Synthesis and biological evaluation of an albumin-binding prodrug of doxorubicin that is cleaved by prostate-specific antigen (PSA) in a PSA-positive orthotopic prostate carcinoma model (LNCaP)

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The prostate-specific antigen (PSA) is a serine protease that is over-expressed in prostate carcinoma and represents a molecular target for selectively releasing an anticancer agent from a prodrug formulation. We have recently investigated a macromolecular prodrug strategy for improved cancer chemotherapy based on 2 features: (i) rapid and selective binding of thiol-reactive prodrugs to the cysteine-34 position of endogenous albumin after intravenous administration, and (ii) enzymatic release of the albumin-bound drug at the tumor site (Mansour et al., Cancer Res 2003, 63, 4062–4066). In this work, we describe an albumin-binding prodrug, EMC-Arg-Ser-Tyr-Tyr—Ser-Arg-DOXO [EMC: e-Mal-emicidocapoic acid; DOXO = doxorubicin; X = amino acid] that is cleaved by PSA. Because of the incorporation of 2 arginine residues, the prodrug exhibited excellent water-solubility and was rapidly and selectively bound to endogenous albumin. Incubation studies with PSA and tumor homogenates from PSA-positive tumors (LNCaP) demonstrated that the albumin-bound form of the prodrug was efficiently cleaved by PSA at the P1–P2 scissile bond releasing the doxorubicin dipeptide H-Ser-Arg-DOXO, which was further degraded to doxorubicin as the final cleavage product. In cell culture experiments, the prodrug was ~100-fold less active against LNCaP cells than the free drug. In contrast, in a mouse model of human prostate cancer using luciferase transduced LNCaP cells orthotopically implanted in SCID mice, the prodrug showed enhanced antitumor efficacy when compared to doxorubicin. Doxorubicin treated at a dose of 2 × 4 mg/kg caused significant weight loss and mortality (−25%), and did not result in a significant antitumor response at the end of the experiment. The prodrug at 3 × 12 mg/kg doxorubicin equivalents, however, was well tolerated and induced a significant reduction in tumor size of 62% (±25%, *p = 0.003) as well as a decrease of the metastatic burden in the lungs as detected in luciferase assays (−50%, SD ± 115%, *p = 0.038).

Key words: doxorubicin; macromolecular prodrug; human serum albumin; PSA; orthotopic animal model; LNCaP; luciferase; in vivo bioluminescence

Hormone-refractory prostate cancer responds unfavorably to chemotherapy. The best results to date are achieved with Taxotere. To improve prostate cancer therapy, tumor-specific delivery of anticancer agents to the primary tumor and metastases is a goal worth pursuing.

For selectively releasing anticancer agents, the prostate-specific antigen (PSA) is especially attractive as a target protease because it is solely expressed in prostate tissue and prostate carcinoma in prostate cancer patients with high levels up to mg/g present in human prostate carcinoma. PSA is a serine protease that belongs to the kallikrein gene family with chymotrypsin-like activity that is involved in the hydrolytic processing of semenogelins (cleavage of the seminal fluid proteins semenogelin I and II), which is required for liquefaction of seminal fluids. Over-expression of PSA has primarily been demonstrated in prostate carcinoma and at low levels in breast cancer.

We have recently developed an albumin-binding prodrug of doxorubicin that is cleaved by PSA, i.e., EMC-Arg-Arg-Ser-Ser-Tyr-Tyr—Ser-Gly-DOXO [EMC = 6-malemidocapoic acid; DOXO = doxorubicin]. Our drug targeting strategy is based on 2 features: (i) in situ binding of a thiol-binding prodrug to the cysteine-34 position of circulating albumin after intravenous administration with subsequent accumulation of the drug albumin conjugate in the tumor because of passive targeting; (ii) release of the albumin-bound drug at the tumor site because of the incorporation of a cleavable bond between the drug and the carrier.

EMC-Arg-Arg-Ser-Ser-Tyr-Tyr—Ser-Gly-DOXO is cleaved efficiently by PSA between Tyr and Ser releasing the doxorubicin dipeptide H-Ser-Gly-DOXO. Two arginine residues were introduced into the peptide linker to render the prodrug water-soluble. The prodrug 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. Incubation studies with CWR22 tumor homogenates revealed, however, that the full potential of EMC-Arg-Arg-Ser-Ser-Tyr-Tyr—Ser-Gly-DOXO had not been exploited considering that a less active doxorubicin dipeptide DOXO-Gly-Ser and not doxorubicin was released in PSA-positive prostate carcinoma tissues.

The goal of the present work was to optimize the structure of this prodrug that would allow doxorubicin to be released in prostate tumors as the final cleavage product. In recent work, we discovered that among a spectrum of doxorubicin amino acid derivatives N-(L-arginine)doxorubicin (H-Arg-DOXO) was the only compound that was efficiently cleaved to doxorubicin in tumor homogenates at pH 7.4. We reasoned that replacing Gly by Arg at the C-terminal position could behave in a similar way and also release doxorubicin through the action of other proteases in tumor homogenates. Consequently, we developed the doxorubicin prodrug EMC-Arg-Ser-Ser-Tyr-Tyr—Ser-Arg-DOXO (PSA5) that is shown in Figure 1.

To test novel antitumor agents in a setting that reflects the human disease better than subcutaneous models, we have developed orthotopic models for pancreas and kidney cancer using luciferase marked tumor cells. Bioluminescence mediated detection and observation of the tumors over the period of a treatment allows the randomization of tumor bearing animals, exclusion of animals without or with aberrantly growing tumors as well as the monitoring of drug effects on the tumor. For our study, the PSA-positive LNCaP prostate tumor cell line, transduced with a construct expressing a Luciferase–Neomycin resistance fusion protein, was implanted orthotopically into the prostates of SCID mice. When implanted orthotopically, this cell line metastasizes to local lymph nodes, lungs and other organs. Thus drug effects on metastases can be investigated as well.

In this work we describe the synthesis of the novel prodrug, its cleavage profile and in vitro and in vivo activity in comparison to free doxorubicin.

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Results

Chemistry

The albumin-binding prodrug PSA5 (see Fig. 1) was synthesized from doxorubicin, using a Fmoc-based strategy. H-ArgDOXO was obtained by reacting doxorubicin with Fmoc-Arg-OH in anhydrous N,N-dimethylformamide (DMF) in the presence of N-ethylisodisopropylamine (DIEA) and O-(azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) as the coupling agent, removing the Fmoc-group with piperidine and isolating the product with diol column chromatography. In a next step, a serine residue was introduced by reacting H-Arg-DOXO with commercially available Fmoc-Ser-OH in anhydrous DMF in the presence of DIEA and HATU. The protecting group was removed with piperidine and H-Ser-Arg-DOXO was isolated through chromatography on a diol column.

In a final step, EMC-Ag-Ser-Tyr-Tyr-OR was coupled to H-SerArg-DOXO in anhydrous DMF in the presence of N,N-diisopropylcarbodiimide, 1-hydroxybenzotriazole hydrate and 4methylmorpholine in a one-step procedure. PSA was purified through size-exclusion chromatography on Sephadex™ LH-20 followed by reverse phase chromatography (C18 column). The lyophilized red product was characterized by mass spectrometry and purity determined by HPLC. PSA5 showed excellent water-solubility with up to 10 mg/mL in buffer solutions in the pH range of 6.0–7.0.

Binding of PSA5 to endogenous albumin in human plasma

To determine the coupling rate and selectivity of PSA5 for endogenous albumin, the prodrug was incubated with human blood plasma at 37°C, and the samples were subsequently analyzed by reverse phase chromatography. Chromatograms after an incubation time of 5 min, 4, and 24 hr are shown in Figure 2. Protein components were detected at 254 nm and the anthracene moiety simultaneously at 495 nm. Figure 2 demonstrates rapid and selective binding of PSA5 to endogenous albumin after 5 min with a distinct single peak at the retention time of ~32 min and with only traces of PSA5 eluting after ~11 min.

The stability of the albumin conjugate of PSA5 in human blood plasma was assessed after PSA5 had been incubated at 37°C with plasma for 5 min, 4, and 24 hr. HPLC chromatograms in Figure 2 shows that the albumin-bound form is relatively stable over 24 hr with ~18% degradation after 24 hr.

To demonstrate that the cysteine-34 position of albumin plays an important role in the coupling step, analogous HPLC experiments were carried out, in which human plasma was preincubated with an excess of EMC (ε-maleimidocaproylic acid) prior to incubation with PSA5 (see Fig. 2). In this experiment, a low degree binding to HSA was observed after incubating plasma with PSA5.

Cleavage of the albumin-conjugate of PSA5 by PSA and in LNCaP tumor homogenates

To investigate whether and how fast the albumin conjugate of PSA5 is cleaved by PSA, the conjugate was incubated with enzymatically active PSA and chromatograms recorded at 495 nm using reverse phase HPLC. Fast and distinct cleavage to H-Ser-Arg-DOXO eluting at 5.4 min was observed after incubating the albumin-conjugate of PSA5 with PSA after 2 hr (see Fig. 3). After 4 hr of incubation, cleavage to H-Ser-Arg-DOXO was essentially complete.

To assess, whether H-Ser-Arg-DOXO is further degraded in prostate tumors, we incubated this doxorubicin dipeptide with homogenates from LNCaP tumors (PSA levels ~5.6 µg/g). Chromatograms after 0, 2 and 5 hr are shown in Figure 4 demonstrating that H-Arg-DOXO and doxorubicin are formed in a time-dependent manner.

When the albumin conjugate of PSA5 was incubated with homogenates from LNCaP tumors, cleavage to H-Arg-DOXO and doxorubicin was detected (see Fig. 5).

In vitro activity of doxorubicin and the albumin-conjugate of PSA5 against the PSA-positive prostate cancer cell line LNCaP LN

LNCaP LN is the PSA-expressing prostate cancer cell line LNCaP with a stably integrated Luciferase–Neomycin (LN) resistance fusion gene cassette. Cells expressing this fusion protein can be selected by G418, and if injected in vivo through bioluminescence imaging. Because of the activation of PSA5 by PSA cleavage, the level of PSA expression by the LNCaP LN cells was measured over a period of 96 hr. It was correlated with the number of cells and the expression of luciferase.

When ~200,000 cells were plated in a 6-well plate, the cells grew exponentially for 72 hr; at this time-point the proliferation rate ceased because of cell density (Fig. 6a, light and dark grey bars). PSA accumulated over the whole 96-hr period, whereas the expression of luciferase decreased with the slow-down of proliferation (Fig. 6a, red and blue bars). PSA levels secreted in the PSA-positive LNCaP cell culture medium was measured using microparticle immunoassay (~100.000 cells/mL: ~21 ng/mL after 24 hr, ~75 ng/mL after 48 hr, 150 ng/mL after 72 hr and ~260 ng/mL after 96 hr).

Next, the sensitivity of the LNCaP LN cell line to free doxorubicin as well as the prodrug was tested in proliferation assays. After an incubation of the cells in the presence of either drug for 72 hr, alamar blue was added to the cells to measure mitochondrial activity. After 3 hr, cells were harvested, and the alamar blue signal was measured. Subsequently, cells were lysed and the luciferase activity of the lysate determined.

Doxorubicin very efficiently killed the LNCaP cells in vitro with IC_{50} values of 70 and 140 nM, respectively. The prodrug, on the other hand was ~100-fold less active with IC_{50} values of 8 and 32 µM (Fig. 6b).

Antitumor activity of PSA5 and doxorubicin in an orthotopic prostate tumor model (LNCaP)—In vivo results

The in vivo efficacy of the prodrug was compared to free doxorubicin in a mouse prostate cancer model using LNCaP LN cells orthotopically implanted into the prostate of SCID mice. This technique allows the tumor cells to grow in an environment related to their origin and thus mimics tumor–stromal cell interactions better than subcutaneous models. Furthermore, since lung metastases are reproducibly observed, antimetastatic activity of test compounds can be evaluated. Bioluminescence measurements using the stably integrated luciferase gene were used to monitor the growth of the otherwise undetectable tumors.

Tumor cells were detected as early as 2 days after implantation (Fig. 7c). However, since this original activity usually decreases during the establishment of the tumor, bioluminescence signals from day 10 were used to randomize the animals into treatment groups (Fig. 7a). The take rate was around 65%, and mice without detectable tumors were excluded from the study.

Based on preliminary toxicity studies, 12 mg/kg doxorubicin equivalents for the prodrug were compared to 4 mg/kg of the free drug. Animals were injected with the drugs once weekly for a pe-
FIGURE 2 – Chromatograms of incubation studies of human plasma after 5 min, 4 and 24 hr with PSA5 at 37°C and chromatograms of incubation studies of human plasma preincubated with an excess of 6-maleimidocaproic acid (EMC) for 2 hr and subsequent incubation with PSA5 after 15 min at 37°C. Concentration of the anthracycline was 250 μM. Chromatographic conditions: see “Experimental” Section.

FIGURE 3 – Chromatograms of incubation studies with the albumin conjugate of PSA5 in the presence of human PSA after 0, 2, 4 and 24 hr at 37°C. The concentration of the albumin conjugate of PSA5 was 110 μM.
period of 3 weeks. The weight of the animals was checked 3 times weekly to monitor the well-being of the animals. After the second injection with free doxorubicin, animal weights in this group decreased drastically, and 2 animals from this group died (Fig. 7b); as a consequence the treatment had to be stopped. No toxicity was observed with PSA5 and 3 × 12 mg/kg could be administered without a decrease in body weight.

Using bioluminescence measurements, the growth of the tumor was checked once weekly. Doxorubicin (until the treatment was stopped), and the prodrug at 12 mg/kg, markedly reduced the growth of the tumor (Fig. 7c). Termination of the doxorubicin treatment resulted in a resumption of tumor growth, and a restoration of the tumor signals to almost the level of the control tumors (Fig. 7c). In contrast, the bioluminescence signal for the PSA5 treated animals remained at a relatively constant level over the period of the treatment indicating stable disease (Fig. 7c).

PSA5 and doxorubicin activity in an orthotopic prostate tumor model—Necropsy results

After 3 weeks of treatment (38 days after implantation) the animals were sacrificed, and tumor sizes were measured. Furthermore, organs that are potential targets for metastases from the LNCaP tumors (kidney, liver, spleen, intestine, the inguinal lymph nodes and lungs as well as a sample of the femur), were harvested, homogenized and assayed for luciferase activity as a measure for tumor cell infiltration of the organs. A part of the primary tumor was also homogenized and subjected to a luciferase assay.

The antitumor activity of PSA-5 at 12 mg/kg noted by the in vivo measurement was confirmed with the caliper measured tumor sizes. Tumor sizes in this group were reduced by 62% (+25%, **p = 0.003) compared to control tumors. Doxorubicin, on the other hand resulted in a reduction of the tumor burden by 33% (-37%, p = 0.08), which was not significant to controls (Fig 8a, left panel). The results from the in vitro luciferase assays generally correlated well with the tumor sizes [Figs. 8a (right panel) and 8b], and further confirmed the effect of the prodrug at 12 mg/kg [-50%, (SD ± 32%), **p = 0.007]. However, the tumor size reduction detected when caliper measuring the tumors of doxorubicin treated animals, although smaller than for PSA5, was not con-

FIGURE 6 – (a) PSA secretion and luciferase activity of LNCaP cells in vitro. LNCaP LN cells were plated into the wells of a 6 well plate. 24, 48, 72 and 96 hr after, the medium was removed and PSA levels were determined. Cells were then trypsinized, counted, lysed in luciferase assay buffer and the luciferase activity was determined. Shown are the cell counts (line), luciferase activity (LU/0.3 μL of lysate; light grey bars) and PSA values (ng/mL, dark grey bars). (b) Determination of the IC50 of doxorubicin and PSA5 on LNCaP LN cells in vitro. LNCaP LN cells were plated into a 96-well plate. 24 hr later, serial dilutions of the drugs were added in triplicates. Survival curves, plotted as percent of control, are shown from the Alamar Blue results (grey line) and the luciferase assay (black dotted line).
and exposed to the Nightowl camera for 5 min. at 10×10 binning. Signals were quantified graphically, and the animals distributed according to the 4 treatment groups (n = 8). (b) Animal weights during treatment (n = 8). To monitor drug side effects, animals were weighed 3 times weekly. Two animals of the doxorubicin group died at days 28–30. (c) Tumor growth curve. In vivo bioluminescence was determined once weekly (as in a), and the results plotted graphically (n = 8). The black arrow indicates the time-point of the randomization, the grey arrows of the treatments. Note that, because of toxicity, doxorubicin could only be applied twice.

In confirmation of our earlier results with PSA cleavable doxorubicin prodrug, the ex vivo luciferase measurements correlated better with the in vivo signals, which also suggested similar tumor sizes between control and doxorubicin treated tumors. As in previous experiments, the luciferase assay mediated analysis of the metastatic spread of tumor cells to the organs of mice bearing LNCAp LN tumors suggested that the lung is the main target organ for metastases, left panel). When compared to lungs from control animals, those originating from mice treated with PSA-5 displayed a significantly reduced luciferase activity [−50%, (SD ± 115%), *p = 0.038; −95%, (SD ± 6%)], suggesting that the treatment either prevented the tumor cells from spreading to the lungs or from proliferating there (Fig. 8c, right panel). Doxorubicin treatment also reduced the spread of tumor cells to the lung, albeit not significantly [−76%, (SD ± 19%), p = 0.059].

In situ binding of prodrugs to albumin turns this potential disadvantage into a therapeutic benefit by incorporating a cleavable bond between the drug and the albumin-binding moiety that ensures a specific release of the drug at its site of action. As a consequence, the therapeutic index of doxorubicin prodrug EMC-Arg-Arg-Ser-Ser-Tyr-Tyr—Ser-Gly-DOXO is significantly enhanced allowing high doses to be administered to tumor-bearing animals with a concomitant increase in antitumor activity compared to free doxorubicin.19

In this work, we developed an albumin-binding doxorubicin prodrug EMC-Arg-Ser-Ser-Tyr-Tyr—Ser-Arg-DOXO (PSA5) that is cleaved by PSA releasing the doxorubicin dipeptide H-Ser-Gly-DOXO, which yielded the doxorubicin dipeptide H-Ser-Gly-DOXO after PSA cleavage which was not, however, further degraded in tumor homogenates.7

In confirmation of our earlier results with PSA cleavable doxorubicin prodrugs,7 PSA5 showed significantly higher IC50 values than doxorubicin in the PSA-positive cell line LNCAp presumably due to low PSA levels secreted in the cell culture medium. In contrast, in subsequent in vivo experiments PSA5 showed superior antitumor efficacy at 3×12 mg/kg doxorubicin equivalents over doxorubicin at a dose of 2×4 mg/kg. It should be noted that PSA5 at this dose schedule showed no noteworthy decrease in body weight in contrast to doxorubicin treated animals that showed a significant decrease in body weight (see Fig. 7b) as well as toxic deaths indicating clearly that the dose of 2×4 mg/kg doxorubicin cannot be exceeded.
Therapy with PSA5, however, was well-tolerated and showed significant antitumor activity on the primary tumor as well as on the number of metastases up to the end of the experiment. The in vivo result thus demonstrates that the therapeutic index of doxorubicin is improved with PSA5. The main reason for this improvement is presumably a more favorable biodistribution concomitant with a relative tumor accumulation of doxorubicin in the primary tumor and metastases. Results with an anti-CD31-antibody indicate a high degree of vascular disorder; together with their dark red appearance, orthotopically implanted LNCaP tumors seem to be highly vascularized and suitable for passive targeting with albumin.

The use of a luciferase marked LNCaP prostate cell line in our study allowed the randomization of the animals at the beginning of drug treatment as well as the exclusion of non-tumor bearing animals. Since the take-rate in this model is only 60–70%, the latter issue is of crucial importance. Moreover, drug effects could be monitored in vivo, despite the orthotopic implantation of the tumors. Up to the time point when the treatment had to be stopped because of toxicity, the efficacy of the free doxorubicin could be shown to be relatively similar to that of the PSA5 produg. At the time of necropsy the in vivo bioluminescence signal suggested a resumption of tumor growth in the doxorubicin group to almost the levels of the controls. Although analysis of the tumor sizes revealed a slight reduction of primary tumor size when compared to the controls, this was not significant. In vitro luciferase assays, however, confirmed the in vivo bioluminescence analysis, suggesting a lack of efficacy of the doxorubicin treatment on the primary tumor. The slight discrepancy between tumor size measurements and luciferase in vivo/in vitro assays might be explained by a faster recovery of tumor cells from the treatment than the surrounding stromal cells, resulting in smaller tumors with higher lu-
ciferase activity. Besides measuring apparent tumor size in vivo and tumor cell content of the primary tumor in vitro, luciferase assays may also be used to measure tumor cell spreading into organs as a measure for metastatic disease. Both the free drug and the prodrug decreased the metastatic spread of LNCaP LN cells to the lung, albeit in the case of doxorubicin this effect was just below statistical significance. The more marked effect of doxorubicin on lung metastases than on the primary tumor suggests that during the treatment period the level of tumor cells infiltrating the lungs was reduced and once treatment was stopped, the resumption of the metastatic process was not as efficient as that of the primary tumor growth.

Besides albumin-binding prodrugs of doxorubicin that aim to exploit PSA as a molecular target, 2 low-molecular weight doxorubicin prodrugs have been developed that are cleaved by PSA.22–24 These doxorubicin derivatives include the peptide sequences Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-OH (Mu = morpholinocarbonyl) and N-glutaryl-(hydroxypropyl)-Ala-Ser-cyclohexylglycyl-Gln-Ser-Leu-OH (abbreviated L-377.202) bound to the amino position of doxorubicin. Both low-molecular weight prodrugs were designed to release N-(i-leucyl)doxorubicin after cleavage by PSA.

The MTD of both prodrugs was ~5–7-fold higher than for free doxorubicin, and at these doses they demonstrated significant antitumor activity in PSA-positive animal models (LNCaP, CWR22, PC 82).23,24 A phase I study has been carried out with L-377.202 with 225 mg/m² being established as the MTD in this study that corresponds to ~90 mg/m² doxorubicin equivalents.

In summary, we have developed a novel albumin-binding prodrug of doxorubicin that is cleaved by PSA releasing doxorubicin as the final cleavage product and shows promising antitumor efficacy in an orthotopic prostate tumor model.

**Experimental section**

**Chemicals, Materials and Spectroscopy**

Chemicals and solvents were purchased from Sigma-Aldrich, Fluka, Merck, KMF, LGC Promochem and Roth and were used without further purification. Doxorubicin hydrochloride was purchased from Yick-Vic Chemicals & Pharmaceuticals (HK) China, EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-Arg-OH was custom-made by JPT Peptide Technologies GmbH, Berlin, Germany. Human serum albumin (5% solution) was purchased from Octapharma GmbH, Langenfeld, Germany that contained ~60% free thiol groups as assessed with the Ellmann’s test. The buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius AG, Göttingen, Germany) and thoroughly degassed with ultrasound prior to use.
Enzymatically active PSA was purchased from Calbiotech (Bad Soden, Germany). Xenograft tumor tissues were gratefully received from Dr. I. Fichtner (EPO Exp. Pharmacol. & Onkol. GmbH, Berlin). Lyophilization was performed with a lyophilizer Alpha 2-4 (Christ, Osterode, Germany).

UV/vis-spectrophotometry was carried out with a double-beam spectrophotometer U-2000 from Hitachi. Mass spectra (ESI-MS) was performed with a Thermoelectron LCQ Advantage.

**Chromatography**

Column chromatographic separations were carried out as flash chromatography using reverse-phase Lichroprep® RP-C18 from Merck with a particle size of 0.040–0.063 mm. HPLC for the determination of purity of PSA5 was performed with a Waters System (pump: Waters 616, detector: Waters 996 Photodiode Array Detector; controller: Waters 600S; auto sampler: Waters 717; Empower PDA software); column: Waters Symmetry® C18 (300 mm × 2.1 mm i.d., 5 μm). Chromatographic conditions: flow: 0.5 mL/min; mobile phase A: 95% acetonitrile, 5% water; mobile phase B: 95% water, 5% acetonitrile. Gradient: 0–20 min 100% mobile phase A, 20–40 min increase to 100% mobile phase B, injection volume: 50 μL. HPLC for incubation and stability studies with PSA5 and the respective albumin-conjugate was performed with 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) or a Lambda 1,000 visible detector from Bischoff (at λ = 495 nm): UV-detection at 280 nm; column: Waters, 300 A, Symmetry C18 (4.6 × 250 mm) with precolumn; chromatographic conditions: flow: 1.0 mL/min; mobile phase A: 70% MeCN, 30% 50 mM sodium potassium phosphate (pH 7.0), mobile phase B: 27.5% MeCN, 72.5% 50 mM potassium phosphate (pH 7.0), gradient: 0–25 min 100% mobile phase B; 25–40 min increase to 100% mobile phase A; 40–50 min 100% mobile phase B; 50–60 min decrease to 100% mobile phase B; injection volume: 50 μL.

**Synthesis of H-Arg-DOXO.** Doxorubicin hydrochloride (200 mg, 0.35 mmol), Fmoc-Arg-OH (305 mg, 0.837 mmol) and triethylamine (191 mg, 0.35 mmol), Fmoc-Ser-OH (391 mg, 1.192 mmol) and DIEA (50 mg, 0.367 mmol) were dissolved in 22 mL of anhydrous DMF. After stirring at room temperature for 5 min, HATU (157 mg, 0.41 mmol) was added. After stirring at room temperature for 5 min, 1.5, 3 and 24 hr samples were collected and 50 μL samples were analyzed with HPLC.

The protecting group was removed by dissolving 400 mg Fmoc-Ser-Arg-DOXO in 5 mL of 20% piperidine solution (DMF) and stirring for 5 min. The product was precipitated with 250 mL of diethyl ether, washed 3 times with diethyl ether and dried in vacuum to afford 323 mg of H-Arg-DOXO as a red powder after precipitating the combined fractions containing the product with diethyl ether. The protecting group was removed by dissolving 400 mg Fmoc-Ser-Arg-DOXO in 8 mL of 20% piperidine solution (DMF) and stirring for 5 min. The product was precipitated with 400 mL of diethyl ether, washed 3 times with ether and dried in vacuum to afford 312 mg of H-Ser-Arg-DOXO. Mass (ESI-MS): 3 kV; m/z 788.2 [M + H]+; HPLC (495 nm): >95%.

**Synthesis of E-MC-Arg-Ser-Tyr-Arg-DOXO (2TFA) (E-MC-PSA5).** H-Ser-Arg-DOXO (128 mg, 0.163 mmol), EMC-Arg-Ser-Tyr-Arg-OH (159.3 mg, 0.184 mmol), HOBr (65.9 mg, 0.487 mmol) and 4-methylmorpholine (71 μL, 0.443 mmol) were dissolved in 10 mL of anhydrous DMF; after stirring at +5°C for 15 min, DIPC (151 μL, 123 mg, 0.975 mmol) was added. After stirring at +5°C for 72 hr, the product was precipitated with diethyl ether, washed 3 times with diethyl ether and dried in high vacuum. The product was dissolved in methanol/water 3:1 and purified first through size-exclusion chromatography on Sephadex® LH-20 (Amersham Pharmacia Biotech AB) using methanol and in a second purification step on a reverse-phase column using acetonitrile/water + 0.1% TFA. HPLC 30:70 to afford 152 mg of PSA5 as a red powder after lyophilizing the combined fractions containing the product. Mass (LC-MS-pos. ESI, 1.5 kV): m/z 1,636.5 [M+COOH]; 1,749.7 [M+CF3COOH]; 1,750.7 [M+CF2COOH]; HPLC (495 nm): >98%.

**Synthesis of the albumin conjugate of PSA5.** 1.9 mg of PSA5 was dissolved in 2 mL human serum albumin (5% solution from Octapharma) and the solution was incubated at 37°C for 1 hr. The albumin conjugate was obtained after subsequent size-exclusion chromatography (Sephacryl® S-100; Tris-buffer: 50 mM Tris-Cl pH 7.4). The content of anthracycline in the sample was determined using the ε-value for doxorubicin [ε295 (pH 7.4) = 10,650 M−1 cm−1]. The concentration of PSA5 in the conjugate was adjusted to 400 ± 50 μM by concentrating the sample with CENTRIPREP®-10-concentrators from Amicon, FRG (4°C and 4,500 rpm). Samples were kept frozen at −20°C and thawed prior to use.

**Incubation studies with human blood plasma.** Human blood plasma (EDTA stabilized) was taken from healthy volunteers. 1.8 mg of PSA5 were dissolved in 200 μL of 10 mM sodium phosphate/5% glucose buffer pH 6.4 to give a 5 mM stock solution of PSA5. 30 μL was added to plasma preincubated at 37°C for 1 h. After 5 min, 4, 20 hr and for 24 hr at 37°C. A 50 μL sample for each time point was analyzed by HPLC.

In a further experiment 500 μL of human blood plasma was preincubated with 0.4 mg of EMC for 1 hr. Subsequently, 10 μL of PSA5 and 190 μL of the preincubated plasma was mixed and incubated for 10 min. 50 μL samples were analyzed with HPLC.

**Incubation studies of the albumin conjugate of PSA5 with PSA at pH 7.4.** 100 μL of the albumin conjugate stock solution (420 μM) of PSA5 was mixed with 50 μL PSA (200 μg/mL) and 50 μL of buffer (Tris-buffer pH 7.4). The samples were incubated at 37°C. After 5 min, 1, 5, 3 and 24 hr samples were collected and 50 μL of each sample were diluted 1:1 to a final concentration of 110 μM and analyzed by HPLC.

**Preparation of LNCaP tumor tissue homogenates at pH 7.4.** For obtaining LNCaP carcinoma tissue homogenates, all steps were carried out on ice where possible. Tissue of LNCaP xenograft tumors were 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). Homogenization was carried out with a micro-dissmembrator at 3,000 rpm for 3 min with the aid of glass balls, and the samples were then centrifuged at 5,000 rpm for 10 min and kept frozen at −78°C prior to use.

**Incubation studies of the albumin conjugate of PSA5 with LNCaP tissue homogenates.** The albumin conjugate of PSA5 was incubated with LNCaP tissue homogenate at 37°C at a final concentration of 100 μM and chromatograms recorded at 495 nm using reverse phase HPLC at the time points stated in the figures.
In vitro cellular experiments. For the PSA/luciferase measurement, cells were plated at 200,000 cells/well into a 6-well plate. After 24, 48, 72 and 96 hr, medium supernatant was removed and stored at -80°C. Cells were then trypsinized, counted and cells frozen as a cell pellet. Once all samples were collected, the cell pellets were lysed in 90 μL of luciferase assay buffer (25 mM Tris-phosphate pH 7.8; 2 mM EDTA; 2 mM DTT; 0.1% Triton X-100), and 10 μL of the undiluted, as well as 1/10 and 1/100 dilutions were measured in a Luminometer (BMG Lumistar) using the luciferase substrate from Promega (Promega E4550), according to the manufacturer’s instructions. PSA values were determined nephometrically with a microparticulate immunoassay from Abbot.

Animal experiments. All animal experiments were performed in accordance to German Animal License Regulations (Tier schutzgesetz) identical to UKCCR Guidelines for the welfare of animals in experimental neoplasia.18 Male SCID (C.B-17/Icr-Hsd129scid) mice were obtained from Harlan Winkelmann GmbH, Germany. Mice were anesthetized with isofluorane, positioned and fixed on the back. The coat of the abdominal area was shaved and the skin was disinfected with alcohol (70%). A 3–4 mm incision was made alongside the linea alba in the lower abdomen using the cranial edge of the bladder for orientation. Seminal vesicles and prostate were pulled out partially and exposed carefully. A 2–3 cm incision was made alongside the linea alba in the lower abdomen using the cranial edge of the bladder for orientation. Seminal vesicles and prostate were pulled out partially and exposed carefully. 2 × 10^5 tumor cells, suspended in 20 μL PBS, were injected into the anterior prostate using a 29-gauge needle syringe. The exteriorized organs were reinserted into the abdomen, the abdominal wall was closed using 5-0 Dexon sutures (DEXON®; B.Braun-Dexon, Spangenberg, Germany), and the skin was again disinfected using a Dibromol tincture (Trommsdorf GmbH, Germany).

Measurement of in vivo bioluminescence. The animals were injected with 100 μL of β-luciferin (Synchem OHG, Germany), and anesthetized using isofluorane. 10 min. after the injection, the mice were transferred into a NIGHTOWL camera (Berthold Technologies) equipped with isofluorane adaptors. An overview picture was acquired, and an aliquot of the lysate was assayed for luciferase activity.

Histology. 7 μm cryo-sections were prepared, and either processed for immunohistochemistry or assayed for luciferase activity, followed by H&E staining. For the former, the sections were fixed using 4% paraformaldehyde and stained for human cytokeratin using the biotinylated mouse monoclonal pan cytokeratin 1–8 antibody from Progen (Cat # 61506) according to the instructions from the manufacturer. In the latter case, the sections were overlaid with a luciferase substrate mix (Promega E4550), exposed under a Nightowl camera (Berthold) for 2 min. at 1 × 1 binning, washed in water and stained with H&E to reveal the morphology of the sections.

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References


