Sigma–Aldrich), a substrate for esterases. After mixing, the rate of conversion to *p*-nitrophenol was measured at a wavelength of 415 nm during 10 min using an ELISA plate reader (BioRad).

2.5. In vitro antiproliferative effects

The human ovarian cancer cell line OVCAR-3 [19], the human breast cancer cell line MCF-7 [20], the human lung cancer cell line A549 (ATCC CLL 185), and the human colon cancer cell line WiDr (ATCC CLL 218) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cells were harvested and seeded in a 96-well microtiter plate at 5000 cells per well. After 24 h, prodrug or drug was added at different concentrations to the culture medium with or without human β-glucuronidase (0.05 U mL^{-1}) in triplicate. After 72 h, the wells were incubated with the cell proliferation reagent WST-1 (Roche) for 1 h at 37 °C. The absorbance was measured at a wavelength of 450 nm. The antiproliferative effects were determined and expressed as a percentage of growth of treated cells as compared to control cell growth, which was set at 100%. The IC_{50} value was expressed as the (pro)drug concentration that gave 50% cell growth inhibition.

2.6. Animals and human xenograft model

The animal experiments were performed in accordance with the institution guidelines after approval by the Institutional Animal Experimental Committee. Female NMRI mice (Harlan) were handled under conventional conditions. Specified pathogen-free conditions were used for female athymic nude mice (Hsd: athymic nude-*nu*; Harlan).

The human ovarian cancer xenograft FMa has been described earlier [21]. It is a poorly differentiated mucinous adenocarcinoma with a mean volume-doubling time of 5.5 days. Xenografts from previous recipients were transferred by implanting tissue fragments with a diameter of 2–3 mm into both flanks of 8–10-week-old mice. Tumors from previous recipients were transferred by implanting tissue fragments with a diameter of 2–3 mm into both flanks of 8–10-week-old mice. Upon growth, tumors were measured twice a week by the same observer. The tumor volume was calculated by the equation length × width × thickness × 0.5, and expressed in mm^3.

2.7. DOX-mGA3 in plasma of non-tumor-bearing mice

DOX-mGA3 was dissolved in Cremophor EL/ethanol (1/1 v/v) at a concentration of 10 mg mL^{-1} and was stable for at least 48 h as analyzed by HPLC. Immediately before injection, one part of this solution was diluted with four parts of 0.9% NaCl giving a concentration of DOX-mGA3 of 2 mg mL^{-1}. Six mice were injected i.v. with DOX-mGA3 or DOX-GA3 at a dose of 20 mg kg^{-1}. Blood samples were collected with the use of heparinized glass capillaries at different time points ranging from 1 to 60 min

(DOX-GA3) and to 90 min (DOX-mGA3) using three mice per time point. The samples were centrifuged at 2700 g for 10 min to separate the plasma. From the plasma, 20 μL was prepared for analysis by HPLC.

2.8. Distribution of DOX-mGA3 and DOX-GA3 in tumor and normal tissues

Nude mice bearing s.c. FMa xenografts with a mean tumor volume of 300 mm^3 were injected i.v. with 20 mg kg^{-1} DOX-mGA3 (in Cremophor EL/ethanol/0.9% NaCl (1/1/8, v/v)) or DOX-GA3 (in water). At different time points ranging from 1 min to 2 h, blood, tumors, liver, heart and kidneys were removed in groups of three mice per time point. Blood samples were collected as described in the previous section. To plasma 1/100 volume of 100 mM of bis(4-nitrophenyl)phosphate (BNPP) was added to prevent hydrolysis of DOX-mGA3 to DOX-GA3 [22]. Furthermore, 1/100 volume of 100 mM D-glucaric acid-1,4-lactone monohydrate (saccharonolactone; Fluka) was added to prevent hydrolysis of DOX-GA3 by β-glucuronidase [6]. Samples were prepared for HPLC analysis as described above. After blood sampling, tumor, liver, heart and kidney tissues were collected and immediately frozen in liquid nitrogen and stored at −80 °C until preparation for HPLC analysis. Tissue samples were pulverized (Mikro-Dismembrator II, B. Braun Biotech International, Nieuwegein, The Netherlands) and diluted (230 mg tissue/770 μL PBS containing 1 mM saccharonolactone and 1 mM BNNP. The homogenate (40 μL) was diluted in 3.3% (w/v) $AgNO_3$ (8 μL) and acetonitrile (32 μL), shaken (15 min, room temperature), sonicated (15 min, room temperature) and centrifuged (16,000 × *g*, 10 min). To the supernatant (50 μL), 117 μL of phosphate buffer (0.5 mM triethylamine/20 mM sodium phosphate) was added. Samples were analyzed by HPLC as described above.

Each HPLC sample was prepared in duplicate. The mean fluorescence of each duplicate sample was used to calculate the prodrug concentration in each animal at a given time. The half-lives ($t_{1/2}$) and area under the concentration versus time curves (AUCs) were calculated using WinNonlin 1.5 (Pharsight).

3. Results

3.1. Characterization of DOX-mGA3

DOX-mGA3 was first examined for its stability in PBS/0.1% BSA at 37 °C. DOX-mGA3 appeared to be gradually hydrolyzed into DOX-GA3 with a $t_{1/2}$ of approximately 10 h, while DOX-GA3 was stable for at least 24 h (data not shown). We then calculated the the logarithm of the octanol/PBS partition coefficient (log *P*) as a measure of the hydrophilicity of the prodrug and the drug. The highly hydrophilic DOX-GA3 had a relatively low value of −0.15 as compared to 0.52 of doxorubicin. These values were similar to those reported before [5]. The log *P* of DOX-mGA3 was 1.27 and considerably higher than that of doxorubicin demonstrating the very lipophilic nature of this prodrug.

We tested whether the methyl ester group of DOX-mGA3 could be hydrolyzed by esterases in blood [23]. Therefore, we incubated DOX-mGA3 with plasma from different species (Fig. 3A). The hydrolysis in mouse plasma ($t_{1/2}$ of approximately 0.5 min) and that in rat plasma ($t_{1/2}$ of approximately 1.5 min) were much faster than that in human plasma ($t_{1/2}$ of approximately 2.5 h). The rate of hydrolysis was in agreement with the esterase activity in plasma of the different species (Fig. 3B). By measurement of the conversion of the substrate *p*NPAc, it was demonstrated that the esterase activity in human plasma was approximately 2.5-fold lower than that in mouse and approximately 2-fold lower than that in rat plasma. Of note: the assay, however, does not discriminate for differences in catalytic rate or esterase content relevant for DOX-mGA3 conversion between species. To confirm that esterase activity present in blood was indeed responsible for the hydrolysis of the methyl group of DOX-mGA3, we incubated DOX-mGA3 with porcine liver carboxylesterase at 37 °C. After 45 min, 93% of DOX-mGA3 was converted into DOX-GA3, while the methyl group was not removed in the absence of the enzyme (data not shown).

In order to show that DOX-mGA3 will ultimately be converted to doxorubicin by human β-glucuronidase, we first incubated DOX-mGA3 in mouse plasma or PBS/0.1% BSA for 30 min at 37 °C. After this time period, 95% of the DOX-mGA3 was converted into DOX-GA3 in mouse plasma (Fig. 4A), while no DOX-GA3 was generated in PBS/0.1% BSA (Fig. 4B). Thereafter, human β-glucuronidase was added. DOX-GA3 generated after incubation of DOX-mGA3 in mouse plasma was completely hydrolyzed to doxorubicin by this enzyme at 120 min. In PBS/0.1% BSA, only 20% of doxorubicin from DOX-mGA3 could be detected at 240 min after addition of β-glucuronidase. This finding can be explained by spontaneous generation of DOX-GA3 as we demonstrated that DOX-mGA3 is not highly stable in PBS/0.1% BSA. Therefore, it can be concluded that DOX-mGA3 itself is not a good substrate for human β-glucuronidase and that conversion into doxorubicin only occurs after hydrolysis of the methyl group.

3.2. In vitro antiproliferative effects

The antiproliferative effects of doxorubicin, DOX-GA3 and DOX-mGA3 were determined in four different human malignant cell lines (Table 1). In all four cell lines IC50 values for DOX-GA3 and DOX-mGA3 were at least 37-fold higher than that of the parent drug doxorubicin. When OVCAR-3 cells were incubated with DOX-mGA3 in

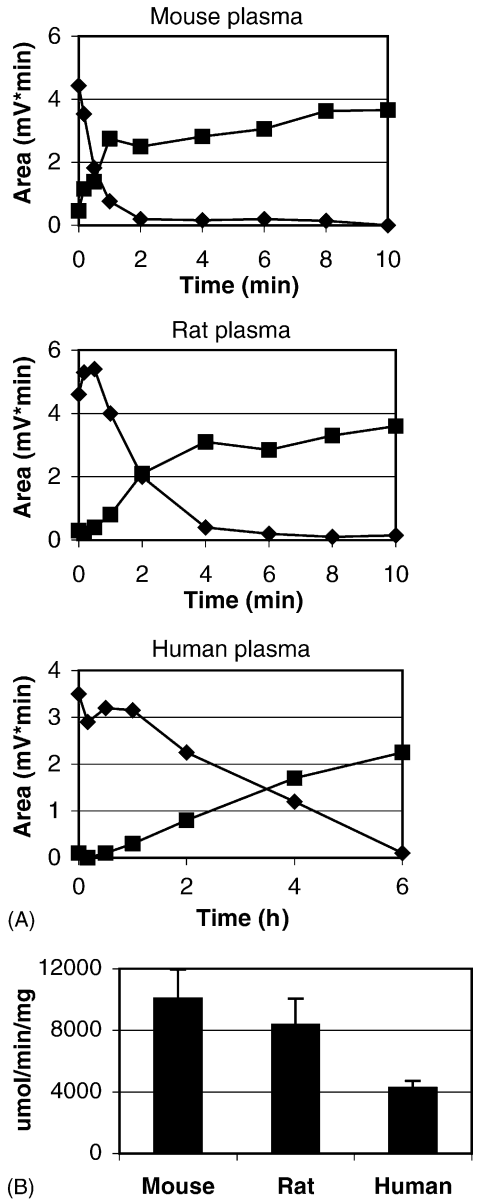


Fig. 3. (A) HPLC analysis of the hydrolysis of the methyl ester bond of DOX-mGA3 (6 μM) in mouse, rat and human plasma at 37 °C. At different time points, samples were taken and analyzed by HPLC. Concentrations of DOX-mGA3 and DOX-GA3 were expressed as the corresponding peak areas (mV × min). DOX-mGA3 (◆) was totally converted to DOX-GA3 (■) in plasma of all three species. (B) Esterase activity in mouse, rat and human plasma in two separate samples per species. Plasma was diluted in PBS and incubated with 1 mM *p*NPAc. Substrate conversion was measured after 10 min. Esterase activity was expressed as the rate of *p*NPAc conversion in μmol min^{-1} mg^{-1}. Bars, ±S.D.

combination with an excess of human β-glucuronidase (0.05 U mL^{-1}), the IC50 value was comparable to that of doxorubicin and DOX-GA3 in combination with human β-glucuronidase (Fig. 5). This finding can be explained by the hydrolysis of the methyl group of DOX-mGA3 in the culture medium during the 72-h assay, whereafter DOX-GA3 can be activated by human β-glucuronidase to doxorubicin.

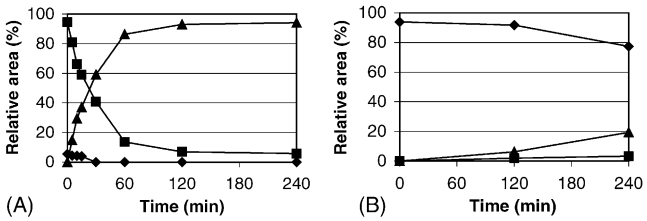


Fig. 4. Conversion of DOX-mGA3 into doxorubicin by human β-glucuronidase after incubation in mouse plasma (A) or PBS/0.1% BSA (B). DOX-mGA3 (100 μM) was first incubated in mouse plasma or PBS/0.1% BSA for 30 min. These mixtures were then diluted 10 times in PBS/0.1% BSA. After addition of human β-glucuronidase (10 μg mL^{-1}), samples were taken at different time points for analysis by HPLC. The peak areas of DOX-mGA3, DOX-GA3 and doxorubicin were expressed as a percentage of the total peak areas. DOX-mGA3 (◆); DOX-GA3 (■); doxorubicin (▲).

Table 1
Antiproliferative effects of DOX-mGA3, DOX-GA3 and doxorubicin expressed as IC_{50}[a] in human malignant cell lines

(Pro)drug	A549 (μM)	MCF-7 (μM)	OVCAR-3 (μM)	WiDr (μM)
DOX-mGA3	>50	22	7	>50
DOX-GA3	26	30	7	13
Doxorubicin	0.7	0.3	0.1	0.2

[a] (Pro)drug concentration that gives 50% growth in treated cells when compared with control cell growth. The values are the mean from two separate experiments performed in triplicate.

3.3. Pharmacokinetics of DOX-mGA3 in plasma of non-tumor-bearing mice

The kinetics of DOX-mGA3 in comparison to that of DOX-GA3 in blood was first tested in non-tumor-bearing mice (Fig. 6). DOX-mGA3 or DOX-GA3 was injected i.v. at a dose of 20 mg kg^{-1}. Orbita punctures were taken at different time points after injection and analyzed by HPLC for the presence of DOX-mGA3 and DOX-GA3. Administration of DOX-mGA3 resulted in a peak concentration of

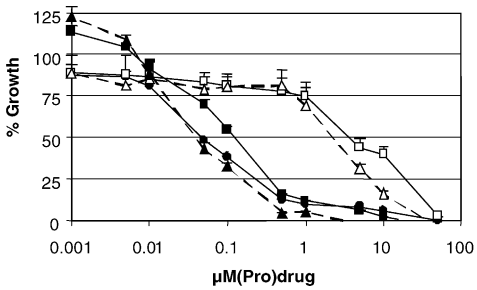


Fig. 5. Antiproliferative effects of DOX-mGA3 in various concentrations with or without human β-glucuronidase in OVCAR-3 cells. Treatment of OVCAR-3 cells with doxorubicin or DOX-GA3 with or without human β-glucuronidase served as controls. After 72 h, cell growth was measured with the cell proliferation reagent, WST-1, and the growth inhibitory effects were expressed as a percentage of control cell growth. The experiment was performed in triplicate wells. Doxorubicin (●); DOX-mGA3 (△); DOX-GA3 (□); DOX-mGA3 with β-glucuronidase (▲); DOX-GA3 with β-glucuronidase (■). Bars, ±S.D.

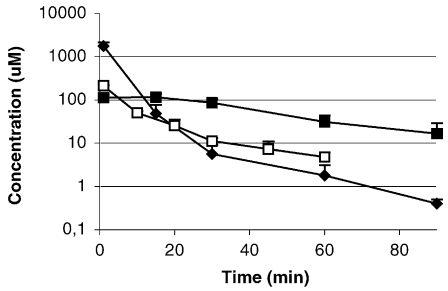


Fig. 6. The concentration-time curves of DOX-mGA3 and DOX-GA3 in plasma as a result of the i.v. administration of 20 mg kg^{-1} DOX-mGA3 or DOX-GA3 to groups of 3 mice each. DOX-mGA3 (◆); DOX-GA3 from DOX-mGA3 (■); DOX-GA3 from DOX-GA3 itself (□). Bars, ±S.D.

this prodrug of 1766 μM (t = 1 min) in plasma and an elimination $t_{1/2}$ of 15.8 min. DOX-GA3 generation showed a plateau phase at a plasma concentration of 103 μM during approximately 30 min followed by an elimination phase with a $t_{1/2}$ of 25.3 min. The $t_{1/2}$ of DOX-GA3 itself after i.v. injection at a dose of 20 mg kg^{-1} was 26.1 min.

The AUC of DOX-GA3 generated from DOX-mGA3 from 1 to 60 min was 4873 nmol min^{-1} mL^{-1}. This was 2.7-fold higher than the AUC of DOX-GA3 itself, which was 1965 nmol min^{-1} mL^{-1}.

3.4. Biodistribution of DOX-mGA3 in FMa-bearing mice

We next investigated whether the concentrations of DOX-GA3 and doxorubicin in plasma and tissues of FMa-bearing mice after i.v. administration of 20 mg kg^{-1} 1 DOX-mGA3 were higher than those after the same dose of DOX-GA3. This information would give an indication whether a higher antitumor efficacy might be expected from DOX-mGA3. Plasma, tumors, liver, heart and kidneys were collected at 0.5, 1 and 2 h after administration of the prodrug. In addition, plasma was collected by orbita puncture at 1 min after prodrug administration. All samples were analyzed by HPLC for the presence of DOX-mGA3, DOX-GA3 and doxorubicin (Fig. 7). The curves of the plasma kinetics of DOX-GA3 generated from DOX-mGA3 and of DOX-GA3 itself were similar in FMa-bearing mice as compared to non-tumor-bearing mice (Fig. 6). The AUC was calculated from the limited number of time points available between 0.5 and 2 h. In general, the AUC of DOX-GA3 after DOX-mGA3 in tumor and normal tissues was 2.5- to 3-fold higher than after DOX-GA3, except for that in liver (Table 2). The generation of

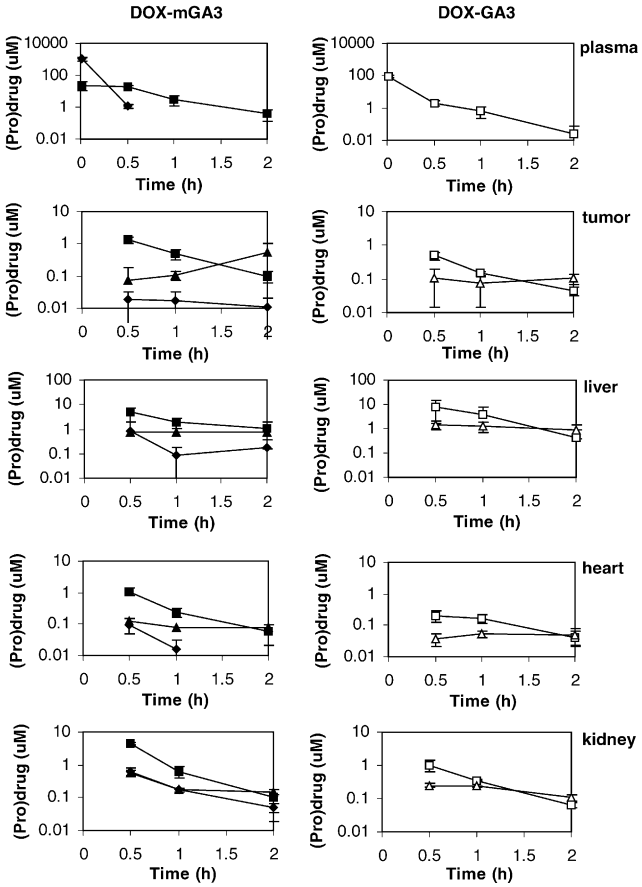


Fig. 7. The concentration-time curves of: DOX-mGA3 (◆), DOX-GA3 (■) and doxorubicin (▲) as a result of the i.v. administration of 20 mg kg^{-1} DOX-mGA3; DOX-GA3 (□) and doxorubicin (△) as a result of the i.v. administration of 20 mg kg^{-1} DOX-GA3 in plasma, tumors, liver, heart, and kidney of FMa-bearing mice. Groups consisted of three mice each. Doxorubicin was not detectable in plasma after administration of the given dose of DOX-mGA3 or DOX-GA3 at any time. Bars, ±S.D.

doxorubicin was most pronounced in tumor tissue, in which the AUC of doxorubicin was 50% of that of DOX-GA3 (Tables 2 and 3). Of interest, the administration of DOX-mGA3 resulted in a 2.7-fold higher AUC of doxorubicin in tumor tissue when compared to the AUC after DOX-GA3 administration.

The ratio between doxorubicin concentrations after DOX-mGA3 and DOX-GA3 administration in FMa tumor tissue increased from almost 1 at 0.5 h after injection to approximately 5 at 2 h (Fig. 8). In normal mouse tissues, the ratio was approximately 1 at all time points except for a higher ratio in heart and kidney at the earliest time point of 0.5 h.

Table 2
AUC[a] of DOX-GA3 in plasma and tissues of FMa-bearing nude mice after i.v. administration of 20 mg kg^{-1} DOX-GA3 or DOX-mGA3

Treatment	Plasma (nmol min^{-1} mL^{-1})	Tumor (nmol min^{-1} g^{-1})	Liver (nmol min^{-1} g^{-1})	Heart (nmol min^{-1} g^{-1})	Kidney (nmol min^{-1} g^{-1})
DOX-mGA3	427.5	44.8	188.7	29.0	100.3
DOX-GA3	59.7	15.3	297.8	12.0	33.4

[a] AUC from 0.5 h until 2 h.

Table 3
AUC[a] of doxorubicin in tissues of FMa-bearing nude mice after i.v. administration of 20 mg kg^{-1} DOX-GA3 or DOX-mGA3

Treatment	Tumor (nmol min^{-1} g^{-1})	Liver (nmol min^{-1} g^{-1})	Heart (nmol min^{-1} g^{-1})	Kidney (nmol min^{-1} g^{-1})
DOX-mGA3	21.6	65.4	7.8	20.6
DOX-GA3	8.1	101.9	4.5	17.5

[a] AUC from 0.5 h until 2 h.

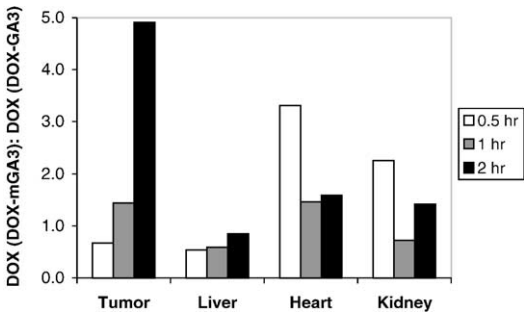


Fig. 8. Ratio of doxorubicin (DOX) concentrations obtained after DOX-mGA3 and DOX-GA3 administration (20 mg kg^{-1} i.v.) in mouse plasma, normal tissue and FMa tumor tissue at 0.5, 1 and 2 h after injection. The ratio was calculated by dividing the doxorubicin concentration obtained after DOX-mGA3 administration by the doxorubicin concentration after DOX-GA3 administration.

4. Discussion

DOX-GA3 is a prodrug with a higher therapeutic index than doxorubicin as shown in human ovarian cancer xenografts, because of tumor-selective activation by β-glucuronidase [6]. The production process of DOX-GA3, however, is very inefficient. The advantage of the synthesis of DOX-mGA3 instead of DOX-GA3 is that in the last deprotection step only the tri-acetate, and not the methyl group, has to be chemically removed. We showed that under less basic conditions for a shorter time period the overall yield of DOX-mGA3 was 60%. For comparison, we have previously reported that complete deprotection yielded 37% of DOX-GA3 [16].

We anticipated that the methyl group of the methylated glucuronide prodrug could be enzymatically removed by esterase activity in blood. Indeed, we showed that DOX-mGA3 was completely hydrolyzed to DOX-GA3 in plasma at a rate dependent on the level of esterase activity. We demonstrated that DOX-mGA3 was not a substrate for human β-glucuronidase. After removal of the methyl group by esterases for formation of DOX-GA3, complete conversion to doxorubicin could be achieved by human β-glucuronidase. Moreover, the antiproliferative effect of DOX-mGA3 in the presence of β-glucuronidase was similar to that of doxorubicin in OVCAR-3 cells in vitro.

We hypothesized that the administration of lipophilic DOX-mGA3 would result in a prolonged circulation of DOX-GA3 in mice as compared to direct administration of the hydrophilic DOX-GA3. Despite the high esterase activity in mouse plasma, we were able to show a 2.7-fold increase of the plasma AUC of DOX-GA3 from DOX-mGA3 as compared to the AUC after administration of DOX-GA3 itself in non-tumor-bearing mice as well as in tumor-bearing nude mice. The level of DOX-GA3 generated from DOX-mGA3 showed a plateau phase of approximately 30 min followed by the elimination phase. The duration of the plateau phase might seem to be long considering the very short conversion $t_{1/2}$ of 0.5 min that was measured in mouse plasma in vitro. The peak concentration of DOX-mGA3 of 1766.4 μM in vivo, however, was considerably higher than the 6 μM used in the in vitro assay. The slower conversion of DOX-mGA3 in vivo might be attributed to the saturation of the esterases in plasma. Indeed, we have observed a longer conversion $t_{1/2}$ of 5 min at a concentration of DOX-mGA3 of 800 μM in mouse plasma in vitro (data not shown). Another possible explanation for the 30-min plateau phase observed in mice will be the rapid uptake of the lipophilic DOX-mGA3 in tissues, whereafter conversion to DOX-GA3 will result in the release of the prodrug in the circulation. The pharmacokinetic behaviour of DOX-mGA3 might even be more favorable in man considering the much lower conversion $t_{1/2}$ of DOX-mGA3 of approximately 2.5 h that we measured in human plasma in vitro (Fig. 3).

In tumor tissue, an increased AUC of DOX-GA3 was reached after administration of DOX-mGA3 as compared to that after administration of DOX-GA3. The AUC of doxorubicin was 50% of the DOX-GA3 AUC in tumor tissue, most probably as a result of the presence of extracellular β-glucuronidase. Therefore, DOX-mGA3 might be expected to give a better tumor growth inhibition than DOX-GA3 at the same dose. In contrast, the higher concentration of DOX-GA3 after the administration of DOX-mGA3 did not lead to a substantially increased concentration of doxorubicin in normal tissues, in which extracellular β-glucuronidase should be absent (Table 3). Since doxorubicin generation in heart tissue after DOX-mGA3 as well as after DOX-GA3 [6] was proportionally lower than that in tumor tissue, we expect that this prodrug will be less cardiotoxic than is normally associated with doxorubicin treatment.

Treatment with DOX-mGA3 is hampered by its low solubility precluding effective doses to be administered. The lipophilic DOX-mGA3 is not soluble in aqueous solutions such as water, phosphate buffer or ethanol. A solution of DOX-mGA3 could be prepared in Cremophor EL/ethanol (1:1) at a concentration of 10 mg mL^{-1} and further diluted in 0.9% NaCl to 2 mg mL^{-1}. In the clinic, some drugs have to be given as a solution in Cremophor EL. For example, administration of paclitaxel in Cremo-

phor EL is a feasible approach when precautions are taken to prevent hypersensitivity reactions [24]. The highest dose of DOX-mGA3 that could be given in a single injection i.v. to mice was 20 mg kg^{-1}. For comparison, we have previously shown that DOX-GA3 gave good tumor growth inhibition in FMa-bearing nude mice at a dose of 250 mg kg^{-1} [6]. Even considering the fact that a 2.7-fold higher concentration of doxorubicin can be reached in tumor tissue after DOX-mGA3 than after the same dose of DOX-GA3, efforts should be taken to formulate the prodrug in different solvents.

In conclusion, in vivo administration of DOX-mGA3 led to improved pharmacokinetics of this prodrug and 2.7-fold higher doxorubicin concentrations in tumor tissue as compared to administration of DOX-GA3 itself. Therefore, better antitumor effects might be expected from a lower dose of DOX-mGA3 than from DOX-GA3. Furthermore, the synthesis of DOX-mGA3 is much more efficient than that of DOX-GA3. The therapeutic usefulness of DOX-mGA3, however, should be improved aiming at better solubility in clinically suitable solvents.

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References

[1] Haisma HJ, Boven E, Van Muijen M, De Jong J, Van der Vijgh WJ, Pinedo HM. A monoclonal antibody-beta-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. Br J Cancer 1992;66:474–8.

[2] Connors TA, Whisson ME. Cure of mice bearing advanced plasma cell tumours with aniline mustard: the relationship between beta-glucuronidase activity and tumour sensitivity. Nature 1966;210:866–7.

[3] Young CW, Yagoda A, Bittar ES, Smith SW, Grabstald H, Whitmore W. Therapeutic trial of aniline mustard in patients with advanced cancer. Comparison of therapeutic response with cytochemical assessment of tumor cell beta-glucuronidase activity. Cancer 1976;38: 1887–95.

[4] De Graaf M, Boven E, Scheeren HW, Haisma HJ, Pinedo HM. Beta-glucuronidase-mediated drug release. Curr Pharm Des 2002;8: 1391–403.

[5] Houba PH, Leenders RG, Boven E, Scheeren JW, Pinedo HM, Haisma HJ. Characterization of novel anthracycline prodrugs activated by human beta-glucuronidase for use in antibody-directed enzyme prodrug therapy. Biochem Pharmacol 1996;52:455–63.

[6] Houba PH, Boven E, Van der Meulen-Muileman IH, Leenders RG, Scheeren JW, Pinedo HM, et al. A novel doxorubicin-glucuronide prodrug DOX-GA3 for tumour-selective chemotherapy: distribution and efficacy in experimental human ovarian cancer. Br J Cancer 2001;84:550–7.

[7] Bosslet K, Straub R, Blumrich M, Czech J, Gerken M, Sperker B, et al. Elucidation of the mechanism enabling tumor selective prodrug monotherapy. Cancer Res 1998;58:1195–201.

[8] Houba PH, Boven E, Van der Meulen-Muileman IH, Leenders RG, Scheeren JW, Pinedo HM, et al. Distribution and pharmacokinetics of the prodrug daunorubicin-GA3 in nude mice bearing human ovarian cancer xenografts. Biochem Pharmacol 1999;57:673–80.

[9] Bosslet K, Czech J, Hoffmann D. A novel one-step tumor-selective prodrug activation system. Tumor Targeting 1995;1:45–50.

[10] Houba PH, Boven E, Erkelens CA, Leenders RG, Scheeren JW, Pinedo HM, et al. The efficacy of the anthracycline prodrug daunorubicin-GA3 in human ovarian cancer xenografts. Br J Cancer 1998;78: 1600–6.

[11] Woessner R, An Z, Li X, Hoffman RM, Dix R, Bitonti A. Comparison of three approaches to doxorubicin therapy: free doxorubicin, liposomal doxorubicin, and beta-glucuronidase-activated prodrug (HMR 1826). Anticancer Res 2000;20:2289–96.

[12] Ghosh AK, Farquhar D. A methyl glucuronate prodrug of phosphorodiamidic mustard. Tetrahedron Lett 1997;38:8795–8.

[13] Papot S, Combaud D, Gesson JP. A new spacer group derived from arylmalonaldehydes for glucuronylated prodrugs. Bioorg Med Chem Lett 1998;8:2545–8.

[14] Bakina E, Wu Z, Rosenblum M, Farquhar D. Intensely cytotoxic anthracycline prodrugs: glucuronides. J Med Chem 1997;40:4013–8.

[15] Florent JC, Dong X, Gaudel G, Mitaku S, Monneret C, Gesson JP, et al. Prodrugs of anthracyclines for use in antibody-directed enzyme prodrug therapy. J Med Chem 1998;41:3572–81.

[16] Leenders RG, Damen EW, Bijsterveld EJ, Scheeren HW, Houba PH, Van der Meulen-Muileman IH, et al. Novel anthracycline-spacer-beta-glucuronide,-beta-glucoside, and -beta-galactoside prodrugs for application in selective chemotherapy. Bioorg Med Chem 1999;7:1597–610.

[17] Mikano M. Facile 1-O-deacylation of per-O-acylaldoses. Carbohydr Res 1989;191:150–3.

[18] De Jong J, Guerand WS, Schoofs PR, Bast A, van der Vijgh WJ. Simple and sensitive quantification of anthracyclines in mouse atrial tissue using high-performance liquid chromatography and fluorescence detection. J Chromatogr 1991;570:209–16.

[19] Hamilton TC, Young RC, McKoy WM, Grotzinger KR, Green JA, Chu EW, et al. Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. Cancer Res 1983;43:5379–89.

[20] Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973;51:1409–16.

[21] Kievit E, Pinedo HM, Schluper HM, Haisma HJ, Boven E. Comparison of monoclonal antibodies 17-1A and 323/A3: the influence of the affinity on tumour uptake and efficacy of radioimmunotherapy in human ovarian cancer xenografts. Br J Cancer 1996;73: 457–64.

[22] Yoshigae Y, Imai T, Taketani M, Otagiri M. Characterization of esterases involved in the stereoselective hydrolysis of ester-type prodrugs of propranolol in rat liver and plasma. Chirality 1999; 11:10–3.

[23] Krisch K. Carboxylic ester hydrolases. In: Boyd PD, Boyd PD, editors. The Enzymes. New York: Academic Press; 1971. p. 59.

[24] Foa R, Norton L, Seidman AD. Taxol (paclitaxel): a novel anti-microtubule agent with remarkable anti-neoplastic activity. Int J Clin Lab Res 1994;24:6–14.