A novel doxorubicin-glucuronide prodrug DOX-GA3 for tumour-selective chemotherapy: distribution and efficacy in experimental human ovarian cancer

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Summary The doxorubicin (DOX) prodrug N-[4-doxorubicin-N-carbonyl (oxymethyl) phenyl] O-β-glucuronyl carbamate (DOX-GA3) was synthesised for specific activation by human β-glucuronidase, which is released in necrotic areas of tumour lesions. This novel prodrug was completely activated to the parent drug by human β-glucuronidase with Vmax = 25.0 μmol min⁻¹mg⁻¹ and Km = 1100 μM. The pharmacokinetics and distribution of DOX-GA3 in nude mice bearing human ovarian cancer xenografts (OVCAR-3) were determined and compared with DOX. Administration of DOX at 8 mg kg⁻¹ i.v. (maximum tolerated dose, MTD) to OVCAR-3-bearing mice resulted in a peak plasma concentration of the drug of 16.4 μM (t = 1 min). A 7.6-times lower peak plasma concentration of DOX was measured after injection of DOX-GA3 at 250 mg kg⁻¹ i.v. (50% of MTD). In normal tissues the prodrug showed peak DOX concentrations that were up to 5-fold (heart) lower than those found after DOX administration. DOX-GA3 activation by β-glucuronidase in the tumour yielded an almost 5-fold higher DOX peak concentration of 9.57 nmol g⁻¹ (P < 0.05) than the peak concentration of only 2.14 nmol g⁻¹ observed after DOX. As a consequence, the area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The anti-tumour effects of DOX-GA3 and DOX were compared at equitoxic doses in OVCAR-3 xenografts at a mean tumour size of 125 mm³. The anti-tumour effects of DOX-GA3 and DOX were compared at equitoxic doses in OVCAR-3 xenografts at a mean tumour size of 125 mm³. The area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The difference in area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The peak concentration of DOX after injection of DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The anti-tumour effects of DOX-GA3 and DOX were compared at equitoxic doses in OVCAR-3 xenografts at a mean tumour size of 125 mm³. The anti-tumour effects of DOX-GA3 and DOX were compared at equitoxic doses in OVCAR-3 xenografts at a mean tumour size of 125 mm³. The peak concentration of DOX after injection of DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The difference in area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The peak concentration of DOX after injection of DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The difference in area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The peak concentration of DOX after injection of DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The difference in area under the curve of DOX calculated in tumour tissue after DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹). The peak concentration of DOX after injection of DOX-GA3 (13.1 μmol min⁻¹g⁻¹) was 10-fold higher than after DOX (1.31 μmol min⁻¹ g⁻¹).

Keywords: anthracyclines; cancer chemotherapy; β-glucuronidase; glucuronide

Doxorubicin (DOX) is an anticancer agent with a wide spectrum of activity. Cumulative dose-related cardiotoxicity, however, is a major side-effect of DOX, in addition to the acute toxicities, such as myelosuppression, nausea and vomiting. The success of DOX, and its limitations in clinical use, have directed research endeavours for the development of analogues of DOX with an improved therapeutic index. Among these are iododoxorubicin, AD-32 and epidoxorubicin (Weiss, 1992). Iododoxorubicin was noted to be promising in phase I trials, but in phase II trials the response rate was too low to warrant further development. AD-32 has greater anti-tumour activity, and less cardiotoxicity, than DOX, but drug formulation and solubility problems prevented its further clinical development. Of the available analogues, only epidoxorubicin appears to have a reduced cardiotoxicity with retention of anti-tumour activity and is in use in current cancer chemotherapy.

Another way to improve the selectivity and efficacy of chemotherapy is the use of non-toxic prodrugs that are preferentially converted into active anticancer agents at the tumour site (Sinhaababu and Thakker, 1996). N-t-leucyl-DOX is a prodrug of DOX, to be activated by tumour peptidases (Deprez-de Campeneere et al, 1982). In human ovarian cancer xenografts, N-t-leucyl-DOX was more effective than DOX (Boven et al, 1992). Clinical studies on N-t-leucyl-DOX, however, have indicated premature activation of the prodrug in the circulation, because of which the selectivity of the prodrug would be reduced (de Jong et al, 1992). Elevated enzyme levels in tumour tissue have been reported for β-glucuronidase (Connors and Whisson, 1966). Bosslet et al (1995) and Schumacher et al (1996) have shown that this enzyme is released in the extracellular space as a result of necrosis in tumours. The enzyme can only be detected in very low concentrations in the circulation (Fishman, 1970). Therefore, β-glucuronidase may be exploited for the specific activation of glucuronide prodrugs in tumour tissue.

We have developed glucuronide derivatives of anthracyclines and showed that these prodrugs, such as epiurubicin-glucuronide (Haisma et al, 1992) and daunorubicin-glucuronides (Leenders et al, 1995; Houba et al, 1996), are relatively non-toxic in vitro and can be activated by β-glucuronidase to yield the active anthracycline. Treatment with the glucuronide prodrug daunorubicin-GA3 (DNR-GA3) induced a better tumour growth delay than daunorubicin (DNR) when studied at equitoxic doses in 3 human ovarian cancer xenografts which were sensitive to DNR (Houba et al, 1998). Comparison of the distribution and pharmacokinetics of DNR and DNR-GA3 demonstrated that the prodrug DNR-GA3 was selectively activated by human β-glucuronidase present in the tumours and resulted in a higher DNR AUC in tumours and lower...
DNR AUCs in normal tissues (Houba et al, 1999). Our treatment results with a DNR prodrug are encouraging, but solid tumour types show better sensitivity to DOX than to DNR. In analogy, this has led to the synthesis of the prodrug of DOX: \textit{N}-\textit{[4-doxorubicin-N-carbonyl (oxymethyl) phenyl] O-\(\beta\)-glucuronyl carbamate (DOX-GA3). After hydrolysis DOX-GA3 is activated to DOX; glucuronic acid and 4-aminobenzyl alcohol (spacers) are released

\textbf{Figure 1} Chemical structure of prodrug \textit{N}-\textit{[4-doxorubicin-N-carbonyl (oxymethyl) phenyl] O-\(\beta\)-glucuronyl carbamate (DOX-GA3)}. After hydrolysis DOX-GA3 is activated to DOX; \(\beta\)-glucuronoyl carbamate (DOX-GA3; Figure 1). DOX-GA3 is a derivative of DOX in which the glucuronic acid moiety is linked to the anthracycline via a synthetic spacer, designed to increase the hydrolysis by human \(\beta\)-glucuronidase. We anticipate that this prodrug may have a broader application in the treatment of cancer.

In the present experiments we compared the antiproliferative effects of DOX-GA3 and DOX in vitro, and calculated the \(K_m\) and \(V_{max}\) of DOX-GA3 hydrolysed by human \(\beta\)-glucuronidase. We studied whether DOX-GA3 was specifically activated to DOX by \(\beta\)-glucuronidase in tumour tissue. To this end, the distribution of DOX released from DOX-GA3 was compared with that of DOX alone in mice bearing OVCAR-3 xenografts in tumour tissue as well as in normal organs at several time-points after injection. After determination of the maximum tolerated dose (MTD) of DOX-GA3 in OVCAR-3-bearing nude mice, we compared the anti-tumour efficacy of DOX-GA3 with that of DOX. As large tumours have more necrosis than small tumours and are therefore expected to contain higher levels of extracellular \(\beta\)-glucuronidase, special attention was paid to the influence of the tumour size on drug effects.

\section*{MATERIALS AND METHODS}

\subsection*{Materials}

The human ovarian cancer cell line NIH:OVCAR-3 (Hamilton et al, 1983) was grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (ICN, Costa Mesa, CA), 50 IU ml\(^{-1}\) penicillin (ICN) and 50 \(\mu\)g ml\(^{-1}\) streptomycin (ICN) in a humidified atmosphere containing 5% CO\(_2\) at 37°C.

Doxorubicin (DOX, Pharmachemie BV, Haarlem, The Netherlands) was purchased as a powder. The prodrug \textit{N}-\textit{[4-doxorubicin-N-carbonyl (oxymethyl) phenyl] O-\(\beta\)-glucuronyl carbamate (DOX-GA3)} was synthesized as described (Leenders et al, 1999). Stock solutions of DOX and DOX-GA3 were prepared in sterile water and stored at \(-20^\circ\)C.

\subsection*{Characterization of DOX-GA3 in vitro}

The purity and stability of DOX-GA3 and the activation of DOX-GA3 in the presence of \(\beta\)-glucuronidase was determined in 0.1% (w/v) BSA/PBS at pH 6.8 and 37°C. Samples were taken after 24 h and were analysed with reversed-phase HPLC as described (Houba et al, 1999). In each cluster of runs standards of DOX-GA3 and DOX were included, at the beginning and at the end of the cluster. The peak areas from the elution peaks in the chromatograms were determined by integration using the program Gyncosoft (Gynkotek, Version 5.3E). Calibration of the system was performed as described (de Jong et al, 1991) and the detection limit was 0.01 \(\mu\)M (pro)drug.

The half-life time of hydrolysis of the prodrug by human recombinant \(\beta\)-glucuronidase (Houba et al, 1996) was determined in PBS at 37°C and pH 6.8, reflecting the tumour interstitial pH (Martin and Jain, 1994), at concentrations of the enzyme (1 \(\mu\)g ml\(^{-1}\)) and the prodrug (100 \(\mu\)M) assumed to be clinically relevant (Houba et al, 1999). The prodrug and the enzyme were diluted in 0.1% (w/v) BSA/PBS at pH 6.8. After incubation at 37°C the reaction was stopped by adding methanol. Samples were analysed by HPLC as described above.

To further characterize the enzymatic conversion of the prodrugs by human \(\beta\)-glucuronidase \(K_m\) and \(V_{max}\) values were determined. A range of prodrug concentrations (5 \(\times\) 10\(^{-5}\) to 6 \(\times\) 10\(^{-3}\) M) was tested against a human \(\beta\)-glucuronidase concentration of 1 \(\mu\)g ml\(^{-1}\) to determine the hydrolysis expressed as \(K_m\) and \(V_{max}\) values. After incubation for 30 min at 37°C the reaction was stopped by adding methanol. Samples were analysed by HPLC and \(K_m\) and \(V_{max}\) values were calculated from direct linear plots as described by Eisenthal and Cornish-Bowden (Eisenthal and Cornish-Bowden, 1974).

\subsection*{Antiproliferative effects}

The in vitro antiproliferative effects of drug and prodrug were determined in OVCAR-3 cells as previously described (Houba et al, 1996). The antiproliferative effect was expressed as the IG\(_{50}\) value, which is the (pro)drug concentration that gives 50% growth inhibition when compared with control cell growth.

\subsection*{Human ovarian cancer xenograft OVCAR-3}

All animal experiments were approved by the Institution Animal Experiments Committee and performed in accord with the institution guidelines. Female athymic nude mice (Hsd: athymic nude-nu; Harlan Cpb, Horst, The Netherlands) were handled under specified pathogen-free conditions. The human ovarian cancer xenograft OVCAR-3 has been described earlier (Molthoff et al, 1991). The histology shows a poorly differentiated serous adenocarcinoma and tumours have a mean volume-doubling time of 5.0 days. Tumours 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, tumours were measured twice a week by the same observer. The tumour volume was calculated by the equation length \(\times\) width \(\times\) thickness \(\times\) 0.5, and expressed in mm\(^3\).

\subsection*{Maximum tolerated dose of DOX and DOX-GA3}

The MTD was defined as the dose that gives a mean reversible loss of approximately 10% of the initial weight within 2 weeks after the first injection. Animals dying within 2 weeks after the final injection were considered as toxic deaths. The MTD of DOX in
Distribution of DOX and DOX-GA3 in tumour and normal tissues

Nude mice bearing s.c. xenografts with a mean volume of 600 mm³ were injected i.v. with DOX (8 mg kg⁻¹) or DOX-GA3 (250 mg kg⁻¹) to determine their distribution and pharmacokinetics. Because of the limited availability of DOX-GA3 we investigated DOX-GA3 at half of the MTD. At different time-points ranging from 1 min to 24 h blood, tumours, liver, heart and kidneys were removed in groups of three OVCAR-3-bearing mice per time-point. Blood was collected in heparinized vials and centrifuged at 16 000 g for 5 min. To plasma 1/100 volume of 100% (T/C%) was assessed on each day of measurement and used to calculate half-life times and area under the concentration versus time curves (AUC). Differences between drug concentrations per time-point were evaluated with Student’s t-test.

Anti-tumour activity of anthracyclines in vivo

Mice bearing OVCAR-3 tumours were used to compare DOX with DOX-GA3 anti-tumour effects. At the start of the treatment (day 0), mice were grouped in order to obtain similarities in the mean tumour volume. For small tumours the mean volume was 125 mm³ and for larger tumours 400 mm³. Control and treatment groups consisted of 6 animals each. DOX was given in a dose of 8 mg kg⁻¹ i.v. weekly × 2 (MTD) to mice with small and larger tumours. DOX-GA3 was studied at 500 mg kg⁻¹ i.v. weekly × 2 (MTD) in groups with small and larger tumours. Mice were weighed twice a week and tumours were measured on the same days.

For evaluation of drug effects the relative tumour volume was expressed by the formula \( V_T/V_C \), where \( V_T \) is the volume on any given day and \( V_C \) is the volume on day 0. The ratio between the mean of the relative volumes of treated tumours and that of control tumours × 100% (T/C%) was assessed on each day of measure-ment and used to calculate the percentage of maximum GI (GI = 100%–T/C%). The relative tumour volume was calculated and normalized for growth of the control tumours. As a measure of the duration of the treatment effects, the days for each tumour to double twice in volume (TD1) was calculated. If a tumour did not reach 2 volume doubling times this volume was extrapolated from the last 2 available measurements. Differences in mean TD1 between groups were evaluated with Student’s t-test. In addition, differences in efficacy between the treatment groups of small vs large tumours were expressed as the specific growth delay (SGD) (Boven et al, 1988). The SGD was calculated according to the following formula:

\[
SGD = \frac{T_{D1\text{-end}} \text{ treated} - T_{D1\text{-end}} \text{ control}}{T_{D1\text{-end}} \text{ control}}
\]

RESULTS

Characterization of DOX-GA3 in vitro

Our initial experiments sought to determine enzymatic activation of the glucuronide prodrug DOX-GA3 to the parent drug DOX. To this end DOX-GA3 was incubated with human \( \beta \)-glucuronidase. HPLC analysis showed that DOX-GA3 was more than 99.9% pure and could be completely hydrolysed to DOX in the presence of excess human \( \beta \)-glucuronidase to DOX, and no spacer-drug intermediate was detectable. A sample chromatogram from an intermediate timepoint (30 min) is shown in Figure 2. The prodrug, incubated at the clinically relevant concentrations of 100 \( \mu \)M for DOX-GA3 and of 1 \( \mu \)g ml⁻¹ for the enzyme, was hydrolysed at pH 6.8 and 37°C with a t1/2 of 140 min.

Based on the in vitro cleavage experiments at different prodrug concentrations at pH 6.8 and at 37°C, \( V_{\text{max}} \) and \( K_m \) were calculated from direct linear plots as described by Eisenthal (Eisenthal and Cornish-Bowden, 1974). For DOX-GA3 a \( V_{\text{max}} \) of 25.0 \( \mu \)mol min⁻¹ mg⁻¹ and a \( K_m \) of 1100 \( \mu \)M was found.

We next analysed the in vitro antiproliferative effects of DOX and DOX-GA3. For DOX the IC₅₀ was 0.12 \( \mu \)M (SD ± 0.06) and the prodrug was more than 100-times less capable of inhibiting cell growth with an IC₅₀ value of 22 \( \mu \)M (SD ± 7.6). This was probably caused by activation of the prodrug through \( \beta \)-glucuronidase released from dead cells because we were unable to detect DOX in the prodrug preparation. When DOX-GA3 was incubated with an excess of \( \beta \)-glucuronidase, the antiproliferative effect returned to a value similar to that of DOX (0.16 \( \mu \)M; SD ± 0.06). This indicated that the prodrug was completely activated to the parent drug under these conditions (Figure 3).

Distribution and kinetics in plasma, tumour and normal tissues

The distribution of DOX (8 mg kg⁻¹) and DOX-GA3 (250 mg kg⁻¹) in plasma, tumour and normal tissues was compared in
OVCAR-3-bearing mice (Figure 4). First, the in vivo stability of the prodrug was tested in non-tumour-bearing mice. Administration of DOX-GA3 (250 mg kg\(^{-1}\)) in these animals resulted in a drug/prodrug ratio of 0.001 (\(t = 30 \text{ min}\)) in plasma and no other major peaks were visible. These findings indicate the lack of activation by endogenous β-glucuronidase in non-tumour-bearing animals. Next, we investigated this effect in tumour-bearing animals. After administration of DOX-GA3, the prodrug peak concentration was 1370 nmol ml\(^{-1}\) (\(t = 1 \text{ min}\)) in plasma and a drug peak concentration of 2.17 nmol ml\(^{-1}\) was found. After administration of DOX, a considerably higher peak concentration of 16.4 nmol ml\(^{-1}\) was detected in plasma. The ratio of drug/prodrug (0.002) was similar to that found in non-tumour-bearing animals, indicating that the systemic activation of prodrug is not increased by the presence of tumour.

DOX administration resulted in DOX peak concentrations in mouse organs that where highest in kidney (90.2 nmol g\(^{-1}\)) followed by liver (46.0 nmol g\(^{-1}\)), and heart (15.9 nmol g\(^{-1}\)). Prodrug injection also resulted in highest peak DOX concentrations in kidney (70.8 nmol g\(^{-1}\)) followed by liver (14.2 nmol g\(^{-1}\)) and heart (3.27 nmol g\(^{-1}\)) at 30 min to 2 h after injection of the prodrug. These values were 2 to 5 times lower than those found after DOX administration, except for kidney tissue where it was slightly lower (Figure 4).

Administration of DOX resulted in a peak concentration of the drug in OVCAR-3 tumour tissue of 2.14 nmol g\(^{-1}\) at 1 min after injection (Figure 4). The prodrug DOX-GA3 had a peak of DOX-GA3 of 44.0 nmol g\(^{-1}\) (\(t = 30 \text{ min}\)). A significantly higher DOX level of 9.57 nmol g\(^{-1}\) (\(t = 2 \text{ h}\)) was found after prodrug injection (\(P < 0.001; t = 2 \text{ h}\)) than after administration of the parent drug. In tumours, DOX was well retained both after DOX and DOX-GA3 administration, and the elimination of DOX from the tumour was not different after drug or after prodrug administration (Figure 4).

As an example for the selective activation of the prodrug at the tumour site, the ratios of the DOX concentrations at 2 h after DOX-GA3 and DOX administration are given for plasma, normal tissues and tumour tissue in Figure 5. At this time, the highest DOX level in tumour tissue was measured after prodrug administration. It is clear that the DOX concentrations in normal tissues were lower after prodrug than after DOX administration, except for the kidney.

**Areas under the concentration versus time curve**

As a measure of drug exposure, the AUC of DOX was calculated for plasma, normal mouse organs and tumour tissue by the trapezoidal rule using the available time-points and the software program Topfit (Table 1). The plasma AUC of DOX after administration of DOX-GA3 (0.342 μmol min ml\(^{-1}\)) was 8-fold higher than the AUC of DOX after injection of DOX-GA3 (0.042 μmol min ml\(^{-1}\)), with DOX given at the MTD and prodrug at half of the MTD. For the liver and the heart the respective AUCs for DOX were 1.8- and 3.1-fold higher and for the kidney the AUC was 2.0 times lower after DOX than after prodrug administration. Of importance, in OVCAR-3 tumour tissue the AUC of DOX from DOX-GA3 was 10-fold higher than that after DOX.

**Drug targeting index (DTI)**

The efficiency of targeting can be described as a drug targeting index (Kearney, 1996). We calculated the DTI for plasma (as a surrogate for bone marrow) and heart tissue because these are the major organs for anthracycline toxicity. For plasma a high DTI of 81.4 was calculated and for heart tissue the DTI was 31.4. This analysis thus confirms the selective activation of the prodrug DOX-GA3 in tumours.

**Anti-tumour activity of DOX and DOX-GA3 in vivo**

The administration of DOX (8 mg kg\(^{-1}\) × 2) resulted in a maximum \(G_0\) of 56% in small OVCAR-3 xenografts. At an equitoxic dose the molar amount of DOX-GA3 (500 mg kg\(^{-1}\) × 2) that could be administered was 38-fold higher when compared with that of the parent drug. In OVCAR-3 xenografts DOX-GA3 induced a maximum \(G_0\) of 87%, which was considerably higher than that of DOX (Table 2, Figure 6). The augmented anti-tumour effect of
DOX-GA3 was also demonstrated by an increase of the duration of the anti-tumour effect, expressed as TD1 \( t_{50} \), from 11.5 to 32.4 days, which was significant \( (P < 0.01, \text{Table 2}) \).

To study the effect of tumour size on the tumour growth inhibition DOX-GA3 was administered to animals bearing small or larger tumours, where larger tumours are expected to have more necrosis and, thus, more \( \beta \)-glucuronidase in the extracellular space. Larger tumours grew much slower than small tumours \( (T_{D1-t_{50}}, \text{small } 8.0 \text{ days, large } 15.4 \text{ days}) \). In animals treated with DOX, the maximum growth inhibition was similar for both small and larger tumours, 56 and 52\% respectively. DOX-GA3 treatment of larger tumours indeed resulted in a better inhibition of growth. The SGD in OVCAR-3 tumours increased from 2.7 to 3.9 for small and larger tumours, respectively.

**DISCUSSION**

In this study we describe a new doxorubicin prodrug, DOX-GA3, designed for specific activation to DOX at the tumour site. In nude mice bearing human ovarian cancer xenografts we showed that equitoxic doses of (pro)drug resulted in better anti-tumour...
effects after DOX-GA3 than after DOX. The administration of DOX-GA3 led to specific activation of the prodrug at the tumour site yielding higher concentrations of DOX in tumour tissue than after DOX injection. The 10-fold higher AUC of DOX from DOX-GA3 thus explains the better anti-tumour effect measured for DOX-GA3 when compared with DOX. Furthermore, the efficacy of DOX-GA3 appeared to be more pronounced in larger tumours when compared with the efficacy in small tumours. In the animals treated with DOX, the larger tumours were not more sensitive than the smaller tumours.

Our findings are consistent with other studies employing this approach. In this regard, as early as 1966 Connors and Whisson (Connors and Whisson, 1966) have shown that glucuronide prodrugs can be activated in tumours by β-glucuronidase. β-Glucuronidase is present extracellularly in necrotic tumour areas found in tumours larger than 3 mm diameter (Bosslet et al, 1995; Schumacher et al, 1996), whereas in normal tissues it is present only intracellularly inside lysosomes and microsomes (Levvy and Conchie, 1966). A clinical trial of aniline mustard was performed in patients with advanced cancer (Young et al, 1976). The drug used, aniline mustard, is transformed into the prodrug p-hydroxy aniline glucuronide which acts as a substrate for β-glucuronidase found in tumours. A correlation was shown between β-glucuronidase activity and patient response. However, the necessary high levels of β-glucuronidase were observed only rarely. The limited success of this trial is possibly due to the slow activation of the aniline glucuronide by the enzyme. The glucuronide prodrugs DNR-GA3 and DOX-GA3 were designed to be rapidly activated by human β-glucuronidase at a pH of 6.8 (Houba et al, 1996).

For DOX-GA3 a Vₘₐₓ of 25.0 μmol min⁻¹ mol⁻¹ and a Kₘ of 1100 μM was found. The relatively high Kₘ may be beneficial for improving tumour:non-tumour ratios of active drug: prodrug activation will

| Table 1  | AUC* of DOX in plasma and tissues of OVCAR-3-bearing mice after i.v. administration of DOX (8 mg kg⁻¹) or DOX-GA3 (250 mg kg⁻¹) |
|----------|------------------------------------------------------------------------------------------------------------------------|
| Treatment | Plasma (μmol min⁻¹ ml⁻¹) | Liver (μmol min⁻¹ g⁻¹) | Heart (μmol min⁻¹ g⁻¹) | Kidney (μmol min⁻¹ g⁻¹) | Tumour (μmol min⁻¹ g⁻¹) |
| DOX      | 0.342                      | 24.5                     | 9.70                     | 33.2                      | 1.31                      |
| DOX-GA3  | 0.042                      | 13.4                     | 3.09                     | 65.0                      | 13.1                      |

*The AUC (0–24 h) using the available time-points.

| Table 2  | Anti-tumour effect with DOX or DOX-GA3 in mice bearing OVCAR-3 xenografts |
|----------|--------------------------------------------------------------------------|
| Treatment | Size | Dose i.v. (mg kg⁻¹) | Days | Tumour volume* mean ± SEM | Weight loss % ± SD | Weight day 14 % ± SD | GI % ± SD | Tₘᵣᵢᵦᵦ* days ± SEM (n) |
| Control  | Small | 128 ± 29 | 0.7 | 123 ± 19 | 1.3 ± 2.0 | 104.4 ± 3.4 | 56 | 8.0 ± 0.6 (10) |
| DOX      | Small | 8 | 0.7 | 123 ± 19 | 5.1 ± 3.4 | 99.0 ± 3.1 | 87 | 32.4 ± 11.3 (10) |
| DOX-GA3  | 500 | 0.7 | 390 ± 97 | 2.8 ± 0.2 | 101.5 ± 2.8 | 52 | 21.5 ± 0.7 (5) |
| Control  | Large | 128 ± 29 | 0.7 | 534 ± 128 | 9.6 ± 7.2 | 91.9 ± 6.5 | 88 | 75.1 ± 11.8 (10) |
| DOX      | Large | 8 | 0.7 | 458 ± 107 | 6.5 ± 1.0 | 101.5 ± 2.8 | 52 | 21.5 ± 0.7 (5) |
| DOX-GA3  | 500 | 0.7 | 390 ± 97 | 2.8 ± 0.2 | 101.5 ± 2.8 | 52 | 21.5 ± 0.7 (5) |

n.a., not applicable; *at start of treatment; *tumour volume doubling time in days from a relative volume of 1 to 4; *maximum growth inhibition; **P < 0.001 when compared with control; ***P < 0.01 when compared with DOX.
be relatively slow at sites with low levels of enzyme. In this regard, a $K_c$ of 1 mM was shown to be optimal by pharmacokinetic modelling by Yuan et al (1991). The half-life of hydrolysis at clinically relevant concentrations of the prodrug and human $\beta$-glucuronidase was 140 min. Although this seems to be far from optimal, the time to reach toxic concentrations of DOX in tumour tissue is much shorter, only a few minutes (Figure 4).

DOX is a lipophilic molecule and, as a consequence, it rapidly penetrates into tissues. Therefore, normal tissue DOX levels are relatively high after DOX administration and may account for unfavourable side-effects (Figure 4). The hydrophilic glucuronide moiety of DOX-GA3 prevents rapid diffusion of prodrug into cells. This is confirmed by the high peak plasma concentrations of DOX-GA3 and its rapid clearance from plasma, tumour and normal tissues.

In plasma, heart and liver tissues the concentrations of DOX were lower after DOX-GA3 administration than after injection of DOX itself. Lower concentrations of DOX in the heart are of advantage, because this organ is the site of cumulative dose-limiting toxicity of DOX. In the kidney, higher concentrations of DOX from DOX-GA3 than after DOX were measured. These higher concentrations may be explained by the presence of $\beta$-glucuronidase in the renal tubular cells. This should not result in increased toxicity as in patients renal insufficiency is rarely observed after DOX administration.

In OVCAR-3 xenografts higher concentrations of DOX from DOX-GA3 were detectable than after administration of DOX. Presumably, DOX-GA3 was activated by $\beta$-glucuronidase present in tumour tissue in the extracellular space, but not in normal tissues. Therefore, higher AUC values of DOX from DOX-GA3 were reached than after DOX administration. These higher AUC values explain the better inhibition of tumour growth in OVCAR-3 xenografts observed after DOX-GA3 administration when compared with DOX, when given at an equitoxic dose. The $T_{90\text{–}100}$ increased significantly ($P < 0.001$) from 11.5 days (8 mg kg$^{-1}$ DOX weekly $\times 2$) to 32.4 days (500 mg kg$^{-1}$ DOX-GA3 weekly $\times 2$).

It was hypothesized that larger tumours contain more necrosis and, thus, more $\beta$-glucuronidase would be available to activate DOX-GA3, as has also been described by the group of Bosslet (Bosslet et al, 1995). Indeed, we calculated a higher SGD for larger tumours (SGD = 3.9) than for small tumours (SGD = 2.7). We reported a similar finding for the prodrug DNR-GA3 where the SGD in OVCAR-3 xenografts observed after DOX-GA3 administration when compared with DOX, when given at an equitoxic dose. The $T_{90\text{–}100}$ increased significantly ($P < 0.001$) from 11.5 days (8 mg kg$^{-1}$ DOX weekly $\times 2$) to 32.4 days (500 mg kg$^{-1}$ DOX-GA3 weekly $\times 2$).

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In conclusion, DOX-GA3 is a non-toxic prodrug which is specifically activated to DOX in tumour tissue by $\beta$-glucuronidase released by necrotic cells. Although the treatment schedule is not yet optimized we have already demonstrated that DOX-GA3 has a higher therapeutic index than DOX. These results are encouraging and warrant clinical development of DOX-GA3.

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