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

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

Tumor Biology Center, Breisacher Straße 117, 79106 Freiburg, Germany [A. M. M., J. D., N. E., C. U., F. K.]; Max-Delbrück Center, Robert-Rössle-Straße 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, 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). Consequently doxorubicin.

In vivo, 1 was 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 LFB Bioproducts (Munich, Germany). TIMP-2 was purchased from Biocytex (Marseille, France). Boehringer Mannheim (Mannheim, Germany). The BIOTRAK Human MMP ELISA Activity Assay for MMP-2 was purchased from Amersham Pharma 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). 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, 1-cyano-4-dihydroxycinnamic 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 sterile isotonic buffer containing 10 mM sodium phosphate and 5% p-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-
A375 Melanoma 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-dissmembrator 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 μg/ml glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Media were routinely changed every 3 days.

Synthesis and Characterization of 1. 1 was prepared by reacting the maleimidodiethylenglycol 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 mg (0.3 mmol) diisopropylcarbodiimide were added as the coupling agent.

Fig. 1. Structure of the albumin-binding doxorubicin produg 1.

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 μM 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 were 50 and 100 μM, respectively. 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 Scha¨rfe 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 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 μl 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 ± 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 ± 2°C room temperature, 50 ± 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 performed to obtain a dose schedule for subsequent studies in xenograft models. For 1, a dose of 2 × 30 mg/kg and 2 × 60 mg/kg doxorubicin equivalents was investigated. 1 was administrated by i.v. application to 2 animals with an interval of 7 days. Two × 30 mg/kg was well tolerated, and no mortality and no body weight loss was observed. However, at the dose of 2 × 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 × 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 10⁶ 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 × (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.
RESULTS AND DISCUSSION

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Dose Selection</th>
<th>Tumor Growth</th>
<th>Hematocrit</th>
<th>Blood Urea</th>
<th>Thrombocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>6</td>
<td>54 mg/kg</td>
<td>Dose (i.v.)</td>
<td>d 8-10</td>
<td>%</td>
<td>(10^6/ml)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.15</td>
<td>2</td>
<td>9.1±1.9</td>
<td>1073±271</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.15</td>
<td>21</td>
<td>8.0±0.0</td>
<td>106±2.7</td>
<td>1033±335</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.15</td>
<td>24</td>
<td>5.8±0.5</td>
<td>6.2±0.2</td>
<td>1391±161</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.15</td>
<td>6</td>
<td>5.8±0.6</td>
<td>1391±123</td>
<td></td>
</tr>
</tbody>
</table>

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 × 133 μmol/kg (= 2 × 8 mg/kg doxorubicin); 1: 2 × 133 μmol/kg (= 2 × 8 mg/kg doxorubicin equivalents), 3 × 39.9 μmol/kg (= 3 × 24 mg/kg doxorubicin equivalents).

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], β-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 produg 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), I 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 sulphydryl 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 λ = 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)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 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 peptides 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. IC50 values that were obtained after a 72-h cell exposure were 0.03 ± 0.01 μM for doxorubicin, 13 ± 2.0 μM for 1, and 10 ± 1.6 μM 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 × 13.3 μmol/kg (= 2 × 8 mg/kg doxorubicin) corresponding to the MTD of doxorubicin in nude mice; and 1: 2 × 13.3 μmol/kg (= 2 × 8 mg/kg doxorubicin equivalents), 3 × 39.9 μmol/kg (= 3 × 24 mg/kg doxorubicin equivalents). The results of this animal experiment are shown in Fig. 4. Doxorubicin at its optimal dose of 2 × 13.3 μmol/kg produced a moderate inhibition in tumor growth that was comparable with the therapeutic results with 1 at 2 × 13.3 μmol/kg. In contrast, therapy with 1 at 3 × 39.9 μmol/kg was well tolerated producing a good antitumor effect with duration of remission for up to 40 days.

The dose-dependency of 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 administrated to tumor-bearing animals with a concomitant increase in antitumor activity compared with free doxorubicin (15–19).

I 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.

ACKNOWLEDGMENTS

We thank Wilhelm Sander-Stiftung for support.

REFERENCES

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


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/14/4062

Cited articles  This article cites 20 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/14/4062.full#ref-list-1

Citing articles  This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/14/4062.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.