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Development of Albumin-binding Doxorubicin Prodrugs that are Cleaved by Prostate-specific Antigen

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Prostate-specific antigen (PSA) is a serine protease that is overexpressed in prostate carcinoma and represents a molecular target for selectively releasing an anticancer agent from a prodrug formulation. In this work, we developed albumin-binding prodrugs with the structures MT-Ser-Ser-Tyr-Tyr−Ser-Gly-DOXO, MT-Asn-Ser-Ser-Tyr−Phe-Gln-DOXO (MT = maleimidotriethyleneglycol acid; DOXO = Doxorubicin) or EMC-Arg-Arg-Ser-Ser-Tyr-Tyr−Ser-Gly-DOXO (EMC = ε-maleimidocaproic acid; X = amino acid). The maleimide Doxorubicin derivatives bound rapidly to the cysteine-34 position of endogenous and exogenous albumin and were efficiently cleaved by PSA at the P_1-P'_1 scissile bond, releasing a respective Doxorubicin dipeptide (Ser-Gly-DOXO or Phe-Gln-DOXO). The derivative containing arginine residues (EMC-Arg-Arg-Ser-Ser-Tyr-Tyr−Ser-Gly-DOXO) exhibited excellent water solubility for intravenous administration. Subsequent biological evaluation was focused on a PSA-negative xenograft model (PC 3) and a PSA-positive xenograft model (CWR22) in order to assess the selectivity of our therapeutic approach. EMC-Arg-Arg-Ser-Ser-Tyr-Tyr−Ser-Gly-DOXO showed no *in vivo* activity in the PSA-negative PC 3 model, but good activity in the CWR22 PSA-positive model that was comparable to Doxorubicin.

Keywords: Doxorubicin; Macromolecular prodrug; Human serum albumin; Prostate-specific antigen (PSA)

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We have recently proposed a macromolecular prodrug strategy for improving the therapeutic index of anticancer agents that is based on two features: (a) rapid and selective binding of thiol-reactive prodrugs to the cysteine-34 position of endogenous albumin after intravenous administration, and (b) acid-sensitive or enzymatic release of the albumin-bound drug at the tumor site [1−3].

Several albumin-binding prodrugs with the anticancer agents Doxorubicin, Camptothecin and platinum(II) complexes have shown promising antitumor activity in preclinical models [1−5]. An acid-sensitive Doxorubicin prodrug, the (6-maleimidocaproyl) hydrazone derivative of Doxorubicin (DOXO-EMCH) has been evaluated in a phase I clinical trial and has demonstrated antitumor efficacy and a fourfold increase in the maximum tolerated dose (MTD) compared to the clinical standard Doxorubicin [6].

The goal of this project was to develop albumin-binding prodrugs of Doxorubicin that are cleaved by the prostate-specific antigen (PSA). PSA is a serine protease that is overexpressed in prostate carcinoma and represents a molecular target for selectively releasing an anticancer agent from a prodrug formulation. Based on our synthetic experience with albumin-binding prodrugs that are cleaved by tumor-associated proteases [3, 7], we set out to develop PSA-specific Doxorubicin prodrugs that bind to the cysteine-34 position of human serum albumin (HSA) after intravenous administration. The general formula of such prodrugs is shown in Figure 1.

The prodrugs consist of a maleimide derivative that can contain an additional water-soluble moiety, a peptide spacer and the anticancer agent Doxorubicin.

When designing the prodrugs, we were aided by the work of other groups that have investigated the sequence specificity of PSA [8−12]. These studies have revealed that distinct oligopeptides, *i.e.* hexa- to decapeptides, show a high

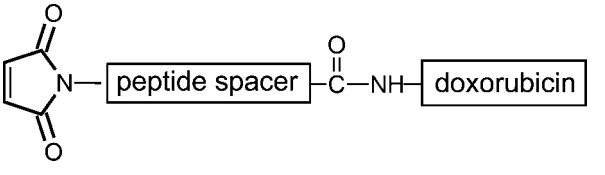

Figure 1. General structure of albumin-binding Doxorubicin prodrugs with incorporated peptide spacers.

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degree of substrate specificity for human PSA. For our studies we selected the three peptide sequences as shown in Figure 2.

The hexapeptide sequence (P_4-P'_2) in these substrates is reported to be cleaved efficiently by PSA at the P_1-P'_1 bond [11, 12]. In this work, we report on the synthesis and antitumor efficacy of albumin-binding Doxorubicin prodrugs incorporating the above-mentioned peptide sequences. Biological evaluation was focused on a PSA-negative model (PC 3) and on PSA-positive models (CWR22, LNCAP) in order to assess the selectivity of our therapeutic approach.

Results

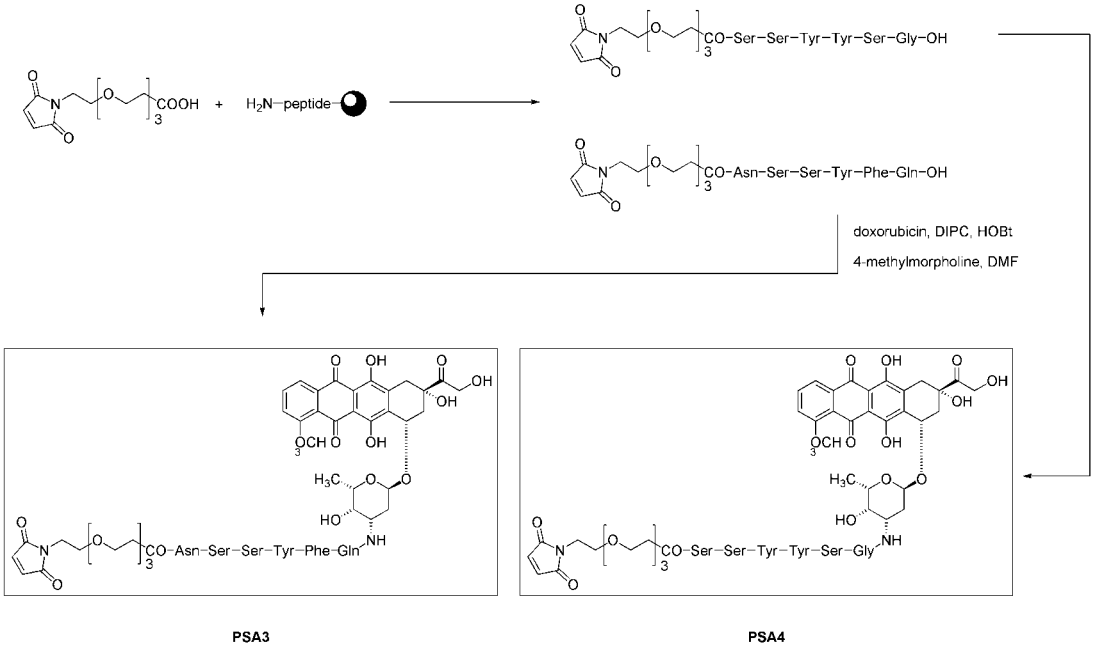
In our previous work, we have synthesized maleimide octapeptide derivatives with Doxorubicin containing maleimidotriethyleneglycol acid that rendered excellent water solubility due to the incorporation of hydrophilic ether bonds [3]. Thus, we used this maleimide derivate for synthesizing the first Doxorubicin derivatives **PSA3** and **PSA4** that incorporated either Asn−Ser−Ser−Tyr−Phe−Gln or Ser−Ser−Tyr−Tyr−Ser−Gly; the synthetic route for preparing **PSA3** and **PSA4** is depicted in Scheme 1.

			Peptide		cleavage site	
P_6	P_5	P_4	P_3	P_2	$P_1 \Downarrow P'_1$	P'_2
		—Asn—	—Ser—	—Ser—	—Tyr—	—Phe—Gln—
		—Ser—	—Ser—	—Tyr—	—Tyr—	—Ser—Gly—
Arg—	—Arg—	—Ser—	—Ser—	—Tyr—	—Tyr—	—Ser—Gly—

Figure 2. Peptide sequences used in the studies.

The maleimide hexapeptide derivatives were obtained by building up the peptide on a solid phase and introducing maleimidotriethylene glycol acid in the final step at the N-terminal position. The maleimide-derivatized peptides were cleaved from the resin and purified by reverse-phase HPLC. Subsequently, the maleimide hexapeptide derivatives were reacted for 18 h with Doxorubicin hydrochloride in DMF in the presence of 1-hydroxybenzotriazole, 4-methylmorpholine, and *N,N'*-diisopropyl carbodiimide (DIPC) as the coupling agent. DMF was removed in high *vacuo*, and the maleimide-Doxorubicin hexapeptide-derivatives **PSA3** and **PSA4** were isolated through chromatography on silica gel. Identity and purity was confirmed by mass spectrometry and reverse-phase HPLC, respectively (see Experimental).

Both, **PSA3** and **PSA4** exhibited water solubility of ~1 mg/mL, which was considerably lower than that observed with analogously constructed maleimide octapeptide derivatives with Doxorubicin (Mal-Gly-Pro-Leu-Gly−Ile-Ala-Gly-Gln-DOXO, Mal = maleimidotriethyleneglycol acid) that are cleaved by matrix metalloprotease 2 (MMP-2), which on the whole did not contain more polar amino acid groups [3]. Nonetheless, we used **PSA3** and **PSA4** for preparing respective albumin conjugates and probing the cleavage profile before designing prodrugs with improved water solubility.



Scheme 1.

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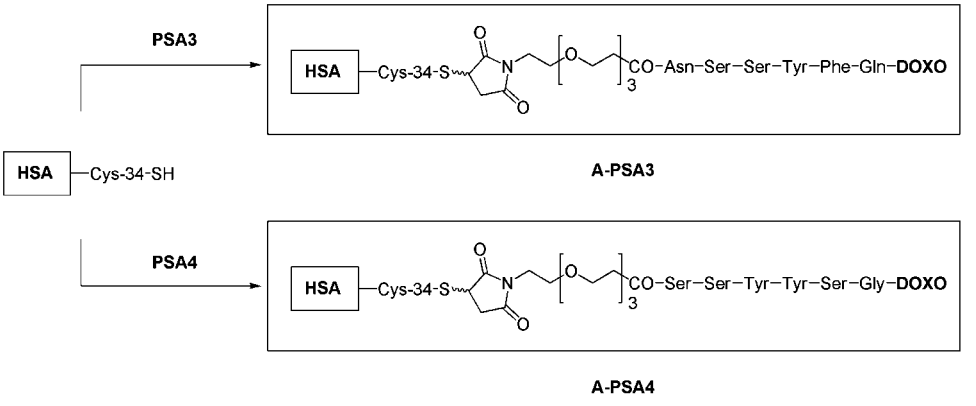

Scheme 2.

For preparing the albumin conjugates, **PSA3** and **PSA4** were coupled to the cysteine-34 position of HSA, thus obtaining **A-PSA3** and **A-PSA4** (Scheme 2).

Cleavage of the albumin conjugates A-PSA3 and A-PSA4 by PSA.

In order to investigate whether and how fast the albumin conjugates **A-PSA3** and **A-PSA4** are cleaved by PSA, the conjugates were incubated with enzymatically active human PSA, and chromatograms were recorded at λ = 495 nm using reverse-phase HPLC. As shown in Figure 3A and B after incubation of **A-PSA3** or **A-PSA4** with PSA for up to 24 h, the initial peak of the albumin conjugate disappears with time and a new distinct peak (R_t ~300 or ~400 s) is observed in each case.

LC-MS detected a mass of 711.2 (Na^+[Ser-Gly-DOXO]) and 842.3 (Na^+[Gln-Phe-DOXO]) for the respective peak corresponding to the cleaved Doxorubicin dipeptide

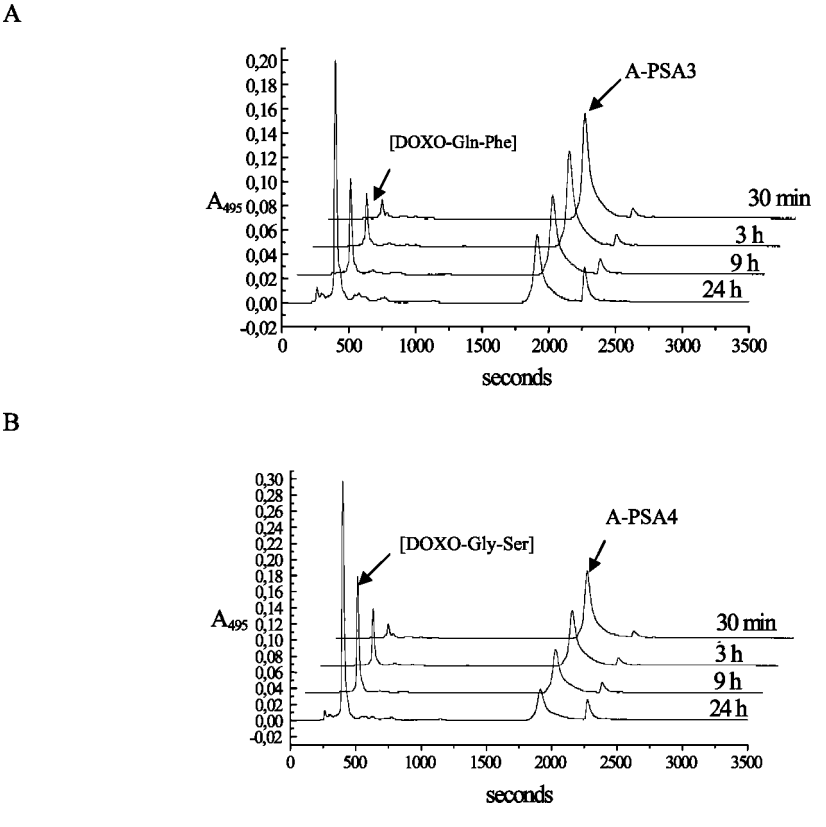

Figure 3. (A, B) Chromatograms of cleavage studies of **A-PSA3** (A) and **A-PSA4** (B) with PSA (20 μg/mL) after 30 min, 3 h, 9 h, and 24 h at 37 °C (pH 7.4). Concentration of the anthracycline was 50 μM. For chromatographic conditions see Experimental.

[Ser-Gly-DOXO] for **A-PSA4** and [Phe-Gln-DOXO] for **A-PSA3**.

Besides the major cleavage products [Gln-Phe-DOXO] and [Ser-Gly-DOXO], a smaller peak eluting behind the main peak for the albumin conjugates was observed, which is unidentified at present.

A-PSA4 demonstrated a faster rate of cleavage than **A-PSA3** (compare Figure 3A and B), and the incorporated peptide sequence [Ser−Ser−Tyr−Tyr−Ser−Gly] was thus chosen for the development of a water-soluble albumin-binding prodrug that could be conveniently administered intravenously.

Development of the water-soluble Doxorubicin prodrug PSA4-Arg

For developing a water-soluble analogue of **PSA4**, we substituted maleimidotriethylene glycol acid through 6-maleimidocaproic acid and introduced two adjacent arginine residues in the sequence (see Figure 4).

PSA4-Arg was synthesized in analogy to **PSA4** (see Scheme 2 and Experimental). In contrast to **PSA4**, **PSA4-Arg** exhibited excellent water solubility (~10 mg/mL) and was consequently selected for further *in vitro* and *in vivo* studies.

Cleavage of the albumin conjugate A-PSA4-Arg by PSA

A further benefit of **PSA4-Arg** over **PSA4** was a faster rate of cleavage by PSA (see Figure 5). Cleavage to [DOXO-Gly-Ser] is essentially complete after 7 h at 37 °C under identical conditions (compare with Figure 3B).

Albumin-binding properties of PSA4-Arg

The HS-group of cysteine-34 of HSA is a unique and accessible functional group of a plasma protein, considering

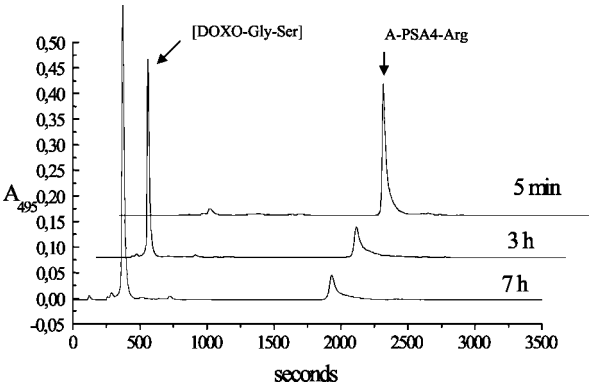

Figure 5. Chromatograms of cleavage studies of **A-PSA4-Arg** with PSA (20 μg/mL) after 5 min, 3 h, and 7 h at 37 °C (pH 7.4). Concentration of the anthracycline was 100 μM. For chromatographic conditions see Experimental.

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 [2]. In order to determine the coupling rate and selectivity of **PSA4-Arg** for this sulfhydryl group, **PSA4-Arg** was incubated with human blood plasma and the samples were subsequently analyzed by reverse-phase chromatography. Chromatograms after an incubation time of 5 and 90 min are shown in Figure 6. Protein components were detected at 280 nm and the anthracycline moiety simultaneously at 495 nm. Binding of **PSA4-Arg** to endogenous albumin is essentially complete after 5 min, with only traces of **PSA4-Arg** eluting at 750 s.

Stability of the albumin conjugate of **PSA4-Arg** in human blood plasma was assessed after **PSA4-Arg** had been incubated at 37 °C with plasma for up to 20 h. Reverse-phase

Structure of PSA4-Arg:

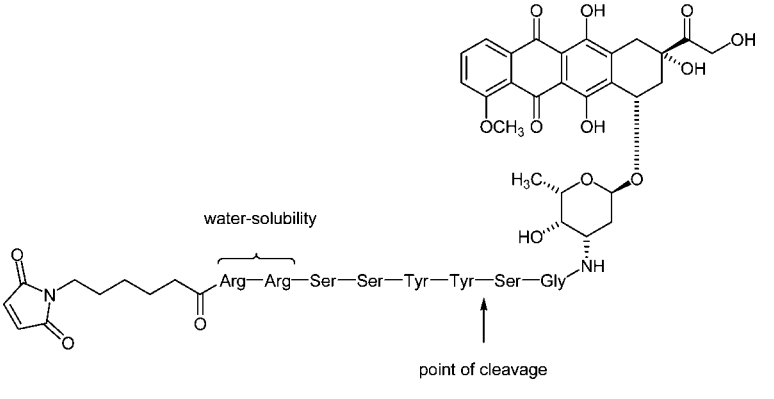

Figure 4. Structure of the water-soluble Doxorubicin derivative **PSA4-Arg**.

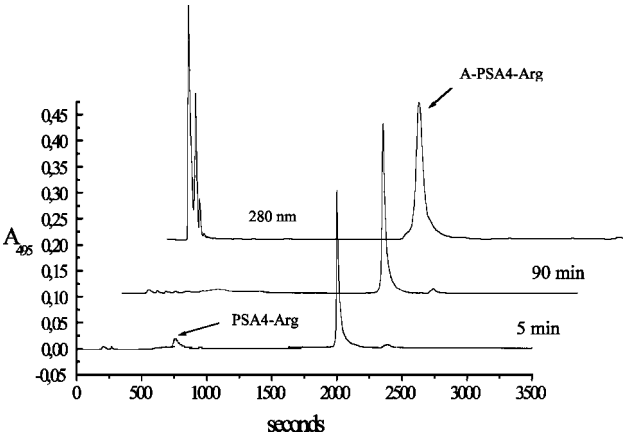

Figure 6. Chromatograms of incubation studies of **PSA4-Arg** with human plasma at 37 °C after 5 and 90 min. Concentration of the anthracycline was 100 μM. For chromatographic conditions see Experimental.

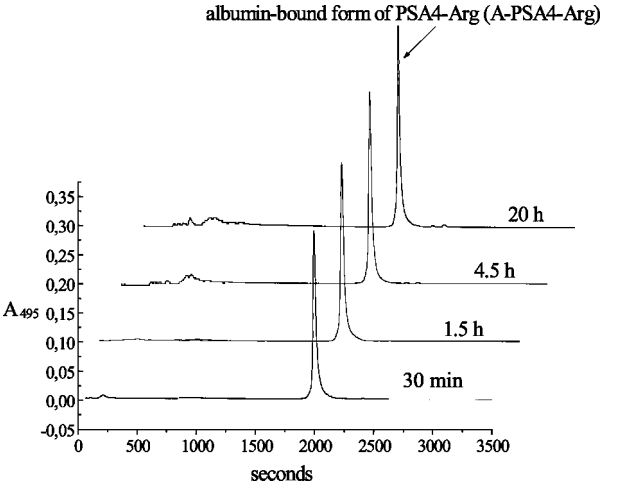

Figure 7. Chromatograms of incubation studies of **PSA4-Arg** with human plasma at 37 °C over 20 h. Concentration of the anthracycline was 100 μM. For chromatographic conditions see Experimental.

HPLC shows that the albumin-bound form of **PSA4-Arg** is highly stable over this time (see Figure 7).

In vitro activity of Doxorubicin, DOXO-Gly-Ser, PSA4-Arg, A-PSA4, and A-PSA4-Arg against LNCAP, CWR22, and PC 3 prostate tumor cells

The antiproliferative activity of **A-PSA4**, **A-PSA4-Arg**, **PSA4-Arg**, free Doxorubicin and of the cleavage product DOXO-Gly-Ser-OH were assessed in the following three tumor cell lines using the MTT assay: LNCAP human prostate carcinoma (PSA positive), CWR22 human prostate carcinoma (PSA positive), and PC 3 human prostate carcinoma (PSA negative).

The IC_{50} values that were obtained after a 72-h cell exposure are summarized in Table 1. The cytotoxicity studies against these three cell lines reveal some salient points:

1. Doxorubicin is the most active compound in all three prostate carcinoma cell lines.
2. **PSA4-Arg** or **A-PSA4-Arg** are an order of magnitude more active than **PSA4**.
3. **PSA4-Arg** in its free form shows similar activity when compared to its albumin-conjugated form.
4. The cleavage product DOXO-Gly-Ser generally shows less cytotoxicity than **PSA4-Arg** or **A-PSA4-Arg** and is considerably less active than free Doxorubicin.
5. **PSA4-Arg**, **A-PSA4-Arg** and **PSA4** are more active in the PSA-positive cell lines, but this is also true for free Doxorubicin.

A critical comparison of the *in vitro* data does not let us conclude that **PSA4-Arg**, **A-PSA4-Arg** and **PSA4** demonstrate selectivity for PSA-positive cell lines. An explanation for this could be the low concentrations of PSA that we found in the cell-conditioned medium of LNCAP cells after 48 h (~28 ng/mL) and CWR22 cells (~10 ng/mL) (no PSA

Table 1. IC_{50} values (μM)† for Doxorubicin, **A-PSA-4**, **A-PSA4-Arg**, **PSA4-Arg** in LNCAP, CWR22 and PC 3 prostate tumor cells.

Compound	IC_{50} value in LNCAP cells μM	IC_{50} value in CWR22 cells μM	IC_{50} value in PC 3 cells μM
Doxorubicin	0.09 ± 0.4	0.17 ± 0.07	1.25 ± 0.64
A-PSA4	6.9 ± 1.2	19 ± 1.4	30 ± 3.6
PSA4-Arg	0.65 ± 0.07	1.1 ± 0.34	3.3 ± 0.67
A-PSA4-Arg	0.88 ± 0.7	1.5 ± 0.14	5.8 ± 1.4
DOXO-Gly-Ser	30 ± 4	17 ± 2	>>60

† IC_{50} values (50% inhibitory concentration) represent the means ± standard deviation of three independent experiments.

was found in cell-conditioned medium of the PSA-negative cell line, PC 3). These levels are not sufficient to cleave large amounts of DOXO-Gly-Ser from the albumin conjugate, which we could demonstrate through incubation studies of **A-PSA4-Arg** with the cell-conditioned medium of these two cell lines (see below). The low levels of PSA in the two PSA-positive cell lines are in striking contrast to the levels that we determined in tumors of CWR22 as well as in native prostate carcinoma specimens of human patients (see below).

Incubation studies of A-PSA4-Arg with cell-conditioned supernatants of LNCAP and PC 3 prostate tumor cells

In order to obtain a picture of the stability and cleavage profile of **A-PSA4-Arg** in supernatants of these biological samples, **A-PSA4-Arg** was incubated with LNCAP and PC 3 cell culture supernatants and samples were analyzed over 24 h using HPLC (see Figure 8A, B).

DOXO-Gly-Ser-OH was not detected in the PSA-negative cell line PC 3, and only small amounts were found in the supernatants of the PSA-positive cell line LNCAP.

In vivo activity of PSA4-Arg in a PSA-negative xenograft model (PC 3) and a PSA-positive xenograft model (CWR22)

In subsequent *in vivo* experiments, the antitumor efficacy of **PSA4-Arg** was evaluated in nude mice in the PC 3 prostate carcinoma model (PSA negative) and the CWR22 prostate carcinoma model (PSA positive) in a strict comparison to free Doxorubicin.

Preliminary toxicity studies in mice showed that the maximum tolerated dose of **PSA4-Arg** was approximately four-

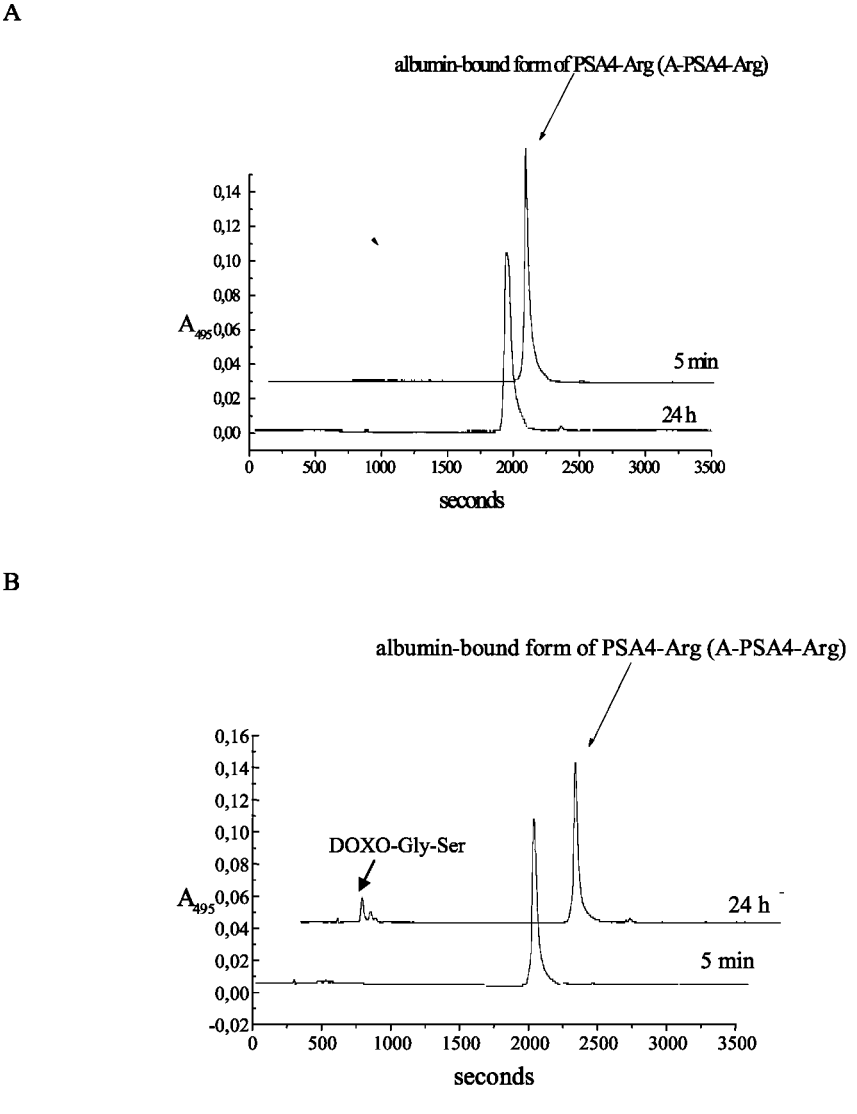

Figure 8. (A, B) Chromatograms of incubation studies of 50 μM **A-PSA4-Arg** in PC 3 cell culture supernatant (A) and in LNCAP cell culture supernatant [c(PSA) ~28 ng/mL)] (B) at 37 °C. For chromatographic conditions see Experimental.

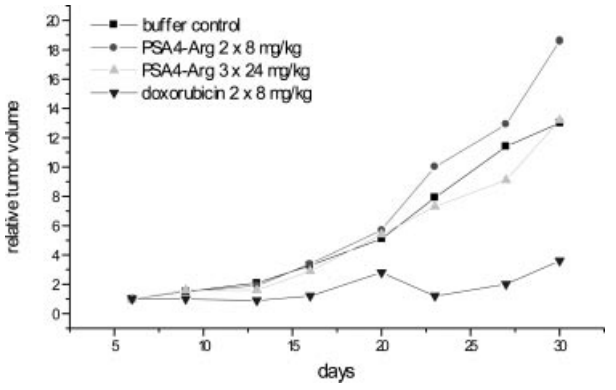

Figure 9. Curves depicting tumor growth inhibition of subcutaneous PC 3 xenografts under therapy with Doxorubicin and **PSA4-Arg**. Dose (i.v.); Doxorubicin: 2 × 13.3 μmol/kg (= 2 × 8 mg/kg Doxorubicin); **PSA4-Arg**: 2 × 13.3 μmol/kg (= 2 × 8 mg/kg Doxorubicin equivalents), 3 × 39.9 μmol/kg (= 3 × 24 mg/kg Doxorubicin equivalents); eight mice per group.

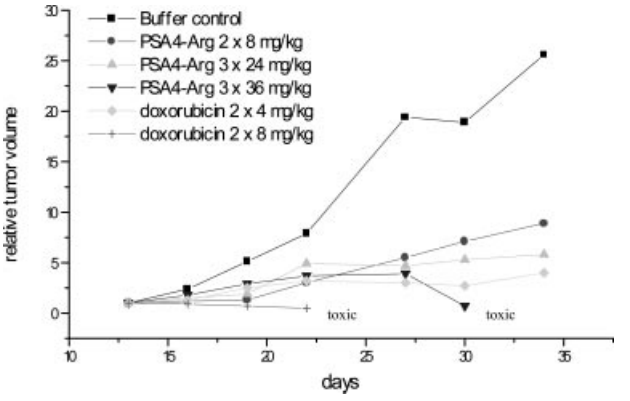

Figure 10. Curves depicting tumor growth inhibition of subcutaneous CWR22 xenografts under therapy with Doxorubicin and **PSA4-Arg**. Dose (i.v.); Doxorubicin: 2 × 6.65 μmol/kg (= 2 × 4 mg/kg Doxorubicin; 2 × 13.3 μmol/kg (= 2 × 8 mg/kg Doxorubicin); **PSA4-Arg**: 2 × 13.3 μmol/kg (= 2 × 8 mg/kg Doxorubicin equivalents), 3 × 39.9 μmol/kg (= 3 × 24 mg/kg Doxorubicin equivalents), 3 × 59.9 μmol/kg (= 3 × 36 mg/kg Doxorubicin equivalents); four mice per group.

fold higher than for free Doxorubicin. Thus, the antitumor efficacy of **PSA4-Arg** was initially compared to that of Doxorubicin in the PC 3 model at the following doses: Doxorubicin: 2 ± 13.3 μmol/kg (= 2 ± 8 mg/kg Doxorubicin) corresponding to the maximum tolerated dose (MTD) of Doxorubicin in nude mice that is routinely used; **PSA4-Arg**: 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 Figure 9 and Table 2. Doxorubicin at its optimal dose of 2 ± 13.3 μmol/kg produced a significant inhibition in tumor growth. In contrast, therapy with **PSA4-Arg** at 2 ± 13.3 μmol/kg and 3 ± 39.9 μmol/kg was well tolerated (no body weight change was noted) but produced a negligible antitumor response in this PSA-negative model.

In contrast, therapy with **PSA4-Arg** in the PSA-positive xenograft model CWR22 at 2 ± 8 mg/kg and 3 ± 24 mg/kg Doxorubicin equivalents produced a good antitumor response (see Figure 10 and Table 3). A dose of 3 ± 36 mg/kg Doxorubicin equivalents was toxic, however. Doxorubicin was tested at two doses: 2 ± 8 mg/kg, which is the dose that is used routinely in nude mice models, and a lower dose of 2 ± 4 mg/kg, since for models such as CWR22, NOD/SCID mice have to be used for successful growth. These mice are more susceptible to cytotoxic therapies compared with nude mice. Indeed, therapy with Doxorubicin at the dose of 2 ± 8 mg/kg proved to be toxic, with all animals dying within 21 days (see Figure 10). In contrast, 2 ± 4 mg/kg produced a good antitumor response, but also a high reduction in body weight (see Table 3).

In order to explain the therapeutic differences of **PSA4-Arg** in the PSA-negative and PSA-positive xenograft model, we first determined the PSA concentrations in PC 3 and CWR22 tumors as well as in three samples of native human prostate carcinoma. In the PC 3 tumors, we were unable

Table 2. Antitumor activity of Doxorubicin and **PSA4-Arg** against human PC 3 prostate xenografts.

Compound	Dose† mg/kg	Treatment day	Mortality	Body weight change,% days 6−16	Optimum T/C %
Buffer		6, 13	None	+5	
PSA4-Arg	8	6, 13	None	+4	93
PSA4-Arg	24	6, 13, 20	None	−1	80
Doxorubicin	8	6, 13	none	−9	15‡

† Dose refers to Doxorubicin equivalents.
‡ Significant to buffer; statistical analysis was performed with the U-test (Mann−Whitney) with $p < 0.05$.

Table 3. Antitumor activity of Doxorubicin and **PSA4-Arg** against human CWR22 prostate xenografts.

Compound	Dose† mg/kg	Treatment day	Mortality	Body weight change,% days 6−16	Optimum T/C %
Buffer		13, 20		+10	−
PSA4-Arg	8	13, 20		−1	28‡
PSA4-Arg	24	13, 20, 27		−1	24‡
PSA4-Arg	36	13, 20, 27	100%	−8	−
Doxorubicin	8	13, 20	100%	−36	−
Doxorubicin	4	13, 20		−28	27‡

† Dose refers to Doxorubicin equivalents.
‡ Significant to buffer; statistical analysis was performed with the U-test (Mann−Whitney) with $p < 0.05$.

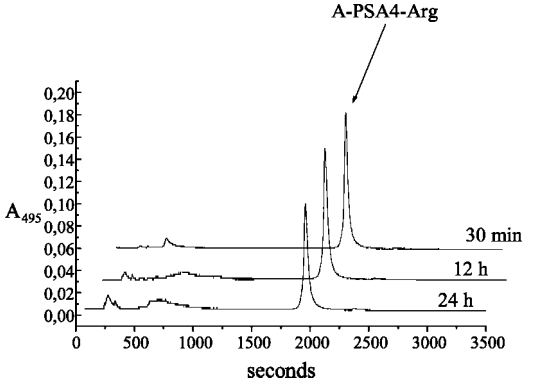

Figure 11. Chromatograms of incubation studies of **A-PSA4-Arg** with PC 3 tissue homogenate at 37°C over 24 h. Concentration of the anthracycline was 50 μM. For chromatographic conditions see Experimental.

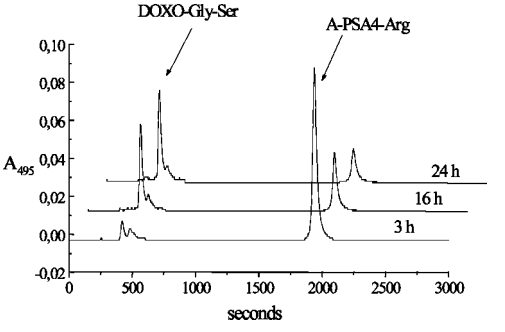

Figure 12. Chromatograms of incubation studies of **A-PSA4-Arg** with CWR22 tissue homogenate (PSA content ~2 μg/mL) at 37°C over 24 h. Concentration of the anthracycline was 25 μM. For chromatographic conditions see Experimental.

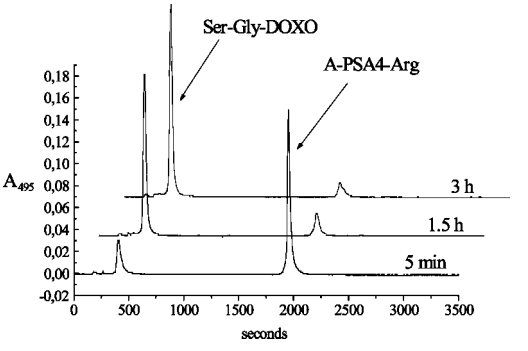

Figure 13. Chromatograms of incubation studies of **A-PSA4-Arg** with human prostate carcinoma tissue homogenate (PSA content ~80 μg/mL) at 37°C over 3 h. Concentration of the anthracycline was 50 μM. For chromatographic conditions see Experimental.
a) IC50 values (50% inhibitory concentration) represent the means ± standard deviation of three independent experiments.

to detect PSA using a microparticle immunoassay; in the CWR22 tumors, a range of 0.2−2.8 μg/mL (0.8−11.2 μg/g) was determined. The highest levels were found in human surgical specimens of human prostate carcinoma with concentrations of 30−500 μg/mL (120−2000 μg/g).

Subsequently, incubation studies were performed with **A-PSA4-Arg** and the respective tissue homogenates. As can be seen in Figure 11, incubation of **A-PSA4-Arg** in homogenates of PC 3 tumors that contained no detectable PSA revealed that the dipeptide DOXO-Gly-Ser was not released over 24 h.

In contrast, incubation studies with homogenates from CWR22 tumors showed a gradual release of DOXO-Gly-Ser over 24 h (Figure 12).

Release of DOXO-Gly-Ser from **A-PSA4-Arg** was considerably faster in the homogenate of a native human prostate tumor that showed levels of ~80 μg/mL, with cleavage being essentially complete within 3 h (see Figure 13). However, further degradation of DOXO-Gly-Ser to Doxorubicin could not be demonstrated in these studies with any of the PSA-positive tumor homogenates.

Discussion

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 enzymes such as β-glucuronidase (HMR 1826) [13], PSA [8, 10, 14−17], to date unidentified peptidases (CPI-0004Na) [18], cathepsin B (PK1 − Doxorubicin conjugated with N-(2-hydroxypropyl) methacrylamide copolymer) [19], or MMP-2 (albumin-binding Doxorubicin prodrug) [3, 20] (reviewed in [20]).

PSA is especially attractive as a target protease because it is primarily expressed in prostate tissue and prostate carcinoma. Besides the albumin-binding prodrug **PSA4-Arg** presented in this paper, two other low-molecular-weight Doxorubicin prodrugs have been developed that aim to exploit PSA as a protease target [10, 14−17]. These Doxorubicin derivatives contain the peptide sequences Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-OH (Mu = morpholinocarbonyl) [L−377,202] and N-glutaryl-(hydoxypropyl)-Ala-Ser-cyclohexaglycyl-Gln-Ser-Leu-OH bound to the amino position of Doxorubicin. Both low-molecular-weight prodrugs were designed to release DOXO-Leu following cleavage of PSA. The dose dependency of L-377,202 and **PSA4-Arg** *in vivo* is similar; *i.e.* a 5−7-fold increase in the maximum tolerated dose (MTD) in mice is noted when compared to Doxorubicin. On the whole, all of the prodrugs have demonstrated good antitumor activity in PSA-positive animal models. Both L-377,202 and **PSA4-Arg** have additionally been tested in PSA-negative models (DuPro-1 and PC 3, respectively) and only marginal activity was seen for the prodrugs in these studies, confirming a lack of cleavage of the incorporated PSA-specific sequence in the prodrugs.

L-377,202 has been evaluated in a phase I study. Nineteen patients with advanced hormone-refractory prostate cancer were treated intravenously with L-377,202 at escalating dose levels from 20 to 315 mg/m^2 of L-377,202 [21]. Dose-limiting grade 4 neutropenia was noted in two of two patients that were treated with a dose of 315 mg/m^2. The recommended dose for phase II studies was 225 mg/m^2, which corresponds to approximately 90 mg/m^2 Doxorubicin equivalents. PK studies demonstrated that L-377,202 was cleaved to N-l-leucyldoxorubicin and Doxorubicin.

In summary, **PSA4-Arg** demonstrated good selectivity when comparing the significant difference in antitumor activity observed in the PSA-negative (PC 3) and the PSA-positive (CWR22) model. Our investigations also emphasize the need to elucidate the cleavage profile of enzymatically cleavable prodrugs in native tumor tissue at physiologically relevant protease concentrations of PSA.

Incubation studies with tumor homogenates reveal, however, that the full potential of **PSA4-Arg** has not been exploited, considering that a less active Doxorubicin dipeptide DOXO-Gly-Ser and not Doxorubicin was released in PSA-positive prostate carcinoma tissues.

Recently, we were able to demonstrate that a MMP-2 specific albumin-binding prodrug initially released a Doxorubicin tetrapeptide [Ile-Ala-Gly-Gln-DOXO] in melanoma tumors and was then degraded to free Doxorubicin by further proteases [3, 16, 17]. This prodrug exhibited enhanced efficacy over Doxorubicin in a melanoma xenograft model.

Thus, our future goal will be to optimize the C-terminal amino acid sequence in **PSA4-Arg** (EMC-Arg-Ser-Ser-Tyr-Tyr−Ser-Gly-DOXO) by substituting Gly for other amino acids that allow free Doxorubicin to be released in PSA-positive prostate tumors as the final cleavage product.

Experimental

Chemicals, materials and spectroscopy

Doxorubicin hydrochloride (MW 580.0) was purchased from Hande Tech Development Co. USA, Inc.; Mal-Ser-Ser-Tyr-Tyr-Ser-Gly-OH, Mal-Asn-Ser-Ser-Tyr-Phe-Gln-OH (Mal = maleimidotriethyleneglycol acid) and 6-maleimidohexanoyl-Arg-Arg-Ser-Ser-Tyr-Tyr-Ser-Gly-OH was custom-made by BACHEM AG (Bubendorf, Switzerland); maleimidotriethylene glycol acid was prepared according to our published procedure[5]; organic solvents: HPLC grade (Merck, Darmstadt, Germany). All other chemicals used were at least reagent grade and obtained from Sigma-Aldrich (Deisenhofen, Germany) or Merck and used without further purification. HSA (20% solution) was purchased from Dessau Pharma, Germany, and contained approximately 30% free thiol groups as assessed with the Ellman·s test. Enzymatically active PSA was purchased from Calbiochem (Bad Soden, Germany). The buffers used were vacuum-filtered through a 0.2-μm membrane (Sartorius, Germany) and thoroughly degassed with argon or nitrogen prior to use. Cell culture media, supplements and fetal calf serum (FCS) were purchased from Bio Whittaker (Serva, Heidelberg, Germany). All culture flasks were obtained from Greiner Labortechnik (Frickenhausen, Germany). ESI-MS spectra were obtained on a Finnigan MAT 312 with associated MAT SS 200 data system, using electron spray ionization at 4.0 kV.ESI. UV/VIS-spectrophotometry was carried out with a double-beam spectrophotometer U-2000 from Hitachi. LC-ESI-MS was performed by bioproof AG (Munich, Germany). PC 3, LNCAP, CWR22 tumor cell lines were obtained from American Type Culture Collection (ATCC). Samples of surgical prostate tumor specimens were supplied by the Department of Urology, University of Freiburg, Germany.

Methods

HPLC for binding and cleavage studies with **PSA3**, **PSA4**, **PSA4-Arg** and the respective albumin conjugates was performed with a BioLogic Duo-Flow System from Biorad (Munich, Germany), which was connected with a Lambda 1000 visible monitor from Bischoff (at λ = 495 nm); UV-detection at 280 nm; column: Waters, 300 Å, Symmetry C18 (4.6 ± 250 mm) with pre-column; 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 μL.

PSA concentration in cell culture supernatants and tumor tissue homogenates was determined nephelometrically with a microparticle immunoassay (MEIA) from Abbott.

*Synthesis and characterization of **PSA3** and **PSA4***

PSA3 or **PSA4** was prepared by reacting the maleimidotriethylene glycol hexapeptide derivative [Mal-Asn-Ser-Ser-Tyr-Phe-Gln-OH] or [Mal-Ser-Ser-Tyr-Tyr-Ser-Gly-OH] with Doxorubicin hydrochloride in DMF using a standard coupling procedure: 58 mg (0.1 mmol) Doxorubicin hydrochloride, 102.8 mg (0.1 mmol) Mal-Asn-Ser-Ser-Tyr-Phe-Gln-OH or 94.6 mg (0.1 mmol) Mal-Ser-Ser-Tyr-Tyr-Ser-Gly-OH, 13.5 mg (0.1 mmol) 1-hydroxybenzotriazole hydrate and 33 µL (30.3 mg, 0.3 mmol) 4-methylmorpholine were dissolved in 20 mL anhydrous DMF; after stirring at +5°C for 15 min, 46.5 µL (37.9 mg, 0.3 mmol) *N,N′*-diisopropyl carbodiimide was added as the coupling agent. After stirring at +5°C for 4 days, DMF was removed by evaporation in high vacuum and the residue dissolved in a minimal amount of chloroform/methanol 3 : 1 (for **PSA3**) or 4 : 1 (for **PSA4**), and the product was purified twice on a silica gel column using chloroform/methanol 3 : 1 or 4 : 1 to afford either 50 mg **PSA4** or 60 mg **PSA4**, respectively, as a red powder after precipitating the combined fractions containing the product with diethyl ether. **PSA3**: Mass (ESI-MS, MW 1553.5): *m/z* 1576 [Na^+ salt adduct], HPLC (495 nm): >98%; **PSA4**: Mass (ESI-MS, MW 1471.5): *m/z* 1494 [Na^+ salt adduct], HPLC (495 nm): > 98%.

*Synthesis and characterization of **PSA4-Arg***

Doxorubicin hydrochloride (50 mg, 0.086 mmol), 100.7 mg (0.086 mmol) 6-maleimidohexanoyl-Arg-Arg-Ser-Ser-Tyr-Tyr-Ser-Gly-OH, 11.6 mg (0.086 mmol) 1-hydroxybenzotriazole hydrate and 37.8 µL (37.8 mg, 0.34 mmol) 4-methylmorpholine were dissolved in 20 mL anhydrous DMF; after stirring at +5°C for 15 min, 39.8 µL (32.5 mg, 0.21 mmol) *N,N′*-diisopropyl carbodiimide was added as the coupling agent. After stirring at +5°C for 4 days, the product was precipitated with diethyl ether, washed three times with 20 mL diethyl ether to afford 130 mg **PSA4-Arg** as a red powder. Mass (ESI-MS, MW 1693.7): *m/z* 1807.6 [TFA salt adduct], HPLC (495 nm): > 97%.

Synthesis of DOXO-Gly-Ser-OH

DOXO-Gly-Ser-OH was prepared by reacting Ser-Gly-OH with trimethylsilyl chloride, diisopropylethylamine and 4-methoxytrityl chloride in DMF using the following procedure: 400 mg (2.47 mmol) Ser-Gly-OH, 653.6 µL (2.1 mmol) trimethylsilyl chloride and 431.1 µL (1.05 mmol) diisopropylamine in 200 mL DMF were heated at 40°C for 4 h until the solution became clear. The solution was left to cool to room temperature and 1273 µL diisopropylamine was added, as well as 800 mg 4-methoxytrityl chloride. Stirring was continued at room temperature for 2 days. The mixture was then kept at +5°C overnight and the formed precipitate was isolated and washed with diethyl ether and dried in vacuum (yield of protected 4-methoxytrityl-Ser-Gly was 400 mg. In a second reaction, 50 mg (0.086 mmol) Doxorubicin hydrochloride, 200 mg 4-methoxytrityl-Ser-Gly, 11.6 mg (0.086 mmol) 1-hydroxybenzotriazole hydrate, and 37.8 µL (0.34 mmol) 4-methylmorpholine were dissolved in 15 mL anhydrous DMF. After stirring at +5°C for 15 min, 39.8 µL (0.21 mmol) *N,N′*-diisopropyl carbodiimide was added as the coupling agent. After stirring at +5°C for 4 days, the product was precipitated with 200 mL diethylether and isolated by centrifugation. The red product was dissolved in 4 mL methanol, and the protective group was removed by adding 1.6 mL dichloroacetic acid for 5 min and subsequent precipitation with 30 mL diethylether (yield 122 mg). Mass [ESI-MS, MW 687.6): *m/z* 686.8 ([M^+-1]), HPLC (495-nm): > 95%.

*Synthesis of the albumin conjugates of **PSA3, PSA4** and **PSA4-Arg***

The albumin conjugates of **PSA3**, **PSA4** and **PSA4-Arg** were prepared by reacting them with commercially available albumin, which contains approximately 30% mercaptalbumin, and isolating the conjugate through size-exclusion chromatography: 10 mL HSA was incubated at 37°C for 1 h with 12.1 mg **PSA3**, with 13.2 mg **PSA4** or with 13.4 mg **PSA4-Arg**. The albumin conjugate was obtained by subsequent size-exclusion chromatography (Sephacryl® HR100; buffer 0.004 M sodium phosphate, 0.15 M NaCl, pH 6.5). 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}) and was adjusted to 400 ± 50 µM by concentrating the sample with CENTRIPREP®-10 concentrators from Amicon, Germany (4°C and 4500 rpm). Samples were kept frozen at −78°C and thawed prior to use.

Incubation studies of the albumin conjugates of PSA3, PSA4 and PSA4-Arg with PSA

Samples (50 or 100 µM) of the albumin conjugates of **PSA3**, **PSA4** or **PSA4-Arg** were incubated with human PSA (20 µg/mL) and chromatograms recorded at λ = 495 nm using reverse-phase HPLC at the time points stated in the figures.

Incubation studies with human plasma

PSA4-Arg was added to human blood plasma (EDTA stabilized) pre-incubated at 37°C at a final concentration of 100 µM, and the samples were incubated for the time periods stated in the figures at 37°C; a 50-µL sample was analyzed by HPLC.

Preparation of prostate tumor tissue homogenates

For obtaining prostate carcinoma tissue homogenates, all steps were carried out on ice where possible. Tissues of PC 3 and CWR22 xenograft tumors as well as tissues from three native human prostate tumors (received from the Department of Urology, Freiburg) were cut into small pieces, and 200-mg samples were transferred in a 2-mL Eppendorf tube to which was added 800 µL homogenate buffer (50 mM Tris-HCl buffer, pH 7.4, containing 1 mM monothioglycerol). 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 until use.

*Incubation studies of the albumin conjugate of **A-PSA4-Arg***

Incubation studies of the albumin conjugate of **A-PSA4-Arg** were performed with supernatants and tissue homogenates of PSA-negative (PC 3) and PSA-positive prostate carcinoma (CWR22, LNCAP, human prostate cancer samples). The albumin conjugate of **PSA4-Arg** was incubated with the biological samples at 37°C at a final concentration of 25 or 50 µM, and chromatograms were recorded at 495 nm using reverse-phase HPLC at the time points stated in the figures.

Cell culture

PC 3 (PSA negative), LNCAP (PSA positive) and CWR22 (PSA positive) prostate carcinoma cells were grown as monolayer cultures in cell culture flasks in RPMI 1640 culture medium without phenol red, supplemented with 10% heat-inactivated FCS, 100 µg/mL glutamine, 100 U/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. For subculture or experiments, cells growing as monolayer cultures were released from the tissue flasks by treatment with 0.05% trypsine/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 [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay was performed in analogy to a literature protocol [21]. Briefly, approximately 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 pre-incubated with various drug concentrations (0.001−60 μM) 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, UK) at a wavelength of 570 nm. Four separate cultures were determined per concentration. Results are shown as means ± standard deviation of three independent experiments ($n = 3$).

Xenograft experiments

The antitumor efficacy of **PSA4-Arg** and Doxorubicin was investigated in a PSA-negative model (PC 3) and in a PSA-positive xenograft model (CWR22); male Ncr nude mice were used for the PC 3 model and male NOD/SCID mice for the CWR22 transplants. NOD/SCID mice were necessary because of the insufficient growth of CWR22 in nude mice. The mice were held in laminar flow shelves under sterile and standardized environmental conditions (25 ± 2°C room temperature, 50 ± 10% relative humidity, 12-h light-dark rhythm). They received autoclaved food and bedding (ssniff, Soest, Germany) and drinking water *ad libitum*.

PC 3 and CWR22 prostate carcinomas were xenografted subcutaneously as fragments according to a literature protocol [22]. Mice were randomly distributed to the experimental groups. When the tumors had reached a palpable size, treatment was initiated. Mice were treated intravenously in a weekly schedule with 10 mM sodium phosphate and 5% d-glucose (pH 6.4), **PSA4-Arg** or Doxorubicin (2.0 mg/mL). **PSA4-Arg** 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; for doses and schedules see Figs. 8 and 9 and Tables 2 and 3. Four to eight mice were used per group for the experiments.

Tumor size was measured twice weekly with a calliper-like instrument in two dimensions. Individual tumor volumes (V) were calculated by the formula $V = (\text{length} \pm [\text{width}]^2)/2$ and related to the values on the first day of treatment (relative tumor volume, RTV). At each measurement day, treated/control values (T/C) were calculated as percentage for each experimental group; the optimum (lowest) values obtained within 4 wk after treatment were used for evaluating the efficacy of the compounds, and optimum T/C-values are presented in Tables 2 and 3. The body weight of mice was determined twice weekly and related to the body weight on the first day of treatment (body weight change, BWC). Statistical analysis was performed with the U-test (Mann−Whitney) with $p < 0.05$.

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