A Novel Macromolecular Prodrug Concept Exploiting Endogenous Serum Albumin as a Drug Carrier for Cancer Chemotherapy

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Introduction. Serum proteins are potential drug carriers of antineoplastic agents due to their accumulation in tumor tissue.[1] Uptake of these proteins in solid tumors is mediated by a number of factors including an increased metabolic activity of tumors, an enhanced vascular permeability of tumor blood vessels for circulating macromolecules, and a lack of a functional lymphatic drainage system in tumor tissue.[2] Recently, a number of acid-sensitive albumin and transferrin conjugates with anthracyclines and the alkylating agent chlorambucil have shown promising in vitro activity.[3−7] In addition, acid-sensitive doxorubicin conjugates with monoclonal antibodies and albumin doxorubicin conjugates prepared by glutaraldehyde cross-linking have shown promising antitumor efficacy in vivo.[8,9]

A selected acid-sensitive doxorubicin albumin conjugate that was developed in our group induced complete remissions of primary kidney tumors in murine renal carcinoma and prevented the formation of metastases in the lungs. In contrast, mice treated with doxorubicin at optimal dose manifested clearly visible kidney tumors at the end of the experiment and large numbers of lung metastases.[10] This albumin doxorubicin conjugate was synthesized by coupling **1**, a maleimide carboxylic hydrazone derivative of doxorubicin (see Figure 1), to thiolated albumin. **1** contains an acid-sensitive linker that allows the drug to be released at the low pH values present in lysosomes and endosomes of tumor cells. We have recently shown that **1** also binds covalently to the cysteine-34 of commercially available human serum albumin which is a mixture of mercaptalbumin and nonmercaptalbumin.[11,12] Approximately 70% of circulating albumin in the blood stream is mercaptalbumin that contains an accessible cysteine-34 which is not blocked by endogenous sulfhydryl compounds such as cysteine or glutathione.[12,13] Considering that free thiol groups are not found on the majority of circulating serum proteins except for albumin, cysteine-34 of endogenous albumin is a fairly unique amino acid on the surface of a circulating protein that could be exploited for developing a novel macromolecular prodrug concept. Since the maleimide group reacts specifically and selectively with thiol groups, we reasoned that it should be possible to preferentially bind maleimide drug derivatives to the HS group of the cysteine-34 position of albumin after intravenous application. In this way a macromolecular prodrug is formed after in situ coupling of a thiol-binding drug derivative to endogenous albumin in the blood circulation. Following this approach, it should be possible to avoid the ex vivo synthesis and characterization of drug albumin conjugates which are costly, are time-consuming, and rely on exogenous and possibly pathogenic albumin.

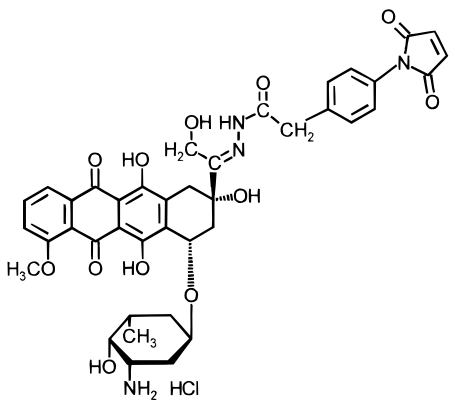

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Figure 1. Structure of **1**, a maleimide phenylacetylhydrazone derivative of doxorubicin.

The objective of the present work was to assess the feasibility and selectivity of our approach by carrying out in vitro and in vivo binding studies using the doxorubicin maleimide derivative **1**. In addition, we wanted to obtain the first in vivo evidence that **1** is superior to free doxorubicin in an animal tumor model, i.e., in murine renal cell carcinoma (RENCA).

In Vitro and in Vivo Binding Studies. To estimate the coupling rate and selectivity of **1** for endogenous albumin, **1** was incubated with human blood plasma at $T = 37$ °C and the samples were subsequently analyzed by anion-exchange chromatography. (The ratio of drug to albumin was approximately 0.3:1; human blood plasma employed contained an albumin concentration of ∼700 μM as determined with a Vitros analyzer from Ortho-Clinical Diagnostics. **1** was synthesized previously;[14] a 2000 μM solution of **1** was freshly prepared in 0.15 M NaCl, 0.004 M sodium phosphate buffer (pH 5.5), and 30 vol % of 1,2-propylene glycol for binding studies and animal experiments; doxorubicin was used as a 3400 μM stock solution in isotonic saline from Pharmacia & Upjohn.) Chromatograms after an incubation time of 5 min for **1** (Figure 2A) and for free doxorubicin (Figure 2B) under identical conditions are available as Supporting Information. Protein components were detected at 254 nm, and the anthracycline moiety was detected by simultaneous fluorescence excitation. In contrast to free doxorubicin, the major amount of **1** is associated with the albumin peak which elutes at around 23.5 min. Longer incubation periods led to a slight increase in albumin binding indicating that the coupling reaction proceeds rapidly within this short period. Incubation studies with total blood and subsequent HPLC analysis of the resulting blood plasma

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after centrifugation led to similar chromatographic profiles – available as Supporting Information (Figure 3).

The following experiment was carried out to demonstrate that the cysteine-34 position of albumin is involved in the coupling step: Human serum was preincubated for 5 min with a 2-fold excess of a nonfluorescent maleimide with respect to the albumin concentration in the blood plasma, i.e., 3-maleimidobenzoic acid, before adding **1** and incubating for a further 5 min. The resulting chromatogram (see Figure 2A) shows that in this case only marginal binding of **1** to albumin takes place and the major amount of **1** elutes with a retention time of approximately 6 min.

As a comparison, the chromatogram of the pure albumin conjugate with **1**, in which the ratio of **1** to albumin is approximately 0.9:1, is shown in Figure 2C which elutes at approximately 23.5 min (see Supporting Information). This albumin conjugate was synthesized by reducing commercially available albumin (from Dessau Pharma, FRG) with dithiothreitol in a first step so that approximately 1 sulfhydryl group per molecule of albumin could be determined using Ellman's reagent (commercially available albumins contain approximately 0.5 free sulfhydryl group per molecule of albumin); in a second step, **1** was coupled to this reduced albumin. (After a 5 min incubation of commercially available albumin or its reduced form with an equivalent of 3-maleimidobenzoic acid, only residual amounts of HS groups can be determined with Ellmann's reagent.) The ESI mass spectra of native albumin, reduced albumin, and the albumin conjugate with **1** are available as Supporting Information (Figure 4A–C). The principal mass peak of human serum albumin is observed in the range 66440–66477. Peaks at 66548 (or 66595) and at 66699 (or 66762) in Figure 4A probably correspond to the oxidized cysteine, homocysteine, or glutathione form of albumin. These signals are not or only weakly observed in the ESI mass spectrum of the reduced form of albumin (see Figure 4B). In the ESI mass spectrum of the albumin conjugate with **1** which was prepared with the reduced form of albumin (see Figure 4C), the principal mass peak is now seen at 67200 which corresponds approximately to the sum of the principal mass of albumin (~66440–66477) and the mass of **1**·HCl (~771; HCl subtracted from the hydrochloride form of **1**).

In a next set of experiments, we investigated whether rapid binding of **1** to endogenous albumin was also taking place in healthy mice after intravenous injection. For this purpose doxorubicin or **1** (10 mmol/kg for both compounds) was administered intravenously to Balb/c mice under anesthesia and blood was drawn from the heart approximately 10 s postinjection. Blood plasma samples were obtained by centrifugation, then frozen, and subsequently analyzed on our HPLC system. The chromatograms for the sample of **1** and free doxorubicin are available as Supporting Information (Figure 5A,B, respectively). As expected, only marginal binding to albumin is seen in the blood plasma of the doxorubicin-treated mouse (Figure 5B); in contrast, significant binding to serum albumin occurred in the blood plasma of the mouse injected with **1** (Figure 5A).

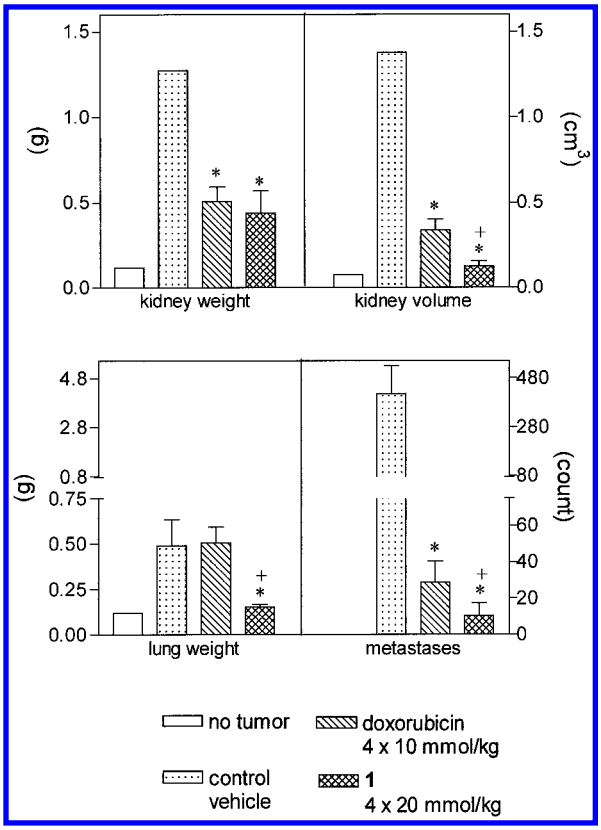

Figure 6. Therapeutic effects of doxorubicin and **1** at an equitoxic dose on kidney weight and volume and on the number of metastases in the lungs compared to the control group receiving the propylene glycol-containing buffer as the control vehicle; statistics: $p < 0.05$ ($n = 10$). *Significant to the group receiving the control vehicle; around 30 lung metastases or more results in lung edema causing a considerable increase in lung weight. $^{+}$Significant to the group receiving doxorubicin.

Biology. On the basis of our previous finding that the acid-sensitive doxorubicin albumin conjugate with **1** was superior to doxorubicin against RENCA, we chose this animal model to compare the antitumor efficacy of **1** with the parent compound doxorubicin.[10] In the RENCA model, primary kidney tumors are induced by subcapsular renal injection of renal carcinoma cells in the left kidney of Balb/c mice with subsequent development of metastases, especially in the lungs. Because the maximum tolerated dose of the albumin doxorubicin conjugate is at least twice that of free doxorubicin,[10] we decided to adopt a similar therapy scheme in this experiment. The doses were thus chosen as follows: 4 × 10 mmol/kg for doxorubicin (equal to 4 × 6 mg/kg, $M_r = 580.0$ g/mol) and 4 × 20 mmol/kg for **1** (equal to 4 × 16.7 mg/kg, $M_r = 807.8$ g/mol). At the end of the experiment after 24 days, body weight loss in both the treated groups (10 mice/group) was almost identical (–15%, curves depicting body weight loss are available as Supporting Information) indicating that these doses are approximately equitoxic.

The therapeutic effects of doxorubicin and **1** on kidney tumor volume and weight as well as on the number of lung metastases are shown in Figure 6 in comparison to a group receiving the propylene glycol-containing buffer as the control vehicle. After 24 days, mice in the doxorubicin-treated group showed distinct kidney tumors which is reflected by a significant increase in

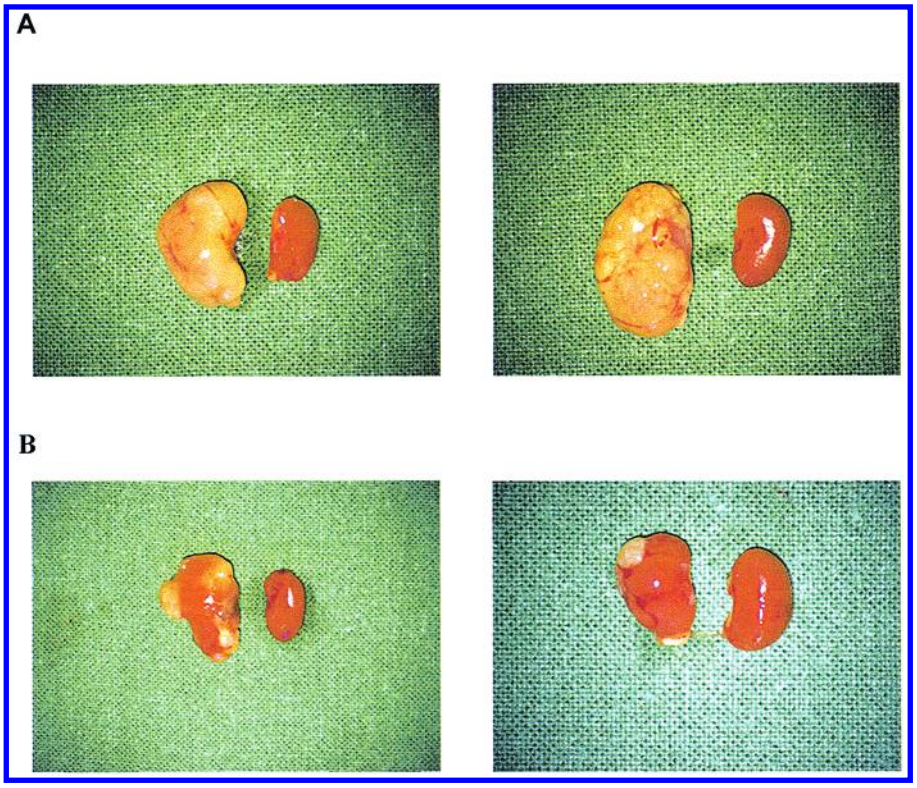

Figure 7. Representative photographic images of healthy kidneys (right) as well as tumor cell-treated kidneys (left) of two mice from the group treated with doxorubicin (A) or **1** (B) after 24 days. The large primary tumor is still clearly visible in the left kidneys of the doxorubicin-treated mice in contrast to mice treated with **1**; differences in wet tumor weights did not differ statistically (for an explanation, see text).

kidney volume compared to healthy kidneys. In the group treated with **1**, however, kidney volumes were increased to a much lesser extent compared to healthy kidneys demonstrating a considerable reduction in tumor size. A significant difference is not seen when comparing the kidney weights in the treated groups in this experiment probably due to the fact that wet weights were taken. In the RENCA model, subcapsular renal injection can lead to hematoma and hydronephrosis influencing the kidney tumor weights, especially when the primary tumor is of small size. As an example, the left (RENCA) and right (healthy) kidneys for two animals of each group are depicted in Figure 7. Whereas the left kidneys of the doxorubicin-treated mice show a large primary tumor, the left kidneys of the mice treated with **1** show only residual amounts of macroscopically visible tumors. On the whole, the therapeutic effect of **1** at the dose of 4 × 20 mmol/kg with respect to the volume of the kidney tumor was similar to that reported by us for the albumin conjugate prepared with **1** at the identical dose.[10]

In addition, a therapeutic difference was observed regarding the development of metastases among the treated groups. Mice in the doxorubicin-treated groups showed about 30 lung metastases; in contrast, only 10 lung metastases were detected on average in the group treated with **1** − see Figure 6.

Summary. In conclusion, we have shown that the doxorubicin hydrazone derivative **1**, which contains a maleimide group as a thiol-binding group, binds preferentially to endogenous serum albumin after incubation with human blood plasma or direct intravenous injection into mice. Preincubation studies with a maleimide compound and coupling reactions with native serum albumin indicate that **1** binds to cysteine-34 of albumin which is an attractive binding site in blood plasma due to the fact that other major plasma proteins do not contain free HS groups.

In addition, **1** shows a superior antitumor effect in murine renal cell carcinoma when compared to doxorubicin at equitoxic dose. This increase in therapeutic efficacy can be best explained by an enhanced permeability of tumor blood vessels for circulating proteins and a subsequent retention due to a lacking lymphatic recovery system in tumor tissue. Studies in the RENCA model have shown that renal cell carcinomas are highly vascularized indicating that circulating macromolecules such as serum albumin and respective conjugates might be trapped by the vascular network of these tumors.[15]

Although a more detailed analysis of the in situ coupling of thiol-binding drug derivatives to endogenous albumin is warranted, we believe that the outlined macromolecular prodrug strategy is an attractive approach of altering the pharmacokinetic profile of clinically established anticancer drugs and increasing their therapeutic index. We are meanwhile improving the resolution of plasma components, e.g., by using electrophoretic techniques, and are also investigating a number of thiol-binding derivatives of doxorubicin that differ in the length of the spacer arm and in the nature of the thiol-binding group with the aim of developing tailor-made drug derivatives with maximum affinity for the cysteine-34 position of serum albumin.

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Supporting Information Available: Detailed description of the incubation procedures and the employed chromatographic method and animal experiments; chromatograms of in vitro and in vivo binding studies of doxorubicin and **1** [with human blood plasma (Figure 2), of mouse blood plasma after iv injection (Figure 3) and with human blood (Figure 5)]; ESI mass spectra of human serum albumin, of reduced albumin, and of the albumin conjugate with **1** (Figure 4A−C); graphical depiction of body weight loss (Figure 8). This material is available free of charge via the Internet at http//pubs.acs.org.

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