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Development and In Vitro Efficacy of Novel MMP2 and MMP9 Specific Doxorubicin Albumin Conjugates

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Abstract—Two doxorubicin albumin conjugates (**A-DP1** and **A-DP2**), which differ in their substrate specificity for the matrix metalloproteinases MMP2 and MMP9, were prepared by binding maleimide doxorubicin peptide derivatives to the cysteine-34 position of human serum albumin. The incorporated octapeptide, Gly-Pro-Gln-Arg–Ile-Ala-Gly-Gln, in **A-DP2** is not cleaved by activated MMP2 and MMP9 in contrast to Gly-Pro-Leu-Gly–Ile-Ala-Gly-Gln incorporated in **A-DP1** that is cleaved efficiently by activated MMP2 and MMP9 liberating a doxorubicin tetrapeptide. **A-DP1** showed antiproliferative activity in a murine renal cell carcinoma line in the low micromolar range (IC_{50} value$\approx$0.2 μM).

Matrix metalloproteinases (MMPs), especially MMP2 (gelatinase A) and MMP 9 (gelatinase B), play a critical role in tumor progression, tumor angiogenesis and metastasis.[1,2] In recent years, several MMP inhibitors have been developed preclinically, and selected candidates are being evaluated in phase I–III studies.[3,4]

In contrast to the original therapeutic approach of inhibiting MMPs, we have assessed a novel drug targeting strategy in which the *protease activity* of MMP2 and MMP9 is exploited to release the anticancer agent doxorubicin from a macromolecular carrier. Bearing in mind that MMPs are primarily secreted in the interstitial stroma of tumor tissue, we selected human serum albumin as the drug carrier because most of the body's albumin is present in extracellular compartments and additionally albumin accumulates in tumor tissue.[5] In the past, doxorubicin polymer conjugates have been developed that are cleaved by intracellular proteases such as cathepsins present in lysosomes,[6–8] but no pertinent work has been reported that targets extracellulary secreted proteases such as MMPs.

In our previous work, we have shown that acid-sensitive doxorubicin albumin conjugates are superior to free doxorubicin in tumor-bearing mice.[9–11] In these conjugates, doxorubicin was derivatized at its C-13 keto-position with maleimide hydrazide spacers and then coupled to serum albumin. Based on this synthetic experience, we set out to develop MMP2 and MMP9 specific doxorubicin albumin conjugates having the general formula shown in Figure 1, in which doxorubicin is bound through a maleimide peptide spacer to the cysteine-34 position of human serum albumin (HSA).

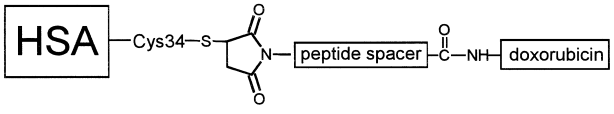

Figure 1.

When designing our conjugates, we were aided by the detailed work of Netzel-Arnett et al.[12,13] who have analyzed the sequence specificity of MMP2 and MMP9 by measuring the hydrolysis rate of over 50 oligopeptides. These and other studies[14] have revealed that distinct octapeptides show a high degree of substrate specificity for MMP2 and MMP9. For our studies we selected the following two octapeptides:

	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4
1a	Gly	Pro	Leu	Gly–	Ile	Ala	Gly	Gln
2a	Gly	Pro	Gln	Arg–	Ile	Ala	Gly	Gln

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1a is cleaved efficiently at the P_1–P'_1 bond (290–330% relative cleavability according to Netzel-Arnett et al.[13]) in contrast to **2a** (<5% relative cleavability[13]).

The synthetic route for preparing the maleimide doxorubicin octapeptide derivatives with **1a** and **2a** is depicted in Scheme 1.

The maleimide octapeptide derivatives were custom-made by Bachem, AG (Bubendorf, Switzerland), by synthesizing the octapeptide on a solid phase and introducing maleimidoacetic acid[15] in the final step at the N-terminal position of the octapeptide. The formed products **1b** and **2b** were cleaved from the resin and purified by reverse phase HPLC. Compounds **1b** and **2b** were subsequently reacted for 18 h with one equivalent doxorubicin hydrochloride in dimethylformamide in the presence of one equivalent 1-hydroxybenzotriazole and two (for **1b**) or four (for **2b**) equivalents 4-methylmorpholine and either 1.2 (for **1b**) or two (for **2b**) equivalents of *N,N'*-diisopropylcarbodiimide (DIPC) as the coupling agent. DMF was removed in high vacuo and the maleimide doxorubicin octapeptide derivatives **1c** and **2c** were isolated through chromatography on silica gel (EE/MeOH 2:1 for **1c** and MeOH/0.1% CF_3COOH for **2c**) and their identity confirmed by mass spectrometry (MALDI-TOF): m/z 1412 (K^+ salt adduct of **1c**), m/z 1431 (M^+ of **2c**).

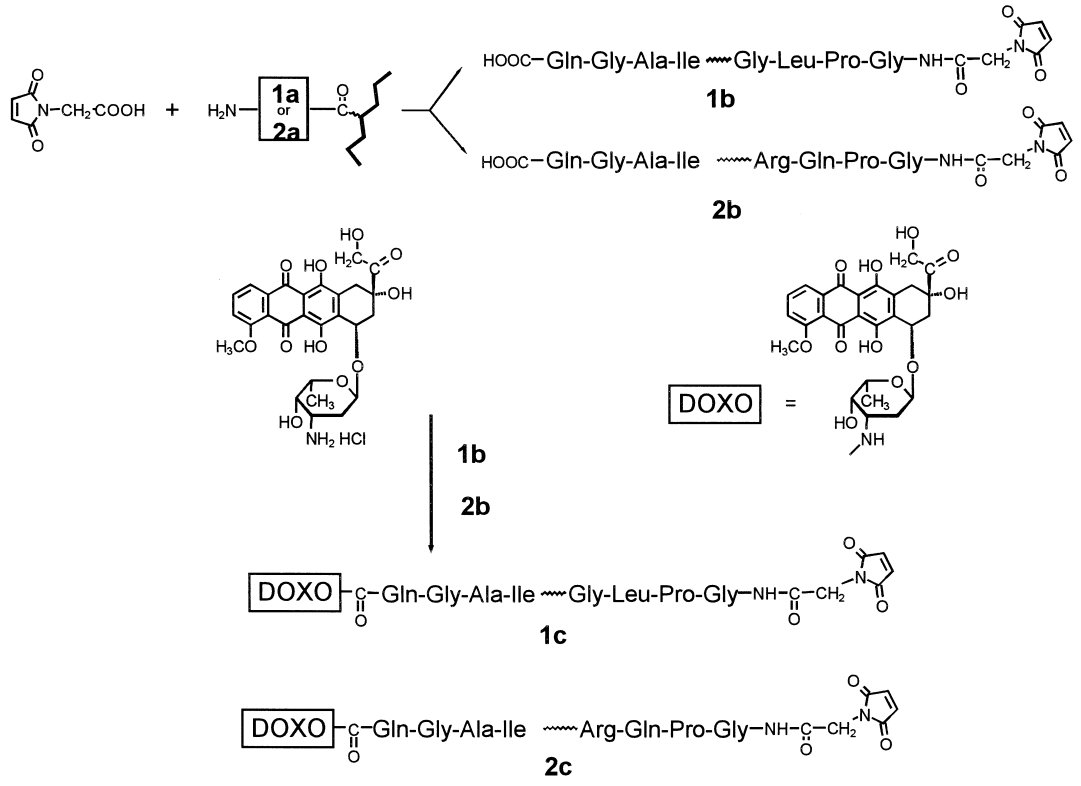
For preparing the albumin conjugates, **1c** and **2c** were coupled to the reduced form of human serum albumin according to Scheme 2.

Commercially available albumins, such as the employed HSA from Dessau Pharma, FRG, is a mixture of mercaptalbumin and nonmercaptalbumin containing approximately 20–60% free sulfhydryl groups per molecule albumin due to the fact that the cysteine-34 position is blocked by sulfhydryl compounds such as cysteine, homocysteine or glutathione.[10,16,17] We have therefore developed a procedure[18] of selectively reducing HSA with suitable agents, such as dithiothreitol (Cleland's reagent), in a first step so that approximately one sulfhydryl group per molecule albumin can be determined using Ellman's reagent; in a second step, **1c** or **2c** were coupled to this reduced albumin and the resulting conjugates (**A-DP1** and **A-DP2**) isolated through size-exclusion chromatography (Sephacryl® HR100).[19]

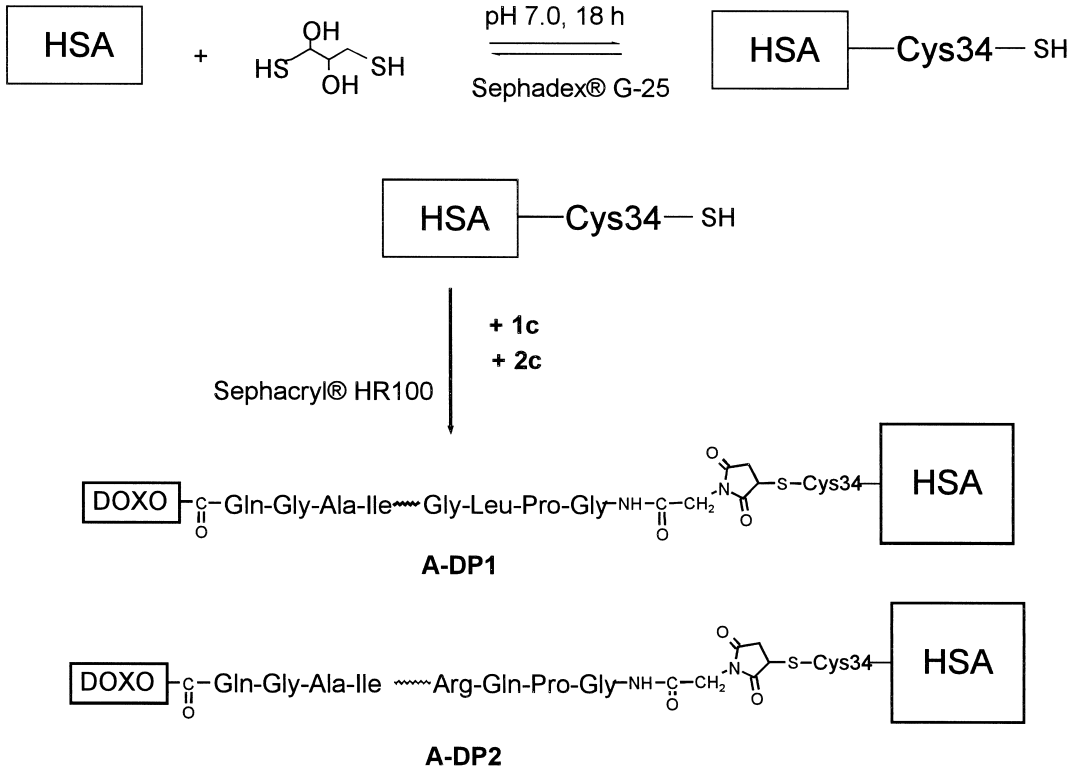
The purity of the conjugates was assessed with an analytical HPLC size-exclusion column (Bio-Sil SEC 250). A typical chromatogram recorded at $\lambda = 495$ nm is shown in Figure 2A for **A-DP1** revealing a distinct main peak eluting at 8.8 min.

Above all, ESI mass spectra of **A-DP1** and **A-DP2** proved to be a valuable tool for evaluating the structure of the conjugates.[20] As an example, the ESI mass spectra of the reduced form of albumin and of **A-DP1** are shown in Figure 3A and B.

The principal mass peak in the reduced form of human serum albumin is observed at 66,449 (see Fig. 3B). In the ESI mass spectrum of the albumin conjugate **A-DP1**, which was prepared with the reduced form of albumin (see Fig. 3B), the principal mass peak is now



Scheme 1.



Scheme 2.

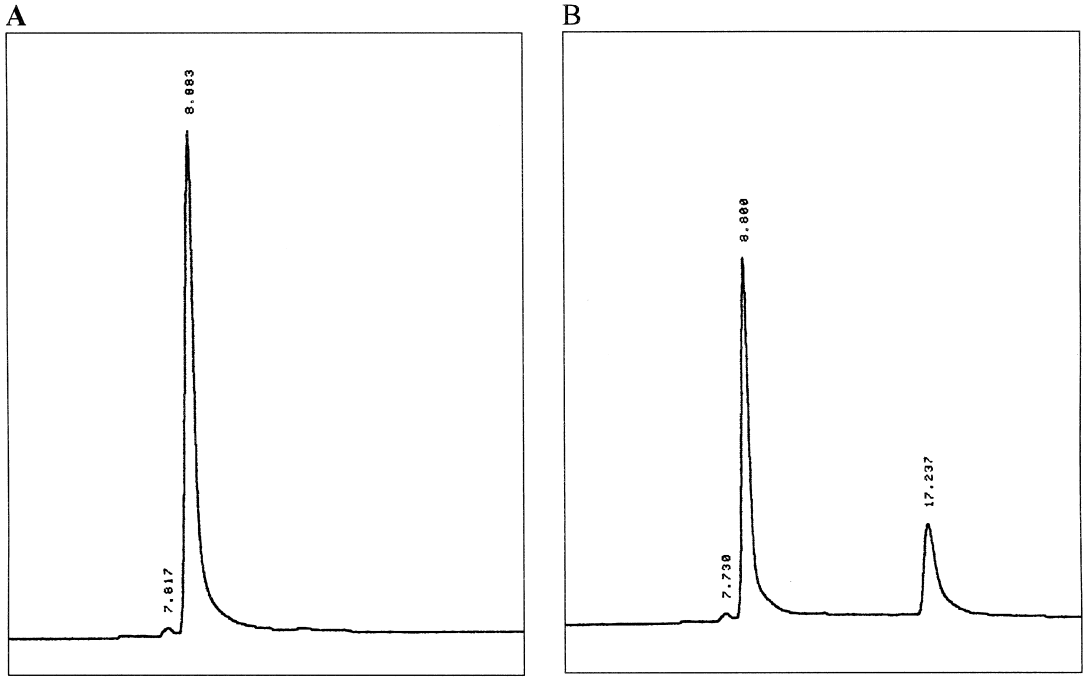


Figure 2. (A) Chromatogram of **A-DP1** on a size-exclusion column recorded at 495 nm: 7.8 min (dimeric albumin conjugate), 8.8 min (monomeric albumin conjugate); (B) chromatogram of **A-DP1** after a 2 h incubation with 1.5 mU activated MMP2. The cleaved doxorubicin tetrapeptide Ile-Ala-Gly-Gln-DOXO is observed at 17.2 min; conditions: (Bio-Sil SEC 250, 300×7.8 mm, mobile phase: 0.15 M NaCl, 0.01 M sodium phosphate, 5 v/v% CH_3CN, pH 7.0, flow: 1.5 mL/min).

seen at 67,829 which corresponds approximately to the sum of the mass of albumin (~66,449) and the mass of the maleimide doxorubicin peptide derivative **1c** (1373). The ESI mass spectrum of **A-DP2** revealed a principal mass peak at 67,920≈66,449 + 1431 (data not shown).

In order to investigate whether and how fast **A-DP1** and **A-DP2** were cleaved by MMP2 and MMP9, 60 μM samples were incubated with activated MMP2 (1.5 mU) or MMP9 (2 mU)[21] and chromatograms recorded at λ = 495 nm on our analytical HPLC size exclusion

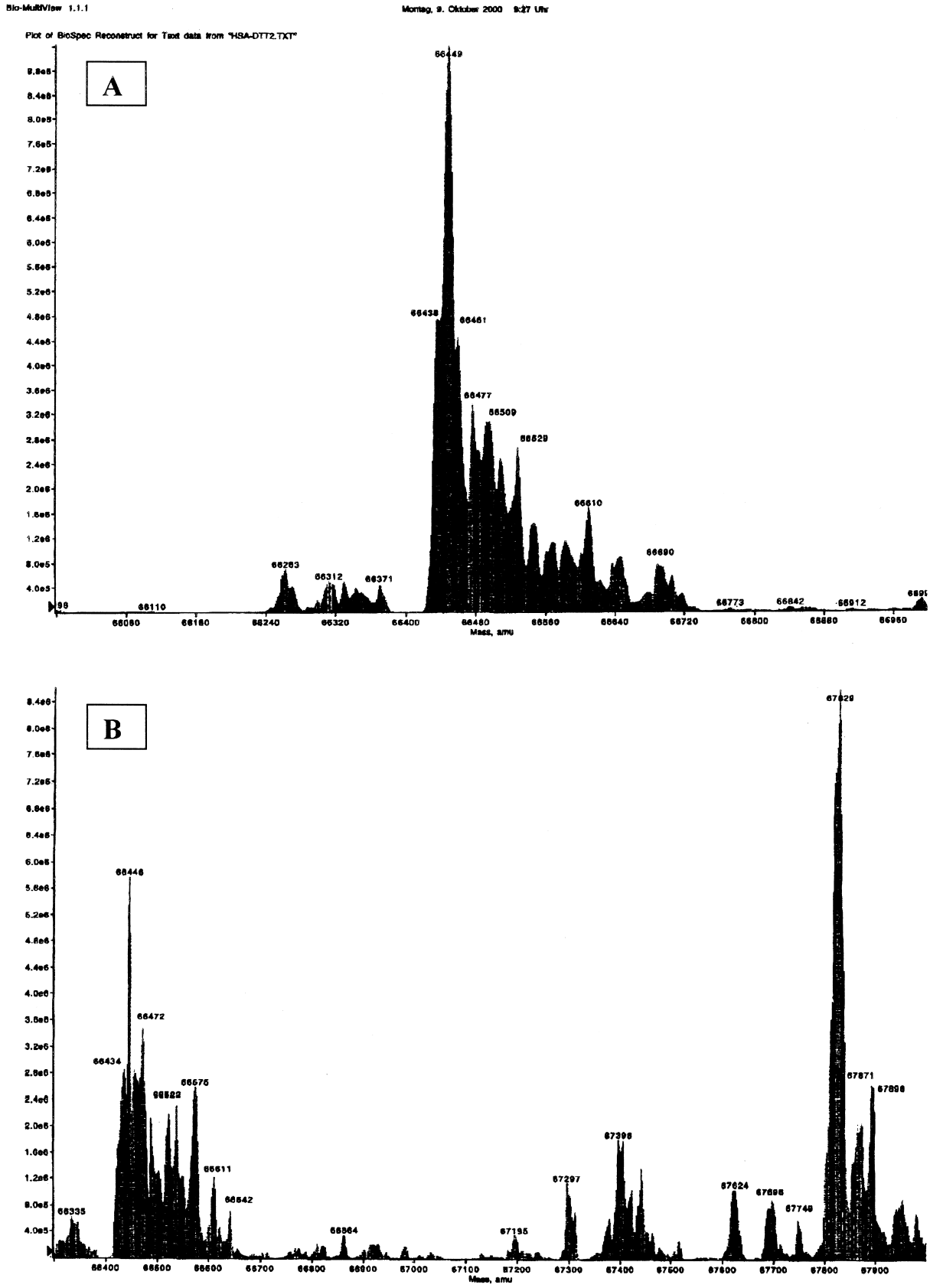


Figure 3. ESI mass spectra of: (A) human serum albumin (from Dessau Pharma, FRG) which was reduced with dithiothreitol so that approximately one sulfhydryl group per molecule albumin could be determined using Ellmann's reagent; and (B) the albumin conjugate **A-DP1** with **1c** which was prepared with the reduced form of albumin.

column. As shown in Figure 2B, after incubation of **A-DP1** with activated MMP2 for 2 h, the initial peak of **A-DP1** at 8.8 min decreases and a new peak is observed at 17.2 min which increases with time (50% cleavage after approximately 5 h). The appearance of this peak was also observed after incubation with activated MMP9 (50% cleavage after a shorter incubation time of ~90 min).

To identify this peak, a 0.9 mL sample of **A-DP1** (300 μM) was incubated with activated MMP2 (from Roche Diagnostics GmbH, Mannheim, FRG) for 4 h and then chromatographed twice on a Sephadex® G-25 column with distilled water. The red band eluting after the albumin fraction was isolated and corresponded to the HPLC peak at 17.2 min (see Fig. 2B). Mass spectrometry of a vacuum-dried sample showed a distinct mass peak at 913.2 corresponding to $[M+1]^+$ of Ile-Ala-Gly-Gln-DOXO.

When activated MMP2 was preincubated with two equivalents of tissue inhibitor of MMP2 (TIMP-2) and then added to **A-DP1**, Ile-Ala-Gly-Gln-DOXO was not cleaved from the conjugate as shown by HPLC demonstrating that the conjugate **A-DP1** is specifically cleaved by active MMP2. Furthermore, incubation studies of **A-DP1** with MMP1 released only negligible amounts of the doxorubicin tetrapeptide. This result indicates that the incorporated peptide sequence in **A-DP1** is selectively recognized and cleaved by MMP2 and MMP9.

In contrast to the results with **A-DP1**, incubation studies with **A-DP2** under identical conditions did not result in a cleavage of the conjugate. This behavior is in agreement with the low rate of cleavability reported by Netzel-Arnett et al. for the incorporated peptide sequence.[13]

In subsequent cell culture experiments, the newly synthesized conjugates **A-DP1**, **A-DP2** and doxorubicin (as the reference compound) were evaluated for inhibitory effects against a murine renal cell carcinoma cell line (RENCA cells) using a BrdU-incorporation assay.[22] In order to assess whether RENCA cells were secreting MMP2 and MMP9 into the cell culture medium, we performed assays of supernatants using the commercially available ELISA kits from Amersham Pharmacia Biotech (BIOTRAK™)[23] for determining the concentration of MMP2 and MMP9 in their active and non-active forms: we found the following concentration in cell culture medium exposed for 2 days to approximately 150,000 RENCA cells/mL: ~6 ng/mL total MMP2, ~3 ng/mL active MMP2, ~1 ng/mL total MMP9, ~0.9 ng/ml active MMP9.

Respective IC_{50} values of the subsequently performed cell culture experiments are summarized in Table 1.

A-DP1, which is cleaved efficiently by MMP2 and MMP9, exhibits an IC_{50} value that is 15-fold lower than the value for **A-DP2**. Although the IC_{50} value for **A-DP1** is higher than for free doxorubicin, it lies in a range (0.2 μM) that is relevant for further preclinical assessment. The decrease in cytotoxic activity of **A-DP1** compared to free doxorubicin indicates that the liberated doxorubicin tetrapeptide Ile-Ala-Gly-Gln-DOXO is less active that doxorubicin per se, which is often the case when peptides are bound to the 3-amino position of doxorubicin as noticed by other researchers.[6] Unfortunately, we are unable to synthesize Ile-Ala-Gly-Gln-DOXO due to the fact that a protected form of Ile-Ala-Gly-Gln could so far not be obtained using solid-phase synthesis. As a consequence, we plan to obtain sufficient amounts of Ile-Ala-Gly-Gln-DOXO after MMP2 cleavage from **A-DP1** as we scale-up our synthesis of the albumin doxorubicin conjugate.

Table 1. IC_{50} values for doxorubicin, **A-DP1** and **A-DP2** against RENCA cells after an incubation time of 48 h using the BrdU-incorporation assay

Compounds	Cytotoxicity IC_{50} (μM)[a]
Doxorubicin	<0.01
A-DP1	0.2
A-DP2	3

[a]Similar results were obtained in a second experiment.

In conclusion, we have demonstrated for the first time that activated MMP2 and MMP9 specifically and rapidly cleave a substrate-specific octapeptide between the anticancer drug doxorubicin and human serum albumin. Furthermore, the substrate specificity of the incorporated peptide sequence correlated with the cleavability and in vitro activity of the doxorubicin albumin conjugates in MMP2 and MMP9 expressing RENCA cells.

At present, we are evaluating the suitability of related peptide sequences using the outlined synthetic approach; in addition, we will examine the therapeutic efficacy of selected conjugates in suitable tumor-bearing models.

Acknowledgements

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19. The method for preparing **A-DP1** and **A-DP2** is described in the following: 3.0 mL of a 1000 μM solution of reduced HSA (sulfhydryl content of 0.95 per molecule of HSA as shown with the Ellmann's test) were reacted with a 1.5-fold excess of either **1c** or **2c** dissolved in 250 μL DMF and the mixture gently shaken at room temperature for 30 min. The sample was centrifuged and the supernatant loaded on a Sephacryl® HR100 column (d = 2.0 cm, l = 20 cm) and the conjugate isolated using a 0.15 M NaCl, 0.004 M sodium phosphate buffer (pH 7.4). The doxorubicin content in the sample was determined using the ε-value for doxorubicin at pH 7.0 ($\varepsilon_{495} = 10{,}650\ M^{-1}\ cm^{-1}$) and adjusted to a standard concentration of 300 μM for the following in vitro studies.
20. Mass spectroscopy (LC-ESI-MS) was performed by A&M GmbH, Bergheim, FRG using a Chrom-Sil Butyl-2FE C4-column (300 Å, 5 μm, 10×2 mm) and a LCQ Classic ion trap (ThermoQuest); capillary voltage: 11–20 V, spray voltage: 4 V.
21. Human MMP2, MMP9 and MMP1 isolated from fibroblasts were purchased from Calbiochem GmbH (Bad Soden, FRG) or Roche Diagnostics GmbH (Mannheim, FRG). Aliquots of the proteases were activated with either APMA (MMP1, MMP2) or trypsin (MMP9) according to the instructions of the manufacturer.
22. The 5-bromo-2′-deoxyuridine cell proliferation kit was obtained from Roche Diagnostics GmbH (Mannheim, FRG), and the assay carried out as previously described by us.[6]
23. The BIOTRAK™ human matrix metalloproteinase ELISA activity assay for MMP2 and MMP9 was purchased from Amersham Pharmacia Biotech, Freiburg, FRG. The immunocapture activity assays are suitable for measuring the concentrations of endogenous (naturally occurring) as well as total (activated by APMA) MMP2 or MMP9. The assays were performed twice according to the instructions of the manufacturer.