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Development of a novel albumin-binding prodrug that is cleaved by urokinase-type-plasminogen activator (uPA)

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Abstract—A water-soluble albumin-binding prodrug of doxorubicin [EMC-Gly-Gly-Gly-Arg-Arg-DOXO (EMC, 6-maleimidocaproic acid)] was developed that is cleaved specifically by the tumor-associated protease urokinase-type plasminogen activator (uPA).

Tumor invasion and metastases are known to be associated with increased activities of several proteolytic enzymes, including the extracellular serine protease urokinase-type plasminogen activator (uPA).[1] As an important part of the plasminogen activation system, uPA is able to catalyze the conversion of inactive plasminogen to plasmin which degrades most substrates in the extracellular matrix (e.g., laminin, fibronectin, and vitronectin) and activates other important proteases such as matrix metalloproteases (MMPs).[2,3] Over-expression of uPA has been found in various malignant tumors, especially in breast,[4,5] ovarian[5–7], and cervical[5,8] carcinoma.

In order to prevent tumor growth and the formation of metastases, a number of inhibitors of uPA have been developed in the past and first inhibitors are undergoing clinical trials.[9] In contrast, no reports based on exploiting the protease activity of uPA for releasing an anticancer agent from a prodrug formulation have appeared in the literature. As a consequence, we set out to develop a uPA-specific prodrug with the anticancer agent doxorubicin. Anthracyclines have been widely used for the development of low- and high-molecular weight prodrugs that are cleaved enzymatically or in a pH-dependent manner.[10] In our previous work, we have developed doxorubicin prodrugs that are cleaved at acidic pH[11] or enzymatically by MMP-2 and MMP-9[12,13] or prostate-specific antigen.[14]

The doxorubicin derivatives were designed as albumin-binding prodrugs that bind in situ to the cysteine-34 position of circulating albumin after intravenous administration and release the albumin-bound drug at the tumor site. Albumin is a promising drug carrier because of its passive accumulation in solid tumors due to their high metabolic turnover, angiogenesis, hypervasculature, defective vascular architecture, and impaired lymphatic drainage.[15] An acid-sensitive albumin-binding prodrug of doxorubicin, that is, 6-maleimidocaproyl hydrazone of doxorubicin, demonstrated superior antitumor efficacy compared to doxorubicin in several preclinical models and exhibited a good safety profile and antitumor efficacy in a recently completed clinical phase I study.[16]

When designing enzymatically cleavable doxorubicin prodrugs, the peptide linker is generally bound to the 3′-NH_2 group through a spacer since direct coupling of the protease substrate to the amino position of doxorubicin impairs cleavage by the protease. This spacer is either self-immolative, for example, *p*-aminobenzyloxycarbonyl (PABC) releasing the anticancer agent spontaneously,[17] or is an amino acid or a short peptide sequence. The latter doxorubicin derivatives are ideally degraded to doxorubicin.[13,18]

In the present work, we decided to develop albumin-binding prodrugs having the following general formula:

Abbreviations: DOXO, doxorubicin; DMF, dimethylformamide; HATU, *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5,-b]pyridino-1-ylmethylene]-*N*-methylmethan-aminium hexafluorophosphate; DIEA, diisopropylethyl amine; DIPC, *N*,*N*′-diisopropylcarbodiimide; AA, amino acid.

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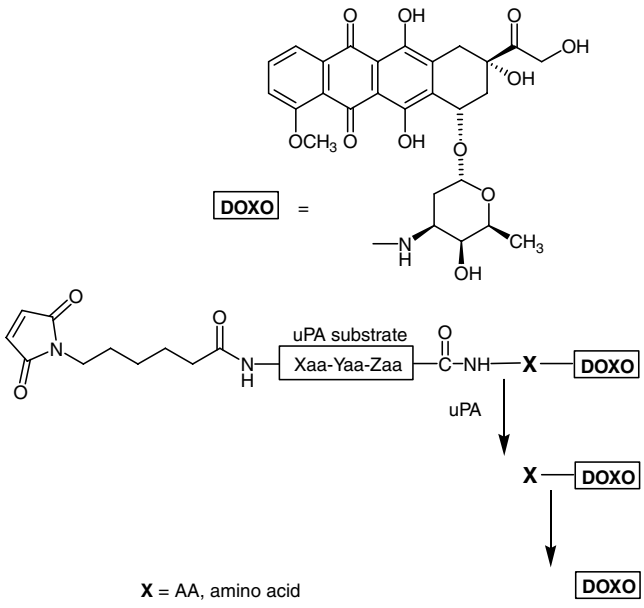


Figure 1. General structure of an albumin-binding prodrug containing a uPA specific substrate.

Figure 1 shows a general stucture of an albumin-binding prodrug in which a suitable uPA substrate is bound at its scissile bond to a doxorubicin amino acid derivative. Since derivatization at the 3′-NH_2 position generally reduces the antitumor potential of doxorubicin,[17] we focused our initial work on discovering an amino acid derivative of doxorubicin that is cleaved efficiently in tumor tissue homogenates, an issue that has not been adequately addressed in the past. For this purpose, several polar and non-polar amino acid derivatives of doxorubicin were synthesized by reacting Fmoc-protected L-amino acids with doxorubicin, and cleaving the protecting group with piperidine (see Ref. 19 and Table 1).

Subsequently, the doxorubicin amino acid derivatives were incubated with tumor tissue homogenates from mamma carcinoma xenografts (MDA-MB 435, MCF-7, M3366),[20] and their cleavage profile was assessed over 24 h with the aid of fluorescence HPLC (see Refs. 21 and 22). For preparing the tumor homogenates two protocols were used, the first at pH 5.0 that primarily liberates intracellular proteases,[23] the second

Table 1. Cleavage of AA-DOXO in tissue homogenates of Mamma carcinoma xenografts (MDA-MB 435, MCF-7, M3366)

Substrate[a]	Cleavage in tumor-tissue homogenates at pH 5.0; after 24 h[c]	Cleavage in tumor-tissue homogenates at pH 7.4; after 24 h[c]
Gln-DOXO	NC[b]	NC
Asn-DOXO	NC	NC
Arg-DOXO	NC	>70%
His-DOXO	NC	<10%
Ser-DOXO	NC	<10%
Ala-DOXO	NC	NC
Val-DOXO	NC	NC
Leu-DOXO	<10%	NC
Met-DOXO	NC	NC

[a] General synthesis of AA-DOXO see Ref. 19; for cleavage study experiments of AA-DOXO at pH 5.0 and 7.4 see Ref. 20.
[b] NC, not cleaved.
[c] The amount of cleavage was assessed by following the decrease of the peak area of the respective AA-DOXO over 24 h; doxorubicin was the sole cleavage product that was detected.

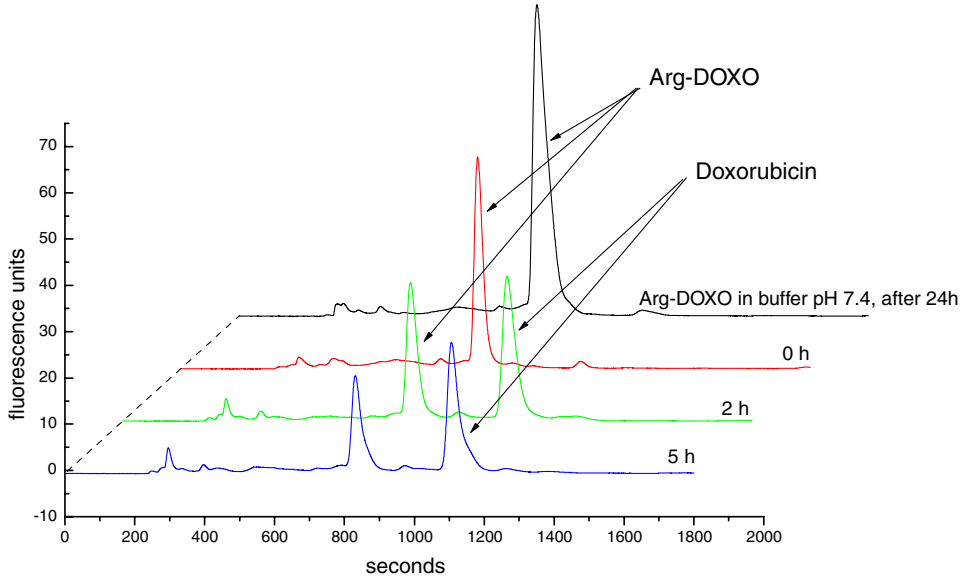


Figure 2. Chromatograms of incubation studies of Mamma 3366 tissue homogenate with Arg-DOXO after 0, 2 and 5 h at 37 °C. Concentration of Arg-DOXO was 125 μM. Chromatographic conditions, see Refs. 21 and 22.

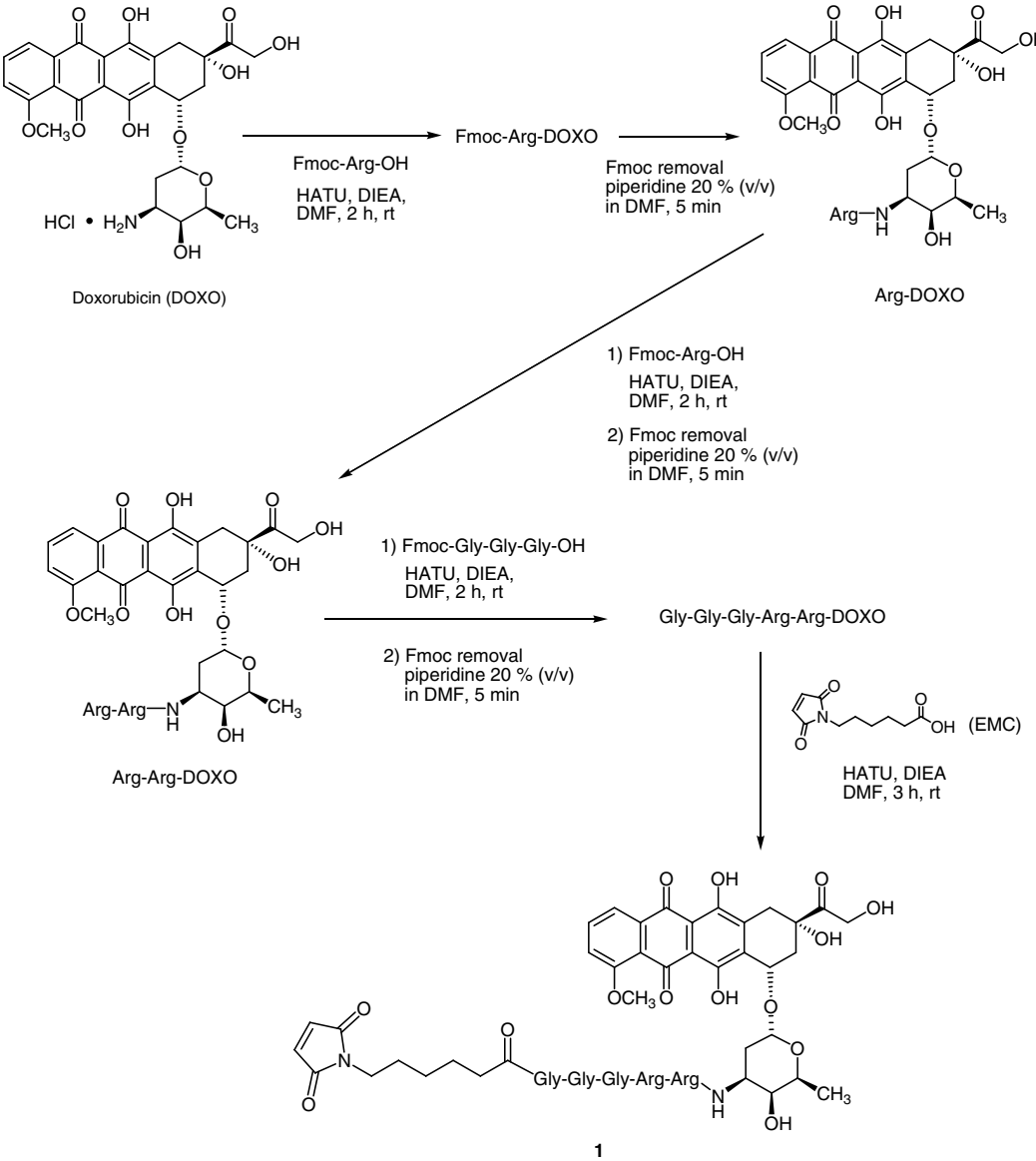
at pH 7.4 that is used for extracting extracellular proteases[24]—see Ref. 20. Among the spectrum of doxorubicin amino acid derivatives, *N*-(L-arginine)doxorubicin (Arg-DOXO) was the only compound that was efficiently cleaved to doxorubicin in tumor homogenates at pH 7.4 (see Table 1) as illustrated in Figure 2, which depicts the time-dependent cleavage profile of Arg-DOXO over 5 h. Marginal cleavage was observed for Leu-DOXO at pH 5.0 and for Ser-DOXO and His-DOXO at pH 7.4 (see Table 1).

Consequently, we selected Arg-DOXO for developing a doxorubicin prodrug that could be cleaved by uPA. A general substrate for uPA is Z-Gly-Gly-Arg-AMC (AMC, 7-amino-4-methylcoumarin)[25–27] which is used in a fluorogenic assay for determining the activity of the enzyme.

In addition, other uPA-specific peptide sequences such as H-Glu-Gly-Arg-benzylamide,[28] H-Glu-Gly-Arg-anilide,[28] and Z-Gly-Gly-Arg-Val-OMe[25] have proven to be good substrates for uPA. The arginine residue is the relevant amino acid in the P_1-position considering that the peptide bond Arg561–Val562, which is the cleavage site of plasminogen, and the bonds Arg83–Ala84 and Arg89–Ser90, which represent bonds between domain 1 and 2 of the human uPA receptor, are specifically cleaved by uPA.[1]

In our prodrug design, we selected the peptide sequence Gly-Gly-Arg as the uPA substrate which has the asset that a further arginine residue is introduced in the prodrug. In our earlier work regarding albumin-binding doxorubicin prodrugs that are cleaved by prostate-specific antigen, two arginine residues rendered excellent water-solubility, an important prerequisite for intravenous application and in situ coupling to endogenous albumin.[14]

The synthesis of the doxorubicin prodrug (**1**) is shown in Scheme 1. Because doxorubicin is not stable in the presence of acids such as trifluoroacetic acid and the



Scheme 1.

maleimide group is not compatible with bases such as piperidine, we opted to build up the prodrug starting with doxorubicin and using a Fmoc-based strategy. Arg-Arg-DOXO was obtained by reacting Arg-DOXO with Fmoc-Arg-OH in anhydrous DMF in the presence of DIEA and HATU as the coupling agent, removing the Fmoc-group with piperidine solution, and isolating the product over diol column chromatography. In a next step, three glycine residues were introduced by reacting Arg-Arg-DOXO with commercially available Fmoc-Gly-Gly-Gly-OH in anhydrous DMF in the presence of DIEA and HATU. The protecting group was removed with piperidine solution and the crude product precipitated after 5 min with diethyl ether. Gly-Gly-Gly-Arg-Arg-DOXO was isolated through chromatography on a reverse phase RP-C18 column.

In a final step, 6-maleimidocaproic acid (EMC) was coupled to Gly-Gly-Gly-Arg-Arg-DOXO in anhydrous DMF in the presence of DIEA and HATU. After precipitation with diethyl ether, **1** was isolated through chromatography on a reverse phase RP-C18 column and the product was lyophilized (see Ref. 29).

Compound **1** showed good water-solubility with up to 10 mg/mL being dissolved in 10 mM sodium phosphate/5% D-glucose buffer in the pH-range of 6.0–7.0.

In order to investigate the protein-binding properties of **1**, incubation studies with this compound and human blood plasma were performed. The HS-group of cysteine-34 of endogenous albumin is a unique and accessible functional group of a plasma protein

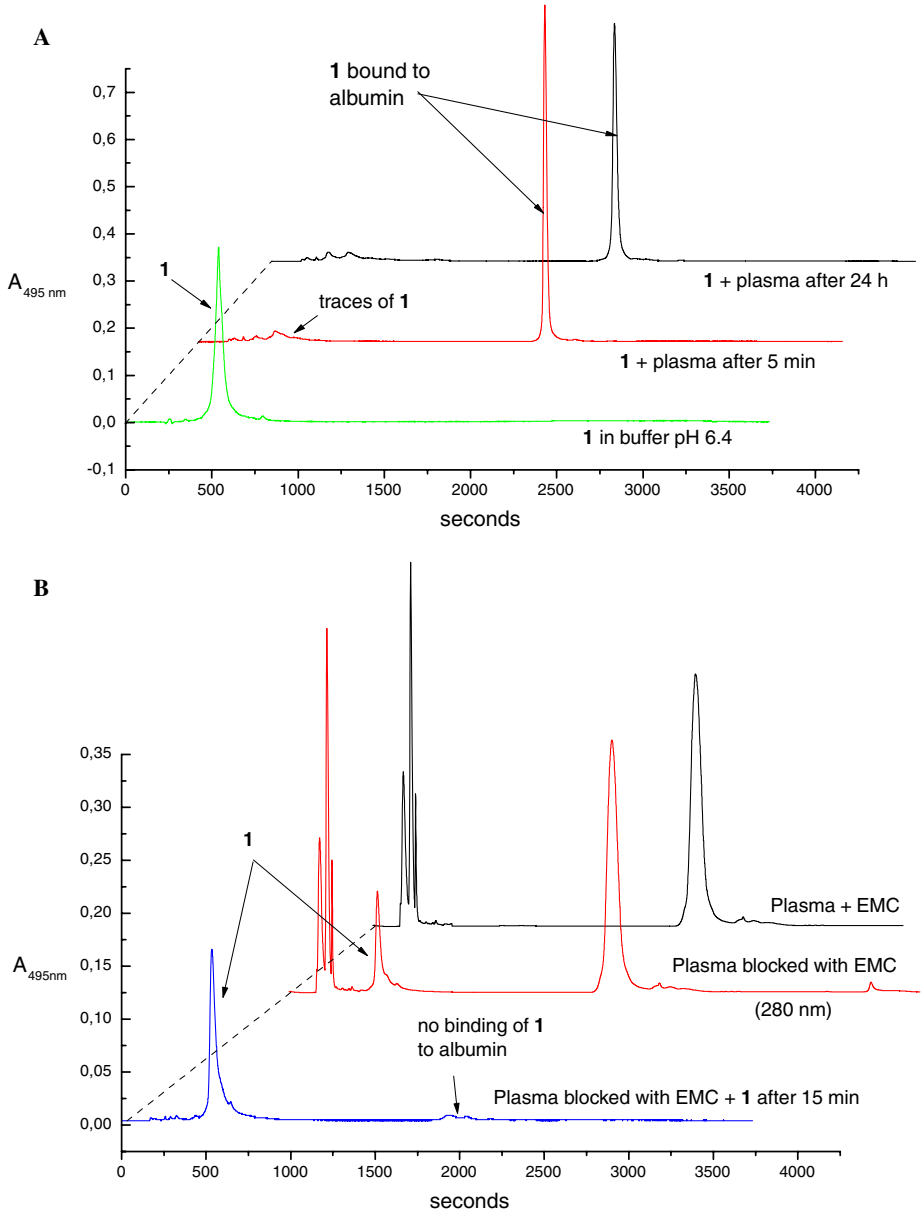


Figure 3. (A) Chromatograms of incubation studies of human plasma after 5 min and 24 h with **1** at 37 °C. (B) Chromatograms of incubation studies of human plasma pre-incubated with an excess of 6-maleimidocaproic acid (EMC) for 2 h and subsequent incubation with **1** after 15 min at 37 °C. Concentration of the anthracycline was 250 μM. Chromatographic conditions, see Refs. 21and 30.

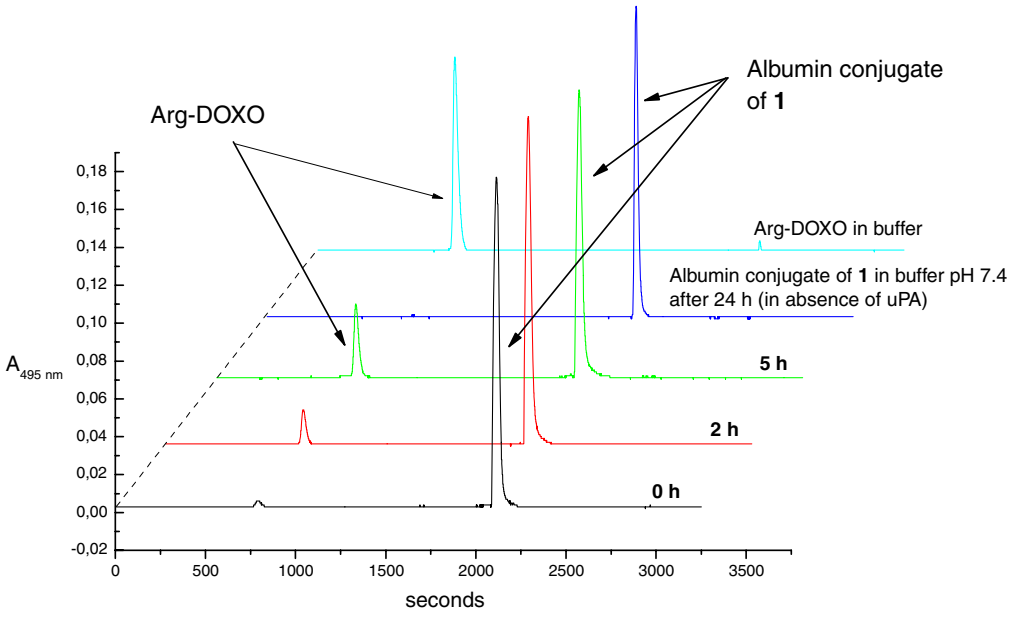


Figure 4. Chromatograms of incubation studies with the albumin conjugate of **1** in the presence of human uPA [(3 KU, 75 μg/mL) Calbiochem, Bad Soden, Germany] after 0, 2 and 5 h at 37 °C. Concentrations of the albumin conjugate of **1** was 125 μM. Chromatograms of the albumin conjugate of **1** at pH 7.4 after 24 h and of Arg-DOXO are shown as references. Chromatographic conditions, see Refs. 21 and 22.

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 abundant thiol group in human plasma.[11] The coupling rate and selectivity of **1** for this sulfhydryl group was studied by incubating **1** with human blood plasma at 37 °C and subsequently analyzing the samples with reverse phase chromatography (see Refs. 21,30). Protein components were detected at 280 nm and the anthracycline moiety simultaneously at 495 nm (see Fig. 3A and B).

Binding of **1** to endogenous albumin is essentially complete after 5 min with only traces of **1** eluting after 500 s (see Fig. 3A) which is in accordance with our previous work on albumin-binding prodrugs bearing a maleimide group.[11,12,14] The following experiment was carried out to demonstrate that the cysteine-34 position of albumin is involved in the coupling step: human plasma was pre-incubated with an excess of a non-fluorescent maleimide with respect to the albumin concentration in the blood plasma, that is, 6-maleimidocaproic acid (EMC), before adding **1**. The resulting chromatogram (see Fig. 3B) after 15 min 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 500 s.

The stability of the albumin conjugate of **1** in human blood plasma was assessed after **1** had been incubated at 37 °C with plasma for 24 h. Reverse phase HPLC shows that the albumin-bound form is highly stable over this time (see Fig. 3A).

In contrast, the albumin conjugate of **1**[31] was cleaved upon incubation with uPA. Figure 4 shows the chromatograms recorded at $\lambda = 495$ nm over 5 h after incubation of human uPA with the albumin conjugate of **1** at pH 7.4 (Tris buffer) at 37 °C demonstrating that Arg-DOXO is released in a time-dependent manner. No cleavage was observed at pH 7.4 over 24 h in the absence of uPA. This result demonstrates that the endopeptidase uPA is able to recognize the Gly-Gyl-Arg substrate attached to albumin and to cleave Arg-DOXO from the macromolecular carrier.

As preparatory work for future in vivo studies in preclinical tumor models, we evaluated the maximum tolerated dose (MTD) of **1** in nude mice in an orientating toxicity study.[32] The MTD proved to be 3×24 mg/kg doxorubicin equivalents (iv, weekly schedule) which is a 4.5-fold increase over the standard dose schedule for doxorubicin that is used in xenograft experiments (2×8 mg/kg). This shift in the MTD for **1** is in accordance with our work on albumin-binding prodrugs with doxorubicin that are either cleaved in an acidic environment[11] or enzymatically by tumor-associated proteases (MMP-2 and -9, PSA).[11,13,14]

In summary, we have developed a prodrug of doxorubicin that is water-soluble, binds rapidly to the cysteine-34 position of endogenous albumin, and is efficiently cleaved by uPA thus representing to the best of our knowledge the first prodrug of a cytostatic agent that is cleaved by this tumor-associated protease.

Acknowledgment

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19. *General synthesis of AA-DOXO*. 0.35 mmol of doxorubicin hydrochloride (Yick-Vic Chemicals and Pharmaceuticals (HK), China), 0.77 mmol of the Fmoc-AA-OH (Bachem AG, Bubendorf, Switzerland), and 1.38 mmol of DIEA were dissolved in 25 mL anhydrous DMF. For the synthesis of Gln-DOXO, Asn-DOXO, and Leu-DOXO, the coupling agent DIPC (1.52 mmol) was added at 5 °C after 5 min. After stirring at 5 °C for 3 days, the product was precipitated with 1000 mL of diethyl ether. For the synthesis of Arg-DOXO, His-DOXO, Ala-DOXO, Ser-DOXO, and Val-DOXO, 0.42 mmol HATU was added as the coupling agent after stirring at room temperature for 5 min. Then the solution was stirred at room temperature for 2 h and the product was precipitated with 1000 mL of diethyl ether.
    The protecting group was removed by dissolving 150 mg product ((Fmoc)AA-DOXO) in 3 mL of 20% piperidine solution (DMF) and stirring for 5 minutes. The violet product was precipitated with diethyl ether, washed three times with diethyl ether, and dried in vacuum.
    Gln-DOXO, Asn-DOXO, and Leu-DOXO were purified on a silica gel column using methanol to afford AA-DOXO as a red powder after precipitating the combined fractions containing the product with diethyl ether. Arg-DOXO and His-DOXO were purified on a LiChroprep® Diol column using first chloroform/methanol 3:1 + 0.1% TFA, and then chloroform/methanol 2:1 + 0.1% TFA.
    Ala-DOXO, Ser-DOXO, and Val-DOXO were purified on a reverse phase RP-C18 column using acetonitrile/water 50:50 at first and then acetonitrile/water 70:30 to afford AA-DOXO as a red powder after lyophilizing the combined fractions containing the product.
    Mass spectrometry: Mass spectra (ESI-MS) were recorded on a Thermoelektron LCQ Advantage; *HPLC*: see Refs. 21 and 22.
    Gln-DOXO: Mass (ESI-MS: 5.5 kV): *m/z* 672.1 $[M+H]^+$, 694.0 $[M+Na]^+$, HPLC (495 nm): >90%; *Asn-DOXO*: Mass (ESI-MS: 5.5 kV): *m/z* 680.1 $[M+Na]^+$, 681.1 $[M+Na+H]^+$, HPLC (495 nm): >90%; *Leu-DOXO*: Mass (ESI-MS; 4.0 kV): *m/z* 657.1 $[M]^+$, HPLC (495 nm): >95%; *Arg-DOXO*: Mass (ESI-MS: 2.5 kV): *m/z* = 700.2 $[M+H]^+$, 722.2 $[M+Na]^+$, HPLC (495 nm): >95%; *His-DOXO*: Mass (ESI-MS: 2.5 kV): *m/z* = 680.9 $[M+H]^+$, 703.1 $[M+Na]^+$, HPLC (495 nm): >90%; *Val-DOXO*: Mass (ESI-MS: 3 kV): *m/z* = 665.1 $[M+Na]^+$, HPLC (495 nm): >95%; *Ser-DOXO*: Mass (ESI-MS: 3 kV): *m/z* = 629.2 $[M+H]^+$, 651.1 $[M+Na]^+$, HPLC (495 nm): >95%; *Ala-DOXO*: Mass (ESI-MS: 3 kV): *m/z* = 637.1$[M+Na]^+$, 638.1 $[M+Na+H]^+$, HPLC (495 nm): >95%.
20. *Cleavage study experiment of AA-DOXO*. One hundred microliters [500 μM] of AA-DOXO was incubated at 37 °C with 100 μL of tumor tissue homogenate from mamma carcinoma xenografts (MDA-MB 435, MCF-7, M3366). The concentration of AA-DOXO was diluted to a final concentration of 125 μM and chromatograms were recorded after 0, 2, 5, and 24 h using fluorescence HPLC. Cleavage studies with the tumor homogenates were performed once; for the AA-DOXO derivatives where cleavage to doxorubicin was observed, the experiments were repeated.
    For obtaining carcinoma tissue homogenates, all steps were carried out on ice where possible. Tissue of xenograft tumors was cut into small pieces, and 200 mg samples were transferred in a 2 mL Eppendorf tube to which was added 800 μL of homogenate buffer (50 mM Tris–HCl buffer, pH 7.4, containing 1 mM monothioglycerol or 50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA · Na_2, pH 5.0, containing 0.1% Brij 35). Homogenization was carried out with a micro-dismemberator at 3000 rpm for 3 min with the aid of glass balls, and the samples were then centrifuged at 5000 rpm for 10 min and kept frozen at −78 °C prior to use.
21. HPLC was performed with a BioLogic Duo Flow System from Bio-Rad (Munich, Germany), which was connected with a Merck F-1050 Fluorescence Spectrophotometer (EX. 490 nm, EM. 540 nm) and a Lambda 1000 visible detector from Bischoff (at λ = 495 nm); UV-detection at 280 nm; column: Waters, 300 Å, Symmetry C18 (4.6 × 250 mm) with precolumn; injection volume: 50 μL.
22. HPLC conditions for the cleavage studies and analysis of **1**, the albumin-conjugate of **1**, and AA-DOXO: flow: 1.2 mL/min, mobile phase A: 22% CH_3CN, 78% 4 mM sodium phosphate buffer (pH 3.0); mobile phase B: 70% CH_3CN, 30% 4 mM sodium phosphate buffer (pH 3.0); gradient: 0–25 min 100% mobile phase A; 25–40 min increase to mobile phase B; 40–50 min 100% mobile phase B; 50–60 min decrease to initial mobile phase.
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29. Synthesis and characterization of **1** (EMC-Gly-Gly-Gly-Arg-Arg-DOXO).
Synthesis and characterization of Arg-Arg-DOXO. Arg-DOXO (150 mg, 0.22 mmol), Fmoc-Arg-OH (85 mg, 0.22 mmol), and DIEA (120 μL, 97 mg, 0.75 mmol) were dissolved in 2 mL of anhydrous DMF. After stirring at room temperature for 5 min, HATU (82 mg, 0.215 mmol) was added as the coupling agent. After stirring at room temperature for 2 h, the product was precipitated with 200 mL diethyl ether, the formed precipitate was isolated and washed three times with diethyl ether and dried in vacuum to yield 180 mg of a red powder.
The protecting group was removed by dissolving 180 mg of the product in 3 mL of 20% piperidine solution (DMF) and stirring for 5 min. The violet product was precipitated with 300 mL diethyl ether, washed three times with 20 mL diethyl ether, and dried in vacuum. The product was purified on a diol column using first chloroform/methanol 3:1 + 0.1% TFA and then chloroform/methanol 2:1 + 0.1% TFA to afford 105 mg Arg-Arg-DOXO as a red powder after precipitating the combined fractions containing the product with diethyl ether. Mass (ESI-MS: 4.0 kV): m/z = 856.1 $[M]^+$, 857.2 $[M+H]^+$, 878.1 $[M+Na]^+$, HPLC (495 nm): >95%.
Synthesis of Gly-Gly-Gly-Arg-Arg-DOXO. Arg-Arg-DOXO (100 mg, 0.12 mmol), Fmoc-Gly-Gly-Gly-OH (53 mg, 0.13 mmol), and DIEA (64 μL, 53 mg, 0.41 mmol) were dissolved in 5 mL of anhydrous DMF; after stirring at room temperature for 15 min, HATU (45 mg, 0.12 mmol) was added as the coupling agent. After stirring at room temperature for 2 hours, the product was precipitated with 400 mL anhydrous diethyl ether and the solvent removed by centrifugation. The precipitate was washed and dried in high vacuum. The product was dissolved in acetonitrile/water 30:70 + 0.1% TFA and purified on a reverse phase RP-C18 column using acetonitrile/water 30:70 + 0.1% TFA and acetonitrile/water 50:50 + 0.1% TFA in this order to afford 150 mg Fmoc-Gly-Gly-Gly-Arg-Arg-DOXO as a red powder after lyophilizing the combined fractions containing the product.
The protecting group was removed by dissolving 150 mg product in 2.5 mL of 20% piperidine solution (DMF) and stirring for 5 min. The violet product was precipitated with 250 mL diethyl ether, washed, and dried in vacuum to afford 110 mg.
*Synthesis of **1**.* 6-Maleinimidocaproic acid (EMC) (56 mg, 0.26 mmol), Gly-Gly-Gly-Arg-Arg-DOXO (90 mg, 0.09 mmol), and DIEA (45 μL, 34 mg, 0.26 mmol) were dissolved in 3 mL of anhydrous DMF; after stirring at room temperature for 15 min, HATU (66 mg, 0.18 mmol) was added. After stirring at room temperature for 3 h, the product was precipitated with 300 mL anhydrous diethyl ether. The precipitate was washed and dried in high vacuum. The product was purified on a reverse phase RP-C18 column using acetonitrile/water 22:78 + 0.1% TFA to afford 20 mg of the product as a red powder after lyophilizing the combined fractions containing the product.
HPLC (Symmetry C18, 495 nm): purity >95%; Mass (ESI-MS, 3.0 kV): m/z 1220.4 $[M]^+$, 1221.4 $[M+H]^+$.
30. HPLC conditions for the separation of human plasma of **1** and the albumin conjugate of **1**: flow: 1.2 mL/min, mobile phase A: 27% CH_3CN, 73% 20 mM potassium phosphate (pH 7.0); mobile phase B: 70% CH_3CN, 30% 20 mM potassium phosphate (pH 7.0); gradient: 0–25 min 100% mobile phase A; 25–40 min increase to mobile phase B; 40–50 min 100% mobile phase B; 50–60 min decrease to initial mobile phase.
31. *Synthesis of the albumin conjugate of* **1**. 1.45 mg of **1** was dissolved in 2 mL human serum albumin (5% solution from Octapharma) and the solution was incubated at room temperature for 2 h. The albumin conjugate was obtained by subsequent size-exclusion chromatography (Sephacryl S-100; Tris-buffer: 50 mM Tris–HCl, 1 mM monothioglycerol, pH 7.4). The content of anthracycline in the sample was determined using the ε-value for doxorubicin [ε_{495} (pH 7.4) = 10,650 M^{-1} cm^{-1}]. The concentration of **1** in the conjugate was adjusted to 500 ± 50 μM by concentrating the sample with CENTRIPREP-10-concentrators from Amicon, FRG (4 °C and 4500 rpm). Samples were kept frozen at −20 °C and thawed prior to use.
32. *In vivo experiment*. For the orientating toxicity study of **1** two female NMRI: nude mice were used. Compound **1** was dissolved in 10 mM sodium phosphate/5% D-glucose buffer, pH 7.0, and was administered at a dose of 3 × 59.95 mg/kg (3 × 24 mg/kg doxorubicin equivalents) by intravenous application to two animals with an interval of 7 days (1th, 8th, and 15th day).