# A New Approach for the Treatment of Malignant Melanoma: Enhanced Antitumor Efficacy of an Albumin-binding Doxorubicin Prodrug That Is Cleaved by Matrix Metalloproteinase 2[1]

Ahmed M. Mansour, Joachim Drevs, Norbert Esser, Farid M. Hamada, Osama A. Badary, Clemens Unger, Iduna Fichtner, and Felix Kratz[2]

*Tumor Biology Center, Breisacher Straβe 117, 79106 Freiburg, Germany [A. M. M., J. D., N. E., C. U., F. K.]; Max-Delbrück Center, Robert-Rössle-Straβe 10, 13122 Berlin, Germany [I. F.]; and Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Nasr City, Cairo, Egypt [A. M. M., F. M. H., O. A. B.]*

## ABSTRACT

The progression of malignant melanoma is characterized by overexpression of a number of matrix metalloproteinases (MMPs), especially MMP-2, which play a critical role in the degradation of basement membranes and the extracellular matrix. Consequently, we assessed a drug targeting strategy in which the protease activity of MMP-2 is exploited to release an anticancer agent from a macromolecular carrier, *i.e.*, circulating albumin. For this purpose, a water-soluble maleimide derivative of doxorubicin (1) incorporating a MMP-2 specific peptide sequence (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) was developed that binds rapidly and selectively to the cysteine-34 position of circulating albumin. The albumin-bound form of 1 was efficiently and specifically cleaved by MMP-2 liberating a doxorubicin tetrapeptide (Ile-Ala-Gly-Gln-DOXO) and subsequently doxorubicin. *In vivo*, 1 was superior to the parent compound doxorubicin in the A375 human melanoma xenograft, which is characterized by a high expression of MMP-2.

## INTRODUCTION

Malignant melanoma characterized by its high capacity for invasion and metastasis is one of the most frequent forms of skin cancer (1). Dissemination of tumor cells is the principal cause of mortality in melanoma patients. Tumor cell invasion as well as metastasis formation are complex and multistep processes. In melanoma progression, different proteolytic enzyme systems, including the plasminogen activator system and MMPs[3] play an important role in the degradation and remodeling of the extracellular matrix and basement membranes (2–6). Recent data indicate that in particular the balance between MMPs and their TIMPs is critical in determining this process (4–6). MMP-2 (gelatinase A) especially plays a critical role in tumor progression, angiogenesis, and metastasis (7). Overexpression of MMP-2 in melanoma has been shown in a number of preclinical as well as clinical investigations (8–10). In disseminated disease, melanoma patients respond marginally to chemotherapy because of the lack of selectivity of anticancer agents (1). Therefore, the development of more selective antitumor agents for treating stage III and IV melanoma is warranted. We have assessed recently a novel drug targeting strategy in which the protease activity of MMP-2 and MMP-9 is exploited to release the anticancer agent doxorubicin from a macromolecular carrier, *i.e.,* serum albumin (11). An albumin doxorubicin conjugate incorporating the octapeptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) between the drug and the carrier is especially cleaved efficiently by activated MMP-2 and MMP-9 liberating a doxorubicin tetrapeptide.

In addition, because of a high metabolic turnover of tumor tissue and the enhanced vascular permeability of the blood vessels of malignant tissue for circulating albumin, we developed recently a macromolecular prodrug strategy based on two features (12, 13): (*a*) *in situ* binding of a thiol-binding prodrug to the cysteine-34 position of circulating albumin after i.v. administration; and (*b*) release of the albumin-bound drug at the tumor site because of the incorporation of a cleavable bond between the drug and the carrier. Proof of concept was obtained with two acid-sensitive doxorubicin prodrugs that are rapidly and selectively bound to circulating albumin and are distinctly superior to the parent compound doxorubicin in a number of animal tumor models. In this work, we combined the advantages of both approaches and developed a water-soluble maleimide derivative of doxorubicin 1 (see Fig. 1) incorporating a MMP-2 specific peptide sequence (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln). We report on the synthesis and preliminary biological evaluation of this doxorubicin prodrug against A375 melanoma, which demonstrated superior *in vivo* efficacy compared with the parent compound.

## MATERIALS AND METHODS

**Chemicals, Materials, and Spectroscopy.** Doxorubicin hydrochloride (*Mr* 580,000) was purchased from Hande Tech Development Co. Inc., Mal-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln acid was custom-made by BACHEM AG (Bubendorf, Switzerland), and organic solvents were HPLC grade (Merck, Darmstadt, Germany). All of the other chemicals used were at least reagent grade and obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck and used without additional purification; human serum albumin (20% solution) was purchased from Dessau Pharma, Dessau, Germany that contained ~30% free thiol groups as assessed with the Ellman's test. MMP-2 and (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid (MMP-2 inhibitor) was purchased from Calbiochem (Bad Soden, Germany). TIMP-2 was purchased from Boehringer Mannheim (Mannheim, Germany). The BIOTRAK Human MMP ELISA Activity Assay for MMP-2 was purchased from Amersham Pharmacia Biotech (Freiburg, Germany). The buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius, Göttingen, Germany) and thoroughly degassed with argon or nitrogen before use. Cell culture medium, supplements, and FCS were purchased from BioWhittaker (Serva, Heidelberg, Germany). All of the culture flasks were obtained from Greiner Labortechnik (Frickenhausen, Germany). Matrix-assisted desorption ionization-time of flight mass spectrometry was performed on a *Bruker* Reflex II spectrometer operating in the reflection mode at 21.50 kV. As matrix, α-cyano-4-hydroxycinnamic acid (10 mg/ml in methanol) was used. UV/VIS-spectrophotometry was carried out with a double-beam spectrophotometer U-2000 from Hitachi. Mass spectroscopy (liquid chromatography-electrospray-mass spectrometry) was performed by Bioproof AG (Munich, Germany). For incubation and animal studies, 1 was dissolved in a sterile isotonic buffer containing 10 mM sodium phosphate and 5% D-glucose (pH 6.4) at a concentration of 6.0 mg/ml.

**Methods.** HPLC for the separation of blood plasma, and analysis of 1 and the albumin conjugate of 1, as well as respective cleavage products was performed with a BioLogic Duo-Flow System from Bio-Rad (Munich, Ger-
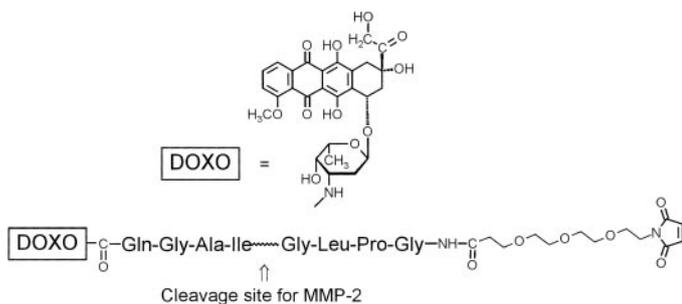
Fig. 1. Structure of the albumin-binding doxorubicin prodrug 1.

many), which was connected with a Merck F-1050 Fluorescence Spectrophotometer (EX. 490 nm, EM. 540 nm) or a $\lambda$ 1000 visible monitor from Bischoff (at $\lambda = 495$ nm); UV-detection at 280 nm; column: Waters, 300 Å, Symmetry C18 (4.6 × 250 mm) with precolumn; chromatographic conditions: flow: 1.2 ml/min, mobile phase: 27.5% $CH_3CN$, 72.5% 20 mM potassium phosphate (pH 7.0), mobile phase B: $CH_3CN$, gradient: 0–25 min 100% mobile phase; 25–40 min increase to 70% $CH_3CN$, 30% 20 mM potassium phosphate; 40–50 min 70% $CH_3CN$, 30% 20 mM potassium phosphate; 50–60 min decrease to initial mobile phase; injection volume: 50 $\mu$l.

**Synthesis and Characterization of 1.** 1 was prepared by reacting the maleimideotriethyleneglycol octapeptide derivative (Mal-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) with doxorubicin hydrochloride in dimethylformamide using a standard coupling procedure: 175.0 mg (0.3 mmol) doxorubicin hydrochloride, 298.5 mg (0.3 mmol) of Mal-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln, 40.5 mg (0.3 mmol) of 1-hydroxybenzotriazole-hydrate, and 98.95 $\mu$l (91.0 mg, 0.9 mmol) 4-methylmorpholine were dissolved in 50 ml of anhydrous $N,N,$-dimethylformamide; after stirring at +5°C for 15 min, 139.36 $\mu$l (113.6 mg, 0.9 mmol) of $N,N'$-diisopropylcarbodiimide were added as the coupling agent. After stirring at +5°C for 3 days, $N,N,$-dimethylformamide was removed by evaporation in high vacuum and the residue dissolved in a minimal amount of chloroform:methanol 4:1 and the product purified twice on a silica gel column using chloroform:methanol 4:1 to afford 250 mg 1 as a red powder after precipitating the combined fractions containing 1 with diethyl ether and drying the product in high vacuum. Mass (matrix-assisted desorption ionization-time of flight; $M_r$ 1,520,700): $m/z$ 1543 ($Na^+$ salt adduct), HPLC (495 nm): >98%.

**Synthesis of the Albumin Conjugate of 1.** The albumin conjugate of 1 was prepared by reacting commercially available albumin that contains ~30% mercaptalbumin with 1 and isolating the conjugate through size-exclusion chromatography; 10 ml of human serum albumin was incubated at 37°C for 1 h with 11.8 mg of 1. The albumin conjugate was obtained by subsequent size-exclusion chromatography [Sephacryl HR100; buffer 0.004 M sodium phosphate and 0.15 M NaCl (pH 6.5)]. The content of anthracycline in the sample was determined using the $\epsilon$-value for doxorubicin [$\epsilon_{495}$ (pH 7.4) = 10,650 $M^{-1}$ $cm^{-1}$]. The concentration of 1 in the conjugate was adjusted to 400 ± 50 $\mu$M by concentrating the sample with CENTRIPREP-10-concentrators from Millipore, Eschborn, Germany (4°C and 4,500 rpm). Samples were kept frozen at −78°C and thawed before use.

**Incubation Studies with Human Plasma.** 1 was added to human blood plasma (EDTA stabilized) preincubated at 37°C at a final concentration of 50 $\mu$M, and the samples were incubated for 2 and 5 min, and for 3, 6, 12, and 24 h at 37°C; a 50 $\mu$l sample was analyzed by HPLC.

**Incubation Studies of the Albumin Conjugate of 1 with MMP-2.** Fifty or 100 $\mu$M samples of the albumin conjugate of 1 were incubated with MMP-2 (2 mU, activated with 4-aminophenylmercuric acetetae for 1.5 h) in the absence or presence of a 2-fold excess of TIMP-2 or a 1000-fold excess of (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid (MMP-2 inhibitor); chromatograms recorded at $\lambda = 495$ nm using reverse-phase HPLC at the time points stated in Fig. 2B.

**Incubation Studies of the Albumin Conjugate of 1 and Cleaved Doxorubicin Tetrapeptide with A375 Melanoma Cell Culture Supernatants and A375 Melanoma Tissue Homogenates.** One-hundred $\mu$M samples of the albumin conjugate of 1 or the cleaved doxorubicin tetrapeptide (albumin conjugate of 1 cleaved with 2 mU MMP-2 activated by APMA for 1.5 h) were incubated with either A375 melanoma supernatant or A375 melanoma tissue

homogenate at 37°C, and chromatograms recorded with fluorescence detection using reverse-phase HPLC at the time points stated in Fig. 3.

**Determination of Concentrations of MMP-2 in Cell Culture Supernatants and Tumor Tissue Homogenates.** The amount of MMP-2 was quantified by ELIZA (BIOTRAK) in cell-conditioned medium (A375 melanoma cells) as well as in tissue of A375 melanoma. RPMI 1640 was exposed to the melanoma cell line A375 in tissue culture flask for a time ranging from 48 to 72 h. For obtaining melanoma tissue, all of the steps were carried out on ice where possible; tissue was cut into small pieces, transferred and weighed in an Eppendorf tube, and diluted with the same volume of homogenate buffer [50 mM Tris-HCl buffer (pH 7.4) containing 1 mM monothioglycerol]. Homogenization was carried out with a micro-dissmemberator at 3000 rotation/min for 3 min with the aid of glass balls, and the samples were then centrifuged at 5000 rpm for 10 min. The amount of active and total MMP-2 was determined in supernatant, and tissue of test samples was determined by comparison with known standard samples of pro-MMP-2 according to the manufacturer's protocol.

**Cell Culture.** A375 melanoma cells were grown as monolayer cultures in cell culture flasks in RPMI 1640 culture with phenol red supplemented with 10% heat-inactivated FCS, 100 $\mu$g/ml glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Media were routinely changed every
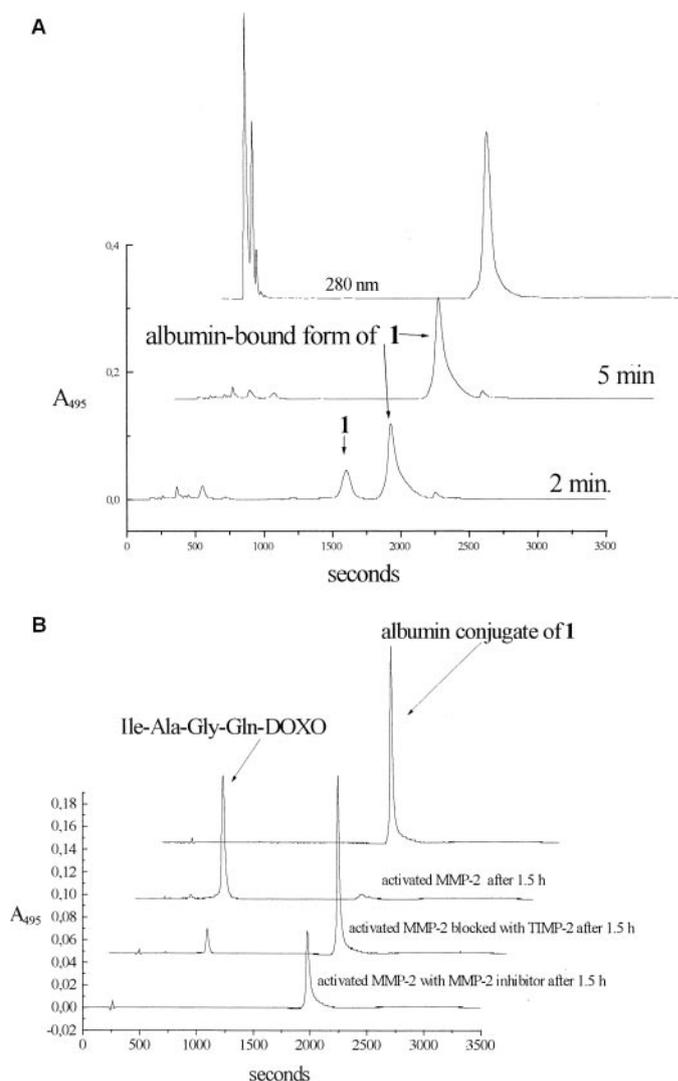


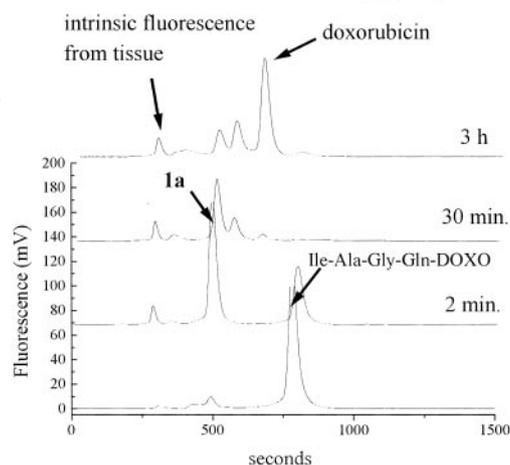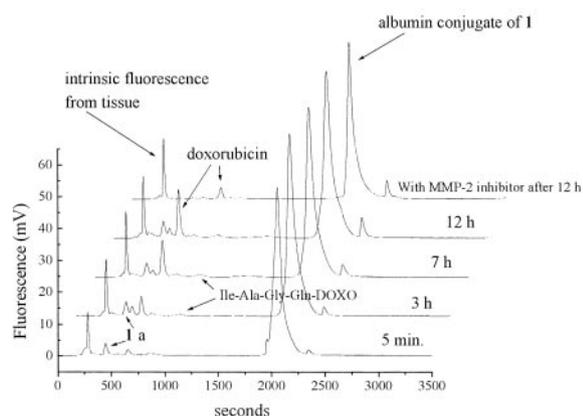Fig. 2. Chromatograms of incubation studies of 1 with human plasma at 37°C after 2 and 5 min (A), and cleavage studies with the albumin conjugate of 1 (B) after a 1.5 h incubation of 2 mU activated MMP-2 in the absence or presence of an excess of TIMP-2 (2-fold) or MMP-2 inhibitor (1000-fold). Concentrations of the anthracycline was 50 and 100 $\mu$M, respectively. Chromatographic conditions: see "Materials and Methods."

**A**



**B**
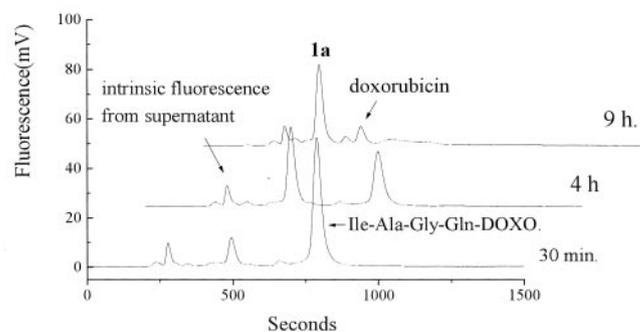


Fig. 3. Chromatograms of incubation studies of A375 melanoma tissue homogenates (*A*) and A375 melanoma cell culture supernatants (*B*) w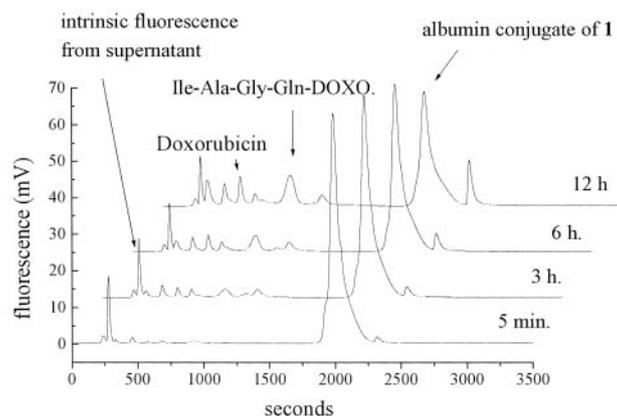ith the albumin conjugate of 1 (*bottom*) and the cleaved doxorubicin tetrapeptide (Ile-Ala-Gly-Gln-DOXO; *top*) at 37°C. Concentrations of the anthracycline was 100 $\mu$M. Chromatographic conditions: see "Materials and Methods."

3 days. For subculture or experiments, cells growing as monolayer cultures were released from the tissue flasks by treatment with 0.05% trypsin/EDTA, and viability was monitored using the cell analyzer system Casy 1 from Schärfe Systems (Reutlingen, Germany). For the experiments, cells were used during the logarithmic growth phase.

**MTT Assay.** The MTT assay was performed in analogy to a literature protocol (14). Briefly, ~5000 cells were plated in each well of a 96-well tissue culture plate. Medium supplemented with 10% FCS was added, and cells were allowed to adhere for 24 h. Subsequently, cells were preincubated with various drug concentrations (0.001–60 $\mu$M) for 72 h and then labeled by adding sterile-filtered MTT (5 mg/ml PBS). Cultures were incubated at 37°C for 2 h. Subsequently, the supernatant was removed and 100 $\mu$l DMSO added to each well. After an incubation time of 10 min, the extinction of the samples was quantified with an ELISA reader (Dynatech Laboratories Inc., Sullyfield, United Kingdom) at a wavelength of 570 nm. Four separate cultures were determined per concentration. Results are shown as means $\pm$ SE of three independent experiments ($n = 3$).

**Xenograft Experiments.** For the *in vivo* testing of 1 in comparison with doxorubicin, female Ncr: nude mice (Taconic Breeding Facility, Germantown, MD) were used. The mice were held in laminar flow shelves under sterile and standardized environmental conditions (25 $\pm$ 2°C room temperature, 50 $\pm$ 10% relative humidity, and 12 h light-dark-rhythm). They received autoclaved food and bedding (ssniff, Soest, Germany) and acidified (pH 4.0) drinking water *ad libitum*. All of the animal experiments were performed under the auspices of the German Animal Protection Law.

An orientating toxicity study in nontumor-bearing mice with 1 was per-

formed to obtain a dose schedule for subsequent studies in xenograft models. For 1, a dose of 2 $\times$ 30 mg/kg and 2 $\times$ 60 mg/kg doxorubicin equivalents was investigated. 1 was administered by i.v. application to 2 animals with an interval of 7 days. Two $\times$ 30 mg/kg was well tolerated, and no mortality and no body weight loss was observed. However, at the dose of 2 $\times$ 60 mg/kg doxorubicin equivalents, the first dose of 60 mg/kg was tolerated, but the animals died after the second administration of 60 mg/kg. For these reasons, a dose of 3 $\times$ 24 mg/kg in the A-375 xenograft model was chosen.

The melanoma cell line A375 was a kind gift of the tumor bank of the National Cancer Institute. Tumor cells were harvested from cell culture, washed with PBS, and $10^7$ cells/0.1 ml were injected s.c. into the left flank region of mice on day 0. Mice were randomly distributed to the experimental groups. When the tumors had reached a palpable size (4–5-mm diameter), treatment was initiated. Mice were treated i.v. in a weekly distance with saline, doxorubicin, or 1; for doses and schedules see Fig. 4. The volume of administration was 0.2 ml/20 g body weight.

Tumor size was measured twice weekly with a caliper-like instrument in two dimensions. Individual tumor volumes (V) were calculated by the formula V = [length $\times$ (width)$^2$]/2 and related to the values on the first day of treatment (relative tumor volume). At each measurement day, treated/control values were calculated as percentage for each experimental group; the optimum (lowest) values obtained within 4 weeks after treatment were used for evaluating the efficacy of the compounds, and optimum treated/control values are presented in Fig. 4. The body weight of mice was determined twice weekly and related to the body weight on the first day of treatment body weight change. Blood was taken from the retro-orbital venous plexus of 5 mice/group
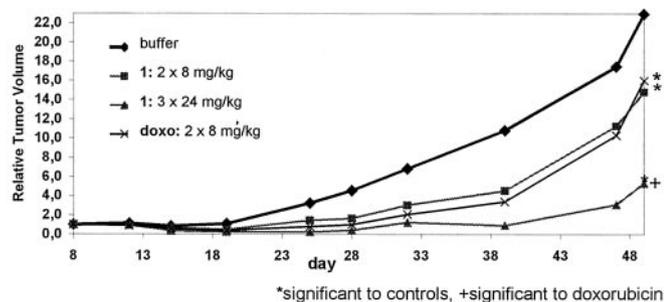
Table of dose schedule , T/C values, mortality, body weight loss and blood parameters of doxorubicin and 1 in the human A-375 melanoma xenograft

| No. Mice | Substance | Treatment (d) | Dose (mg/kg/inj.) | Doxorubicin equivalents (mg/kg/inj.) | Toxic Deaths(d) | BWC (%) d 8-19 | optimum T/C (%) | WBC ($10^6$/ml) | Thromb. ($10^6$/ml) |
|---|---|---|---|---|---|---|---|---|---|
| 8 | Glucose-phosphate buffer | 8, 15 | | | | 2 | | 9.1+/-1.9 | 1075+/-213 |
| 8 | 1 | 8, 15 | 21 | 8 | 0 | 0 | 36* | 10.4+/-2.7+ | 1033+/-334 |
| 8 | 1 | 8, 15, 22 | 63 | 24 | 0 | 5 | 8*+ | 6.2+/-2* | 1291+/-151 |
| 8 | Doxorubicin | 8, 15 | 8 | 8 | 0 | -2 | 22* | 5.7+/-0.6* | 1285+/-123 |

*significant to controls, +significant to doxorubicin, p < 0.05

Fig. 4. Curves depicting tumor growth inhibition of s.c. A-375 melanoma xenografts under therapy with doxorubicin and 1 [dose (i.v.); doxorubicin: $2 \times 13.3$ $\mu$mol/kg (= $2 \times 8$ mg/kg doxorubicin); 1: $2 \times 13.3$ $\mu$mol/kg (= $2 \times 8$ mg/kg doxorubicin equivalents), $3 \times 39.9$ $\mu$mol/kg (= $3 \times 24$ mg/kg doxorubicin equivalents), $3 \times 39.9$ $\mu$mol/kg (= $3 \times 24$ mg/kg doxorubicin equivalents).

at day 4 after first treatment and blood parameters (WBCs and thrombocytes) were determined with a Coulter counter. Statistical analysis was performed with the U-Test (Mann and Whitney) with $P < 0.05$.

## RESULTS AND DISCUSSION

**Albumin-binding Properties of 1 and Release Studies.** In recent years a number of low- and high-molecular weight prodrugs with doxorubicin have been developed that were designed to be cleaved by tumor-associated proteases such as cathepsin B [PK1: doxorubicin conjugated with $N$-(2-hydroxypropyl) methacrylamide copolymer; Ref. 15], $\beta$-glucuronidase (HMR 1826; Ref. 16), prostate-specific antigen (17, 18), or to-date unidentified peptidases (CPI-0004Na; Ref. 19).

In this work, we set out to develop an albumin-binding prodrug of doxorubicin that is cleaved by MMP-2. Biological evaluation was focused on a melanoma model (A375) considering that among a panel of human xenografts, MMP-2 was highly expressed in human melanomas (7, 10), and MMP-2 expression correlated with hematogeneous metastasis and prognosis in melanoma patients (6, 9).

In contrast with our previously synthesized derivative that contained maleimidoacetic acid instead of Mal (11), 1 demonstrated excellent water-solubility because of the incorporation of hydrophilic ether bonds. The incorporated maleimide group reacts specifically with thiol-groups in the pH range 6.5–7.5.

The HS-group of cysteine-34 of human serum albumin is a unique and accessible functional group of a plasma protein considering that free thiol groups are not present in the majority of circulating serum proteins except for albumin, and that the HS-group of cysteine-34 of endogenous HSA is the most reactive thiol group in human plasma (12, 13). To determine the coupling rate and selectivity of 1 for this sulfhydryl group, 1 was incubated with human blood plasma, and the samples were subsequently analyzed by reverse-phase chromatography. Chromatograms after an incubation time of 2 and 5 min are shown in Fig. 2A. Protein components were detected at 280 nm and the anthracycline moiety simultaneously at 495 nm. Binding of 1 to endogenous albumin is complete after 5 min with the major amount of 1 associated with the albumin peak, which elutes at 33–34 min in accordance with our previous work (12, 13). Doxorubicin shows no binding to endogenous albumin even after 90 min (13).

Stability of the albumin conjugate of 1 in human blood plasma was assessed after 1 had been incubated at 37°C with plasma by reverse-phase HPLC. The albumin-bound form of 1 had a half-life of ~16 h under these conditions.

To investigate how fast the albumin conjugate of 1 was cleaved by MMP-2, the conjugate was incubated with activated MMP-2 and chromatograms recorded at $\lambda = 495$ nm using reverse-phase HPLC. As shown in Fig. 2B after incubation of the albumin conjugate of 1 with activated MMP-2 for 1.5 h, the initial peak of the albumin conjugate of 1 at 33–34 min almost disappears and a new distinct peak is observed at ~14 min. Liquid chromatography-MS detected a mass of 914 for this peak corresponding to the cleaved doxorubicin tetrapeptide, [Ile-Ala-Gly-Gln-DOXO; $H^+(M +1)^+$]. When activated, MMP-2 was preincubated with an excess of TIMP-2 or (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid (MMP-2 inhibitor), a potent inhibitor of MMP-2, only small amounts of Ile-Ala-Gly-Gln-DOXO were cleaved from the conjugate as shown by HPLC (see Fig. 2B) demonstrating that activated MMP-2 specifically cleaves the conjugate of 1.

The concentration of MMP-2 in A375 melanoma cell culture supernatants and A375 melanoma tissue homogenates in their active and nonactive forms were determined by ELISA. Using several biological samples, we found a range of 70–100 ng/ml total MMP-2 (approximately 0.75–1.0 ng/ml active MMP-2) for A375 melanoma supernatants and of 370–430 ng/ml total MMP-2 (approximately 0.75–1.5 ng/ml active MMP-2) for A375 melanoma homogenates.

To obtain a first picture of the cleavage profile in these biological samples, we incubated the conjugate of 1 or the cleaved doxorubicin tetrapeptide (Ile-Ala-Gly-Gln-DOXO) with A375 melanoma cell culture supernatants and A375 melanoma tissue homogenates, and analyzed the cleavage products over 12 h using fluorescence HPLC (see Fig. 3, A and B). A major difference was observed in the cleavage profile between cell-conditioned supernatant and tissue homogenate. Whereas Ile-Ala-Gly-Gln-DOXO was cleaved within minutes to an additional doxorubicin peptide 1a and then to doxorubicin as the major cleavage product in A375 melanoma tissue homogenates (Fig. 3A), Ile-Ala-Gly-Gln-DOXO was cleaved primarily to 1a over a period of several hours in the cell-conditioned supernatant with only small amounts of doxorubicin being liberated (Fig. 3B). Incubation studies with the albumin conjugate of 1 confirmed that doxorubicin is the end product of cleavage in tissue homogenate with only small amounts of the tetrapeptide being observed because of its rapid degradation (see Fig. 3A). Furthermore, when the conjugate of 1 was incubated with A375 melanoma tissue homogenate that was preincubated with (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid, only small amounts of doxorubicin could be detected indicating that cleavage by MMP-2 is the primary step in the degradation of the conjugate of 1 in the A375 melanoma tissue homogenate (Fig. 3A). The cleavage profile observed in cell-conditioned supernatant is more complex: peaks for Ile-Ala-Gly-Gln-DOXO, 1a, and doxorubicin are observed besides several fluorescent signals.

These studies indicate that two additional peptidases are present in the biological samples that catalyze the two degradation steps, which cannot be explained by mere hydrolysis considering that cleavage

occurred at very different rates, and the pH values of the biological samples were in a narrow range (pH 7.4 for homogenates, pH 7.8–8.2 for supernatants).

***In Vitro* and *in Vivo* Activity of Doxorubicin, of 1, and of the Albumin Conjugate of 1.** The antiproliferative activity of 1, of the albumin conjugate of 1, and of free doxorubicin were assessed in the A375 melanoma cell line using the MTT assay. $IC_{50}$ values that were obtained after a 72-h cell exposure were $0.03 \pm 0.01$ $\mu$M for doxorubicin, $13 \pm 2.0$ $\mu$M for 1, and $10 \pm 1.6$ $\mu$M for the albumin conjugate of 1.

In subsequent *in vivo* experiments, the antitumor efficacy of 1 was evaluated in nude mice in the A375 melanoma model in a strict comparison to free doxorubicin. Preliminary toxicity studies in mice showed that the MTD of 1 was ~4-fold higher than for free doxorubicin (see "Materials and Methods"). Thus, the antitumor efficacy of 1 was compared with that of doxorubicin at the following doses: doxorubicin: $2 \times 13.3$ $\mu$mol/kg (= $2 \times 8$ mg/kg doxorubicin) corresponding to the MTD of doxorubicin in nude mice; and 1: $2 \times 13.3$ $\mu$mol/kg (= $2 \times 8$ mg/kg doxorubicin equivalents), $3 \times 39.9$ $\mu$mol/kg (= $3 \times 24$ mg/kg doxorubicin equivalents). The results of this animal experiment are shown in Fig. 4. Doxorubicin at its optimal dose of $2 \times 13.3$ $\mu$mol/kg produced a moderate inhibition in tumor growth that was comparable with the therapeutic results with 1 at $2 \times 13.3$ $\mu$mol/kg. In contrast, therapy with 1 at $3 \times 39.9$ $\mu$mol/kg was well tolerated producing a good antitumor effect with duration of remission for up to 40 days.

The dose-dependency of 1 *in vivo* is similar to other enzymatically cleavable doxorubicin prodrugs that aim at exploiting cathepsin B or prostate-specific antigen as molecular targets (15, 18). Equivalent doses of the prodrugs and doxorubicin do not produce a significant difference in antitumor response but in toxicity suggesting that comparable amounts of pharmacologically active doxorubicin reach the tumor cells of the tumor when tumor-bearing mice are treated with either formulation at the same dose. A major difference between doxorubicin and enzymatically cleavable doxorubicin prodrugs is the substantial increase in the MTD. As a consequence, their therapeutic index is significantly enhanced allowing high doses to be administered to tumor-bearing animals with a concomitant increase in antitumor activity compared with free doxorubicin (15–19).

1 exploits circulating albumin as a macromolecular carrier. The mechanism by which macromolecules such as serum proteins accumulate in tumor tissue has been termed "enhanced permeability and retention-effect" and serves as a working model for explaining the targeting potential of macromolecules for solid tumors (20). Incorporating the MMP-2 cleavable octapeptide (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln) between the drug and the carrier seems to ensure an effective release of doxorubicin in melanoma tissue that takes place in a multistep process.

In this work we developed a water-soluble albumin-binding prodrug of doxorubicin (1) that is cleaved by MMP-2. Against A375 melanoma that expressed high amounts of MMP-2, 1 was distinctly superior to doxorubicin at equitoxic dose. Our investigations emphasize the need to elucidate the cleavage profile of enzymatically cleavable prodrugs in native tumor tissue and demonstrate that for 1, additional proteases besides MMP-2 are important for liberating doxorubicin as the final cleavage product. Our next steps will be to study the therapeutic efficacy and cleavage profile in a panel of melanoma xenografts to elucidate whether 1 is a potential candidate for the treatment of malignant melanoma.

## REFERENCES

1. Sun, W., and Schuchter, L. M. Metastatic melanoma. Curr. Treat. Options Oncol., *2:* 193–202, 2001.
2. Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu. Rev. Cell Biol., *9:* 541–573, 1993.
3. de Vries, T. J., van Muijen, G. N., and Ruiter, D. J. The plasminogen activation system in melanoma cell lines and in melanocytic lesions. Melanoma Res., *6:* 79–88, 1996.
4. Chambers, A. F., and Matrisian, L. M. Changing views of the role of matrix metalloproteinases in metastasis. J. Natl. Cancer Inst., *89:* 1260–1270, 1997.
5. Curran, S., and Murray, G. I. Matrix metalloproteinases in tumour invasion and metastasis. J. Pathol., *189:* 300–308, 1999.
6. Hofmann, U. B., Westphal, J. R., van Muijen, G. N. P., and Ruiter, D. J. Matrix metalloproteinases in human melanoma. J. Investig. Dermatol., *115:* 337–344, 2000.
7. Hofmann, U. B., Westphal, J. R., Waas, E. T., Zendman, A. J. W., Cornelissen, I. M. H. A., Ruiter, D. J., and van Muijen, G. N. P. Matrix metalloproteinases in human melanoma cell lines and xenografts: increased expression of activated matrix metalloproteinase-2 (MMP-2) correlates with melanoma progression. Br. J. Cancer, *81:* 774–782, 1999.
8. Kurschat, P., Zigrino, P., Nischt, R., Breitkopf, K., Steurer, P., Klein, C. E., Krieg, T., and Mauch, C. Tissue inhibitor of matrix metalloproteinase-2 regulates matrix metalloproteinase-2 activation by modulation of membrane-type 1 matrix metalloproteinase activity in high and low invasive melanoma cell lines. J. Biol. Chem., *274:* 21056–21062, 1999.
9. Väisänen, A., Tuominen, H., Kallioinen, M., and Turpeenniemi-Hujanen, T. Matrix metalloproteinase-2 (72 kD type IV collagenase) expression occurs in the early stage of human melanocytic tumour progression and may have prognostic value. J. Pathol., *180:* 283–289, 1996.
10. Fiebig, H. H., Klostermeyer, A., Schüler, J. B., and Burger, A. Characterization of matrix metalloproteinases in 47 human tumor xenografts show high expression of MMP-2 in melanomas and sarcomas. Proc. Am. Assoc. Cancer Res., *40:* 463, 1999.
11. Kratz, F., Drevs, J., Bing, G., Stockmar, C., Scheuermann, K., Lazar, P., and Unger, C. Development and *in vitro* efficacy of novel MMP-2 and MMP-9 specific doxorubicin albumin conjugates. Bioor. Med. Chem. Lett., *11:* 2001–2006, 2001.
12. Kratz, F., Müller-Driver, R., Hofmann, I., Drevs, J., and Unger, C. A novel macromolecular prodrug concept exploiting endogenous serum albumin as a drug carrier for cancer chemotherapy. J. Med. Chem., *43:* 1253–1256, 2000.
13. Kratz, F., Warnecke, A., Scheuermann, K., Stockmar, C., Schwab, J., Lazar, P., Drückes, P., Esser, N., Drevs, J., Rognan, D., Bissantz, C., Hinderling, C., Folkers, G., Fichtner, I., and Unger, C. Probing the cysteine-34 position of endogenous serum albumin with thiol-binding doxorubicin derivatives: improved efficacy of an acid-sensitive Doxorubicin derivative with specific albumin-binding properties compared with the parent compound. J. Med. Chem., *45:* 5523–5533, 2002.
14. Cole, S. P. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. Cancer Chemother. Pharmacol., *17:* 259–263, 1986.
15. Loadman, P. M., Bibby, M. C., Double, J. A., Al-Shakhaa, W. M., and Duncan, R. Pharmacokinetics of PK1 and doxorubicin in experimental colon tumor models with differing responses to PK1. Clin. Cancer Res., *5:* 3682–3688, 1999.
16. Murdter, T. E., Sperker, B., Kivisto, K. T., McClellan, M., Fritz, P., Friedel, G., Linder, A., Bosslet, K., Toomes, H., Dierkesmann, R., and Kroemer, H. K. Enhanced uptake of doxorubicin into bronchial carcinoma: $\beta$-glucuronidase mediates release of doxorubicin from a glucuronide prodrug (HMR 1826) at the tumor site. Cancer Res., *57:* 2440–2445, 1997.
17. Denmeade, S. R., Nagy, A., Gao, J., Lilja, H., Schally, A. V., and Isaacs, J. T. Enzymatic activation of a doxorubicin-peptide prodrug by prostate-specific antigen. Cancer Res., *58:* 2537–2540, 1998.
18. DeFeo-Jones, D., Garsky, V. M., Wong, B. K., Feng, D. M., Bolyar, T., Haskell, K., Kiefer, D. M., Leander, K., McAvoy, E., Lumma, P., Wai, J., Senderak, E. T., Motzel, S. L., Keenan, K., Van Zwieten, M., Lin, J. H., Freidinger, R., Huff, J., Oliff, A., and Jones, R. E., A peptide-doxorubicin 'prodrug' activated by prostate-specific antigen selectively kills prostate tumor cells positive for prostate-specific antigen *in vivo*. Nat. Med., *6:* 1248–1252, 2000.
19. Dubois, V., Dasnois, L., Lebtahi, K., Collot, F., Heylen, N., Havaux, N., Fernandez, A. M., Lobl, T. J., Oliyai, C., Nieder, M., Shochat, D., Yarranton, G. T., and Trouet, A. CPI-0004Na, a new extracellularly tumor-activated prodrug of doxorubicin: *in vivo* toxicity, activity, and tissue distribution confirm tumor cell selectivity. Cancer Res., *62:* 2327–2331, 2002.
20. Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Controlled Release, *65:* 271–284, 2000.