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Development of a novel prodrug of paclitaxel that is cleaved by prostate-specific antigen: An *in vitro* and *in vivo* evaluation study

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ABSTRACT

In developed countries, prostate cancer is the third most common cause of death from cancer in men. Unfortunately, whilst accumulating clinical data have suggested that taxanes may prolong the survival in a subset of men with prostate carcinoma, the dose and duration of administration of these drugs are limited by their significant systemic toxicities due to a lack of tumour selectivity. In an attempt to improve both the water solubility and tumour-targeting properties of paclitaxel (Taxol®), we set out to develop a water soluble paclitaxel prodrug that is activated specifically by prostate-specific antigen (PSA) which is almost exclusively expressed in prostate tissue and prostate carcinoma making it an ideal molecular target for prodrug strategies. Using albumin as a drug carrier, we describe a novel albumin-binding prodrug of paclitaxel, EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-Leu-PABC-paclitaxel [EMC: ε-maleimidocaproyl; PABC: *p*-aminobenzyloxycarbonyl] that was synthesised by reacting EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-OH with H-Leu-PABC-paclitaxel. This prodrug was water soluble and was bound to endogenous and exogenous albumin. Moreover, incubation studies with PSA demonstrated that the albumin-bound form of the prodrug was cleaved rapidly at the P1–P1′ scissile bond releasing the paclitaxel-dipeptide H-Ser-Leu-PABC-paclitaxel. Last but not least, due to the incorporation of a PABC self-eliminating linker, this dipeptide was rapidly degraded to liberate paclitaxel as a final cleavage product within a few hours in prostate tumour tissue homogenates. Of note was that the albumin-bound form of the prodrug was approximately three-fold more active in killing PSA-positive LNCaP cells than paclitaxel. In addition, orientating toxicity studies in mice showed that the maximum tolerated dose of the novel paclitaxel prodrug was twice that of conventional paclitaxel. When tested *in vivo* in an orthotopic mouse model of human prostate cancer using luciferase-transduced LNCaP LLN cells, both paclitaxel and the new paclitaxel prodrug showed distinct antitumour efficacy on the primary tumour and metastases that was significantly better than the effect of doxorubicin which was used as a comparison and showed no antitumour efficacy. The new paclitaxel prodrug (3 × 24 mg paclitaxel equivalents) showed comparable antitumour activity on the primary tumour to paclitaxel at its

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maximum-tolerated dose (3 × 12 mg/kg), reduced circulating PSA levels and demonstrated a better antitumour effect on lung metastases but not on bone metastases.

1. Introduction

In developed countries, prostate cancer is the second most frequently diagnosed cancer and the third most common cause of death from cancer in men.[1] At present, there is no effective therapy for men with metastatic prostate cancer,[2] and the best results to date are achieved with taxotere.[3] Androgen ablation, although of substantial palliative benefit, is ultimately not curative and becomes ineffective as soon as the disease progresses to an androgen independent stage. Whilst accumulating clinical data have suggested that chemotherapy may prolong survival in a subset of men, the benefit of therapy with conventional chemotherapy is limited due to the systemic toxicity and the lack of tumour selectivity of antineoplastic agents.[2] Thus, there is an urgent medical need to design drug delivery systems that selectively transport the anticancer agent to the primary tumour and metastases. Besides the development of suitable galenic formulations such as liposomes or micelles, macromolecular prodrugs are being intensively studied in order to reduce the toxicity and to improve the efficacy and selectivity of the chemotherapeutic agents.[4] Over the past decades, several promising prodrug approaches have been developed in which the drug is bound to a suitable macromolecular carrier through a linker molecule that incorporates a pre-determined breaking point to ensure a tumour-specific release of the drug payload.[4] An endogenous carrier for drugs with increasing clinical relevance is human serum albumin (HSA) (66.5 kDa).[5] HSA is capable of passively targeting solid tumours mediated by the EPR effect (enhanced permeability and retention of macromolecules).[5,6] In the first step, the entry of macromolecules into tumour tissue is facilitated by a leaky vasculature, in a second an impaired lymphatic drainage system results in an accumulation of macromolecules in solid tumours.[6] Over the past years, we have investigated a targeting strategy that is based on two features:[7–9] (a) *in situ* binding of a thiol-binding prodrug to the cysteine-34 position of circulating albumin after intravenous administration; (b) release of the albumin-bound drug at the tumour site due to the incorporation of a cleavable bond between the drug and the carrier. The first and the most advanced prototype of this type of prodrug is the (6-maleimidocaproyl)hydrazone derivative of doxorubicin (INNO-206, formerly DOXO-EMCH), an acid-sensitive prodrug of doxorubicin that is currently under phase II clinical development[10] (see also http://www.cytrx.com). Inspired by the translational research with DOXO-EMCH, a broad spectrum of albumin-binding prodrugs has been developed by Kratz and co-workers that incorporate an enzymatically cleavable peptide linker. Examples include doxorubicin prodrugs that are cleaved by matrix metalloproteases 2 and 9,[9] cathepsin B,[11] urokinase[12] or prostate-specific antigen (PSA),[13,14] methotrexate prodrugs that are cleaved by cathepsin B and plasmin,[15] and camptothecin prodrugs that are cleaved by cathepsin B or unidentified proteases.[11,16,17] Amongst these proteases, PSA is especially attractive as a target protease because it is almost exclusively expressed in prostate tissue and prostate carcinoma. PSA is a serine protease with chymotrypsin-like activity that belongs to the kallikrein gene family and 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.[18,19] Over-expression of PSA has primarily been demonstrated in prostate carcinoma and at lower levels in breast cancer.[13,20] PSA is secreted in an active form in prostate cancer but forms two stable complexes with α_1-antichymotrypsin and α_2-macroglobulin in the blood. PSA complexed to α_1-antichymotrypsin is the predominant fraction of PSA. Only a minor fraction of serum PSA is not associated with proteinase inhibitors and it is unknown whether this free fraction still has enzymatic activity.[21] In addition, it has recently been shown that PSA forms complexes in mice bearing PSA-positive LNCAP tumours similar to those in man, but the major immunoreactive complex contains α_1-antitrypsin rather than α_1-antichymotrypsin.[22] Consequently, we and others have investigated prodrug strategies that exploit PSA as a molecular target for releasing an anticancer drug in prostate tumours.[14,23–25] Recently, a PSA cleavable albumin-binding prodrug of doxorubicin EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-Arg-DOXO (**PSA5**) was developed in our group[14] that is depicted in Fig. 1.

PSA5 was shown to rapidly bind to circulating albumin and was superior over doxorubicin in an orthotopic PSA-positive model (LNCaP) with respect to its antitumour efficacy and

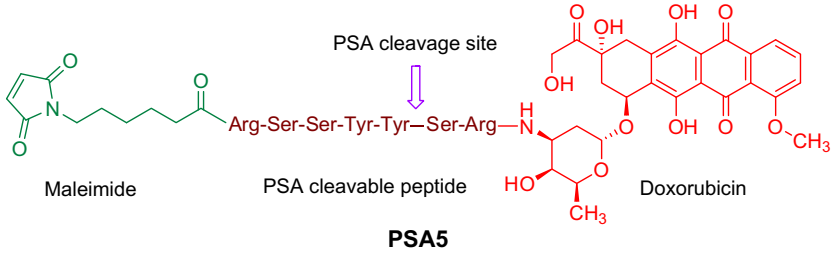


Fig. 1 – Structure of the PSA-cleavable doxorubicin prodrug PSA5.

tolerability, but did not induce tumour remissions.[14] Cleavage studies of the albumin-bound form of **PSA5** (**HSA–PSA5**) demonstrated an efficient cleavage between Tyr and Ser releasing the doxorubicin dipeptide H-Ser-Arg-DOXO as a final cleavage product within 24 h. This dipeptide, however, was cleaved very slowly to H-Arg-DOXO in LNCaP tumour homogenates and only minute amounts of doxorubicin were released. These results revealed that the full potential of the prodrug had not been exploited considering that only small amounts of the active agent doxorubicin were liberated in PSA-positive prostate carcinoma tissue.[14] As a consequence and as the main goal of the present work, we set out to optimise the release characteristics of PSA cleavable prodrugs with albumin-binding properties that would rapidly liberate the free drug as the final cleavage product in PSA-expressing prostate tumours. In addition, we substituted doxorubicin for paclitaxel as a cytotoxic agent due to the greater clinical relevance of taxanes in the treatment of prostate cancer.[3,26–28]

2. Materials and methods

2.1. Chemicals

Paclitaxel was purchased from *Yick-Vic Chemicals and Pharmaceuticals* (HK, China). EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-OH; purity >95%, 220 nm, was custom-made by *JPT Peptide Technologies GmbH* (Berlin, Germany). Human serum albumin (5% solution), containing approximately 45% free thiol groups as assessed with the Ellmann's test, was purchased from *Octapharma GmbH* (Langenfeld, Germany). The buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius, Germany) and thoroughly degassed with ultrasound prior to use. Enzymatically active PSA was purchased from *Calbiochem* (Bad Soden, Germany). LNCaP LLN prostate carcinoma cells were purchased from *ATCC* (Wesel, Germany). LNCaP prostate tumour tissues were received from Dr. N. Esser, *ProQinase GmbH* (Freiburg, Germany). All other chemicals, reagents, and solvents were of analytical grade and obtained from standard suppliers and were used without further purification. Electron Spray Ionization Mass spectra (ESI-MS) were obtained on a Thermo Electron LCQ Advantage with associated MAT SS 200 data. Mass spectra of the key compounds H-Ser-Leu-PABC-paclitaxel (**6**) [PABC: *p*-aminobenzyloxycarbonyl] and the paclitaxel prodrug (**7**) are depicted in the Supplementary Data.

2.2. Chromatography

2.2.1. TLC and flash chromatography

Unless otherwise indicated, analytical TLC was performed on Merck pre-coated plates using TLC silica gel 60F254 (normal phase) and RP-18 F254S (reversed phase). Purifications of the synthesised compounds were carried out by flash chromatography (FC) using silica gel 60 with a particle size of 0.063–0.100 mm which was purchased from *Merck KGaA* (Darmstadt, Germany).

2.2.2. HPLC

The reversed-phase HPLC studies were carried out using the following HPLC methods:

2.2.2.1. *HPLC method-1*. Analytical reversed-phase HPLC of synthetic compounds as well as cleavage, binding and stability studies of the key compounds were performed on a Kontron System (Munich, Germany) using a solvent delivery system (HPLC Pump 422), a variable wavelength UV–Vis detector (HPLC Detector 254 nm and 220 nm) and a Nucleosil® C18-column (100-5, 250 × 4 mm, Macherey-Nagel), *Geminyx* software (v 1.91 by Goebel Instrumentelle Analytik, Germany) for data analysis; HPLC conditions: flow rate: 1 mL/min; mobile phase A: 20% MeCN, 80% water and 0.1% TFA; mobile phase B: 80% MeCN, 20% water and 0.1% TFA: gradient: 0–5 min 100% mobile phase A; 5–40 min increase to 100% mobile phase B; 40–49 min 100% mobile phase B; 49–53 min decrease to 100% mobile phase A; 53–60 min 100% mobile phase A; injection volume: 50 μL.

2.2.2.2. *HPLC method-2*. The preparative purification of prodrug **7** was achieved on a BioLogic Duo-Flow System from Biorad (Munich, Germany) which was connected with a UV-detection at λ = 254 nm from Biorad; column: 25 cm Nucleosil C18-column (100-7, 250 × 21 mm) with a precolumn (100-7, 50 × 21 mm) from Macherey-Nagel using the following HPLC conditions: flow rate: 10 mL/min; mobile phase A: 30% MeCN, 70% water and 0.1% TFA; mobile phase B: 70% MeCN, 30% water and 0.1% TFA: gradient: 0–5 min 100% mobile phase A; 5–35 min increase to 100% mobile phase B; 35–40 min 100% mobile phase B; 40–45 min decrease to 100% mobile phase A; 45–50 min 100% mobile phase A; injection volume: 5 mL.

2.3. Chemical synthesis

2.3.1. Synthesis of H-Ser-Leu-PABC-paclitaxel (6)

4-Aminobenzyl alcohol (PAB-OH) (310 mg, 2.59 mmol) was added to a solution of Fmoc-Leu-OH (830 mg, 2.35 mmol) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (630 mg, 2.59 mmol) in anhydrous dichloromethane (DCM) (50 mL) and the reaction solution was stirred at RT for 24 h. DCM was then removed under reduced pressure and the residue was purified by FC eluting with $CHCl_3$/MeOH 50:1 to afford **1** (1.00 g, 92%) as a colourless powder. MS (APCI, 5 μA, MeCN): *m*/*z* 459.0 ($[M+H]^+$, 100); HPLC analysis (λ = 220 nm) > 95% of peak area. To a solution of **1** (700 mg, 1.52 mmol) and bis(*p*-nitrophenyl) carbonate (bis-PNP) (2.32 g, 7.63 mmol) in anhydrous *N*,*N*-dimethylformamide (DMF) (20 mL), *N*,*N*-diisopropylethylamine (DIEA) (779 μL, 4.57 mmol) was added at 0 °C and the reaction was stirred at RT for 48 h. Subsequently, the volatiles were removed under reduced pressure, and the residue was purified by FC eluting with $CHCl_3$/MeOH 50:1 to afford **2** (800 mg, 84%) as a colourless powder. MS (ESI, 5 kV, MeCN): *m*/*z* 623.9 ($[M+H]^+$, 100), 646.0 ($[M+Na]^+$, 88); HPLC analysis (λ = 220 nm) > 95% of peak area. In the next step, paclitaxel (370 mg, 440 μmol) and 4-(dimethylamino)pyridine (DMAP) (50.0 mg, 440 μmol) were added to a solution of **2** (270 mg, 440 μmol) in anhydrous DCM (15 mL), and the reaction was stirred at RT for 24 h. Afterwards, the volatiles were removed under reduced pressure, and the residue was purified by FC eluting with ethyl acetate/hexane 1:1 to afford **3** (550 mg, 93%) as a colourless powder. MS (ESI, 5 kV, MeOH): *m*/*z* 1360.1 ($[M+Na]^+$, 100), 1338.0 ($[M+H]^+$, 60); HPLC analysis (λ = 254 nm) > 95% of peak area. The Fmoc group was then

removed by treating **3** (500 mg, 370 μmol) with 1% 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) in tetrahydrofuran (THF) (10 mL) at RT for 45 s and the product was precipitated by 1 M HCl in diethyl ether (400 mL) then it was purified by FC eluting with $CHCl_3$/MeOH 7:1 to afford **4** (350 mg, 84%) as a colourless powder. MS (ESI, 5 kV, MeOH): *m*/*z* 1116.1 ($[M+H]^+$, 100), 1138.1 ($[M+Na]^+$, 88); HPLC analysis ($\lambda = 254$ nm) > 95% of peak area. Subsequently, DIEA (15 μL, 90.0 μmol) was added to a solution of Fmoc-Ser-OH (29.0 mg, 90.0 μmol) and 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (34.0 mg, 90.0 μmol) in anhydrous DMF (3 mL). After stirring the reaction mixture at RT for 30 min, **4** (100 mg, 90.0 μmol) and DIEA (15 μL, 90.0 μmol) were added and the reaction was stirred for 2 h. The volatiles were removed under reduced pressure, and the residue was purified by FC eluting with ethyl acetate/hexane 2:1 to afford **5** (115 mg, 90%) as a colourless powder. MS (ESI, 5 kV, MeOH): *m*/*z* 1447.1 ($[M+Na]^+$, 100), 1424.9 ($[M+H]^+$, 58); HPLC analysis ($\lambda = 254$ nm) > 95% of peak area. The Fmoc group was then cleaved by treating **5** (100 mg, 70.0 μmol) with 1% DBU in THF (3 mL) at RT for 45 s and the product was precipitated by 1 M HCl in diethyl ether (200 mL) then it was purified using reverse-phase preparative HPLC chromatography (HPLC method-2) to afford **6** (75.0 mg, 89%) as a colourless powder. MS (ESI, 5 kV, MeOH): *m*/*z* 1225.1 ($[M+Na]^+$, 100), 1203.1 ($[M+H]^+$, 83); HPLC analysis ($\lambda = 254$ nm) > 94% of peak area, retention time of ~30 min in addition to a small peak of ~6% impurity eluted at retention time of ~32 min (the mass spectrum is shown in the Supplementary Data).

2.3.2. *Synthesis of EMC-Arg-Ser-Ser-Tyr-Tyr–Ser-Leu-PABC-paclitaxel (**7**)*

EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-OH (84.0 mg, 88.7 μmol), **4** (90.0 mg, 80.6 μmol), 1-hydroxybenzotriazole (HOBt) (30.0 mg, 242 μmol) and 4-methylmorpholine (35.0 μL, 322 μmol) were dissolved in anhydrous DMF (10 mL). After stirring for 15 min at 0 °C, *N*, *N*′-di-isopropylcarbodiimide (DIPC) (75.0 μL, 484 μmol) was added and the reaction was stirred at 5 °C for 72 h. The final product was precipitated with diethyl ether (100 mL) and purified using reverse-phase preparative HPLC chromatography (HPLC method-2) to afford **7** (75.0 mg, 45%) as a colourless powder after lyophilisation. MS (ESI, 3 kV, MeCN): *m*/*z* 1027.2 ($[M+2H]^{2+}$, 100); HPLC analysis ($\lambda = 254$ nm) > 95% of peak area (the mass spectrum is shown in the Supplementary Data).

2.4. *Cleavage, binding and stability studies*

2.4.1. *Preparation of the LNCaP prostate tumour tissue homogenates*

During the tissue homogenisation, all steps were carried out on ice where possible; with the aid of forceps and scalpel, fresh samples from LNCaP positive prostate carcinoma were cut into small pieces, and the dissected tissue was minced into small pieces (1 mm^3) with the aid of a pair of sharp scissors. About 200 mg of the minced tissue was transferred in a 2 mL Eppendorf tube to which was added 800 μL of 50 mM Tris buffer, pH 7.8, containing 1 mM monothioglycerol. Homogenisation was carried out with a micro-dissmemberator at 2000 rpm for 10 min with the aid of glass balls. The glass balls were removed from the homogenate solution and the samples were then centrifuged at 5000 rpm for 10 min at 5 °C. The supernatants, containing the finely crushed and broken-cell material of the samples, were collected, re-centrifuged, aliquoted to 200 μL each and frozen at −80 °C until use. The PSA level in the tissue homogenate was measured with a nephelometric immunoassay from Abbott.

2.4.2. *Cleavage studies of **6** in the LNCaP tumour tissue homogenates*

About 20 μL of **6** [1.5 mM in 5% glucose solution (pH 3.5–5.5)] was incubated with 580 μL LNCaP prostate tumour tissue homogenates (PSA level ~4 μg/mL) at 37 °C and aliquots of 50 μL were removed at various time points and analysed by HPLC [method-1]. Moreover, stability of **6** in the tissue homogenisation buffer was monitored using HPLC over 24 h.

2.4.3. *Binding of **7** to the endogenous HSA of human plasma*

In order to determine the coupling rate and selectivity of **7** for endogenous albumin, the prodrug was incubated with human blood plasma (EDTA stabilised), taken from healthy volunteers, according to the following procedures: 200 μL of **7** [1 mg in poly(ethylene glycol)–400 (50 μL) + 200 μL 5% glucose solution (pH 3.5–5.5)] was added to 1280 μL human plasma pre-incubated at 37 °C in a final concentration of 250 μM and the mixture was incubated at 37 °C. Subsequently, 50 μL samples were analysed at different time points using HPLC [method-1]. Moreover, plasma stability of **7** was monitored using HPLC over 20 h.

2.4.4. *Binding of 7 to exogenous HSA: preparation of the albumin conjugate (**HSA7**)*

HSA7 was prepared by adding 400 μL of **7** [2 mg in poly (ethylene glycol)-400 (100 μL) + 400 μL 5% glucose solution (pH 3.5–5.5)] to 2560 μL human serum albumin in a final concentration of 250 μM and the mixture was incubated at 37 °C. Complete albumin conjugation was confirmed using HPLC [method-1]. **HSA7** was kept frozen at −20 °C and thawed prior to use.

2.4.5. *Cleavage studies of HSA7 with PSA*

About 200 μL of **HSA7** (250 μM) were incubated with enzymatically active PSA (200 μL, 40 μg/mL Tris buffer, pH 7.8, protein concentration: 1.37 mg/mL, purity by SDS–PAGE: >95%) at 37 °C. At various time points aliquots of 50 μL were removed and analysed by HPLC [method-1]. Moreover, stability of **HSA7** in the PSA cleavage buffer was monitored using HPLC over 24 h.

2.4.6. *Cleavage studies of HSA7 with LNCaP prostate tumour tissue homogenates*

About 100 μL of **HSA7** (250 μM) was incubated with 200 μL LNCaP prostate tumour tissue homogenates (PSA level ~4 μg/mL) at 37 °C and samples of 50 μL were removed at various time points and analysed by HPLC [method-1]. Moreover, stability of **HSA7** in the tissue homogenisation buffer was monitored using HPLC over 24 h.

2.5. *In vitro cellular experiment*

The anti-proliferative activities of the key compounds **6** (H-Ser-Leu-PABC-paclitaxel) and **HSA7** in comparison to conventional paclitaxel were tested in a PSA-positive LNCaP LLN prostate carcinoma cell line. The LNCaP LLN is a PSA-expressing prostate cancer cell line with a lentivirally transduced stably integrated luciferase–neomycin (LLN) resistance fusion gene cassette. In the *in vitro* experiments, the LNCaP LLN cells were grown as a monolayer in cell culture dishes in DMEM culture medium with glutamax-1 and phenol red, supplemented with 10% FCS, 100 μg/mL Pen-Strep. Cells were cultured in a humidified atmosphere of 95% air and 10% CO_2 at 37 °C. For subculture or experiments, cells growing as monolayer cultures were released from the tissue flasks by treatment with 0.05% trypsine/EDTA. For the experiments, cells were used during the logarithmic growth phase.

For determining the PSA concentrations in cell culture supernatants over time, cells were plated at 200,000 cells/well into 6-well plates. After 24, 48, 72 and 96 h, medium supernatant was removed and stored at −80 °C. PSA levels secreted in the cell culture medium were measured with a nephelometric immunoassay from Abbott yielding the following results: 100,000 cells/mL: ~26.9 ng/mL after 24 h, ~88.6 ng/mL after 48 h, ~152.6 ng/mL after 72 h and ~312.0 ng/mL after 96 h.

The cytotoxicity of the key compounds **6** and **HSA7** in the LNCaP LLN cell line was compared to that of paclitaxel and doxorubicin using a luciferase proliferation assay. For IC_{50} measurements, 2.5×10^3 cells in 150 μL were plated per well in 96-well plates. About 24 h later, serial dilutions of the drugs to be tested were added in 50 μL medium in triplicates. The medium was removed after 96 h, cells were lysed in 100 μL of luciferase lysis buffer, and the 96-well plates were carefully shaken for 10 min at room temperature. An aliquot (10 μL) of the diluted lysate was transferred to a 96-well plates and luciferase activity was assayed in a BMS Luminometer after injection of 40 μL of luciferin substrate (Promega E4550) per well.

2.6. *In vivo animal experiments*

All animal experiments were performed in accordance to German Animal License Regulations (Tierschutzgesetz) identical to UKCCCR Guidelines for the welfare of animals in experimental neoplasia.[14,29] Male SCID (C.B-17/IcrHanHsd-Prkdc-scid) mice were obtained from *Harlan Winkelmann GmbH* (Borchen, Germany).

2.6.1. *Orthotopic implantation of LNCaP LLN cells into prostates of male SCID mice*

Mice were anesthetised with isofluorane, positioned and fixed on the back. The coat of the abdominal area was shaved and the skin was disinfected with 70% alcohol. 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. About 2×10^6 tumour cells, suspended in 20 μL PBS, were injected into the anterior part of the prostate using a 29 gauge needle syringe. The exteriorised organs were reinserted into the abdomen, the abdominal wall was closed using 5-0 Dexon sutures from *DEXON®*, *B.Braun-Dexon* (Melsungen, Germany) and the skin was again disinfected using a dibromol tincture from *Trommsdorf GmbH* (Alsdorf, Germany). Paclitaxel (Taxol®) was used dissolved with cremophor/ethanol in isotonic saline solution. Adrimedac® from *medac,* (Germany) was used as the doxorubicin reference (2 mg/mL). **7** was dissolved at 4.6 mg mL in 10% PEG-400 + 90% glucose 5% solution (pH 3.5–5.5).

2.6.2. *Measurement of in vivo bioluminescence*

The animals were injected with 100 μL of a 20 mg/mL solution of D-luciferin (*Synchem OHG*, Germany) in H_2O, and anesthetised using isofluorane. About 10 min after the injection, the mice were transferred into a NIGHTOWL camera (*Berthold Technologies*, Germany) equipped with isofluorane adaptors. An overview picture of the mice as well as two exposures, 5 min at 10×10 binning, and 1 min at 2×2 binning were taken. The resulting images were quantified using the instrument software, yielding the amount of light emitted over the whole animal in ph/s.

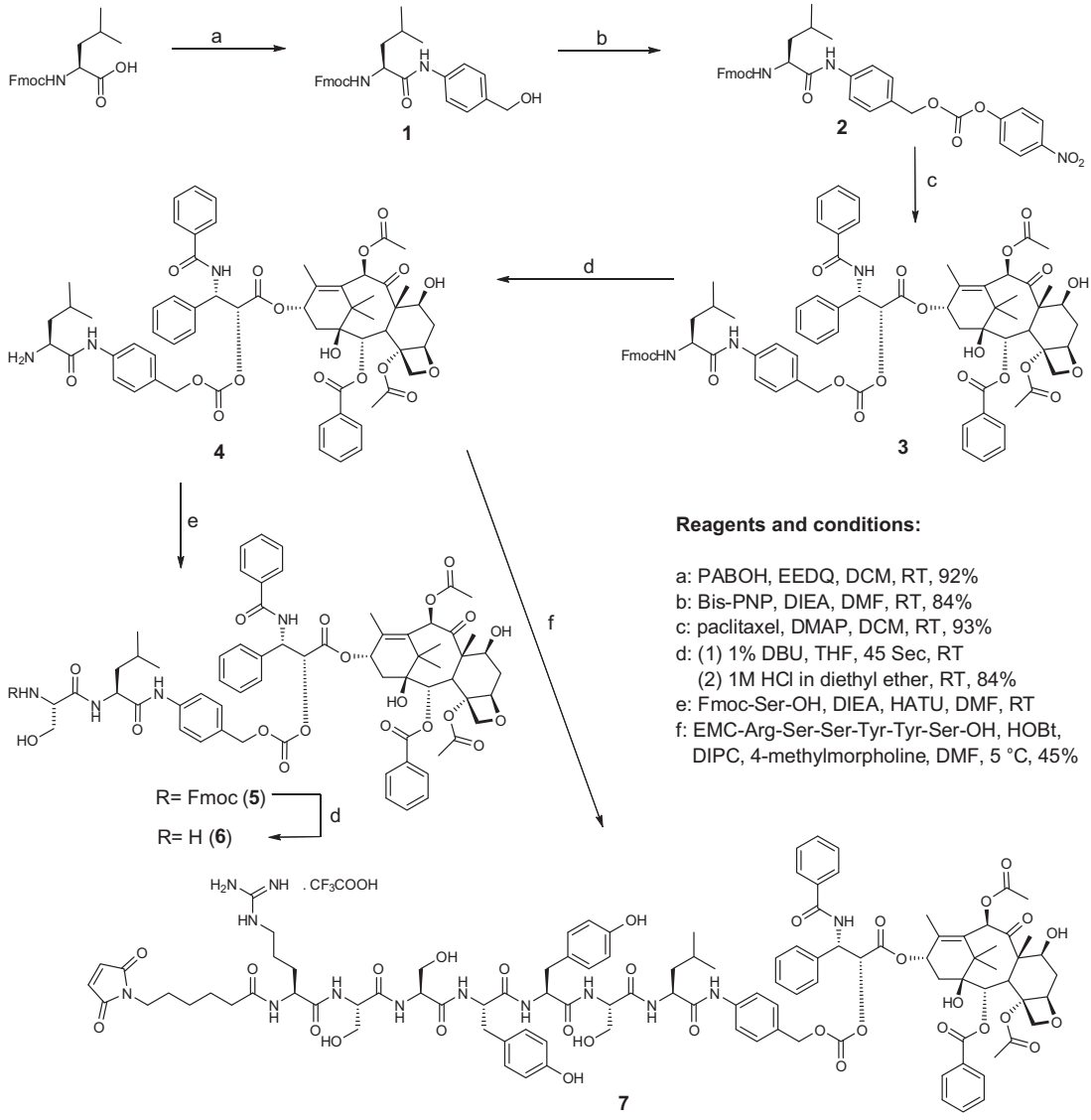
2.6.3. *Necropsy and luciferase assays of mouse organs*

At the end of the experiment, mice were sacrificed, blood was taken and serum was separated for the PSA assay; the abdominal cavity was opened, and a picture of the tumour *in situ* was taken. The primary tumour was resected, measured and its volume was calculated using the formula $V = a^2 \times A/2$ (*a*: small diameter, *A*: large diameter). Tumours were weighed and homogenised in 5 mL of luciferase lysis buffer. To obtain a quantitative analysis of the metastatic spread into potential target organs, the lungs as well as samples from the lumbar spine, the liver and inguinal lymph nodes were resected from the animals taking great care to prevent cross-contamination of the tissues. After homogenisation in 1 mL (liver: 5 mL) lysis buffer, insoluble materials were centrifuged, and 5 μL of the cleared lysate were checked for protein concentration using a Bradford assay (Sigma B6916) with BSA serving as a standard protein and 10 μL were finally measured in a luciferase assay (Promega E4550).

3. Results and discussion

In our previous studies with doxorubicin amino acid derivatives we had shown that H-Arg-DOXO was cleaved more efficiently in tumour homogenates than other doxorubicin amino acid derivatives.[12] Thus, we initially intended to develop a novel prodrug of paclitaxel with the same PSA cleavable peptide sequence that was already used for the doxorubicin prodrug **PSA5**, i.e., with an Arg residue in the P2′ position. Paclitaxel was preferred over docetaxel because the latter compound has four hydroxyl groups and selective activation of one of these HO-groups is synthetically difficult thus no prodrugs of docetaxel have been reported. In contrast, the 2′-hydroxy group of paclitaxel can be selectively derivatised with suitable linkers.

By incorporating a self-eliminating linker between Arg and paclitaxel we aimed at reducing the steric influence of the drug on the enzymatic cleavage. As a self-eliminating linker we selected the *p*-aminobenzyloxycarbonyl (PABC) system



Scheme 1 – Synthesis of H-Ser-Leu-PABC-paclitaxel (6) and EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-Leu-PABC-paclitaxel (7).

introduced by Carl et al.[30] that proved to be capable of reducing the steric hindrance of bulky drugs during the enzymatic cleavage process.[31] Upon deacylation, the PABC spacer decomposes in a rapid 1,6-benzylelimination and spontaneously releases the free drug.[30] Unfortunately, all our synthetic efforts to obtain the precursor H-Ser-Arg-PABC-paclitaxel failed as a result of the chemical incompatibility of the Arg moiety in this position. Alternatively, we synthesised the paclitaxel-dipeptide H-Ser-Leu-PABC-paclitaxel (**6**). Leucine is known to occupy this position in some natural PSA peptide substrates[32] and has also been used by DeFeo-Jones and coworkers in the development of their low-molecular weight doxorubicin prodrug L-377,202 Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-DOXO (Mu = morpholinocarbonyl).[24] In this way, the paclitaxel-dipeptide H-Ser-Leu-PABC-paclitaxel (**6**) could be synthesised in good yields as depicted in Scheme 1. After incubation with LNCaP tumour homogenate (Fig. 2), **6** was cleaved within a few hours by adventitious and as yet unknown proteases releasing paclitaxel as the final cleavage product, the effective release of paclitaxel presumably being

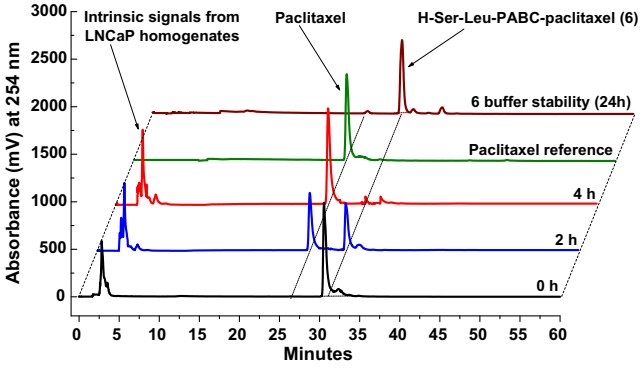


Fig. 2 – Chromatograms of incubation studies with H-Ser-Leu-PABC-paclitaxel (6) (50 μM) in the presence of LNCaP tumour tissue homogenates at 37 °C. A chromatogramme of paclitaxel is shown as a reference. After 2 h a new peak for paclitaxel appears (RT ~ 26 min) and the peak of H-Ser-Leu-PABC-paclitaxel (6) (RT ~ 30 min) decreases over time and disappears after 4 h.

Table 1 – IC_{50} values of doxorubicin, paclitaxel, H-Ser-Leu-PABC-paclitaxel (6) and HSA7 in the LNCaP LLN cell line (dose response curves are shown in the Supplementary Data – Fig. 3S).

Compound	IC_{50} value (nM)	*p*-Values*
Doxorubicin	110 ± 8.00	
Paclitaxel	2.12 ± 0.55	
H-Ser-Leu-PABC-paclitaxel (6)	0.74 ± 0.06	$^{*}p = 0.0103$
HSA7	0.82 ± 0.05	$^{*}p = 0.0107$

* *p*-Values of these IC_{50} values in correlation to that of paclitaxel.

promoted in the final steps by the self-immolative PABC spacer that decomposes spontaneously in a 1,6-benzylelimination.

In subsequent cell culture experiments using the PSA-expressing prostate cancer cell line LNCaP LLN, the cytotoxicity of the paclitaxel-dipeptide, H-Ser-Leu-PABC-paclitaxel (**6**), correlated well with its cleavage rate. H-Ser-Leu-PABC-paclitaxel that rapidly released paclitaxel in LNCaP cell lysates exhibited an IC_{50} value of 0.74 nM that was significantly lower than that of paclitaxel (IC_{50} = 2.12 nM). In contrast, doxorubicin was far less active in this cell line with an IC_{50} value of 110 nM (see Table 1).

These encouraging results prompted us to synthesise a novel albumin-binding prodrug of paclitaxel that incorporates the PABC self-immolative spacer in addition to the heptapeptide Arg-Ser-Ser-Tyr-Tyr–Ser-Leu as a PSA substrate (Fig. 3). The new prodrug EMC-Arg-Ser-Ser-Tyr-Tyr–Ser-Leu-PABC-paclitaxel (**7**) [EMC: ε-maleimidocaproyl] was obtained through coupling of H-Leu-PABC-paclitaxel (**4**) with EMC-Arg-Ser-Ser-Tyr-Tyr-Ser-OH in anhydrous DMF in the presence of HOBt, 4-methylmorpholine and DIPC (Scheme 1).

Prodrug **7** was purified using reverse-phase preparative HPLC chromatography. The lyophilised colourless product was characterised by mass spectrometry and its purity was determined by HPLC (see Experimental Section and Supplementary Data). Prodrug **7** exhibited good water-solubility (~3 mg/mL in 5% glucose solution which could be increased to ~4.6 mg/mL by adding 10% PEG-400). **7** was bound to cysteine-34 of endogenous and exogenous albumin in accordance with our previous work on the albumin-binding properties of maleimide-bearing prodrugs and showed a shift from ~20 min retention time to ~22 min after binding of **7** to albumin (see Supplementary Data – Fig. 1S). In addition, the prodrug showed good stability in human plasma over 20 h (see Supplementary Data – Fig. 1S). Incubation studies with PSA, however, demonstrated that the albumin-bound form of the prodrug **(HSA7)** was rapidly cleaved at the P1–P1′ scissile bond releasing the paclitaxel-dipeptide H-Ser-Leu-PABC-paclitaxel (Fig. 3). This paclitaxel-dipeptide as shown above liberates paclitaxel as a final cleavage product in prostate tumour homogenates (Fig. 2).

Furthermore and as a confirmation of the expected fate of the prodrug inside the prostate tumour tissues, **HSA7** was rapidly cleaved in the LNCaP prostate tumour tissue homogenates liberating paclitaxel quantitatively as a final cleavage product within a few hours as detected by HPLC (Fig. 4).

The overall reactions that are involved in the cleavage process of **HSA7** in PSA-positive carcinoma tissue are depicted in Scheme 2.

In the cell culture experiments, **HSA7** exhibited antiproliferative activity in the low nanomolar range with an IC_{50} value of 0.82 nM against LNCaP cells (see Table 1). Interestingly, **HSA7** was significantly more active than paclitaxel against the PSA-positive LNCaP LLN cells (IC_{50} = 2.12 nM) with an IC_{50} value practically identical to the cleavage product of **HSA7** by PSA, i.e., H-Ser-Leu-PABC-paclitaxel (IC_{50} = 0.74 nM) (see Table 1). These promising *in vitro* results prompted us to test the compound in a mouse model of prostate cancer using PSA-positive, luciferase expressing LNCaP LLN cells orthotopically implanted into the prostates of SCID mice. This technique allows the tumour cells to grow in an environment related to their origin and thus mimics tumour–stromal cell interactions better than subcutaneous models. Furthermore, since lung and bone metastases are reproducibly observed, antimetastatic activity of test compounds can be evaluated. The otherwise undetectable tumours and also metastases may be monitored via bioluminescence measurements of the stably integrated luciferase gene in the LNCaP LLN cells. Ten days after orthotopic implantation, tumour cells were first measured. About 14 d later, mice were randomised into treatment groups according to their *in vivo* bioluminescence

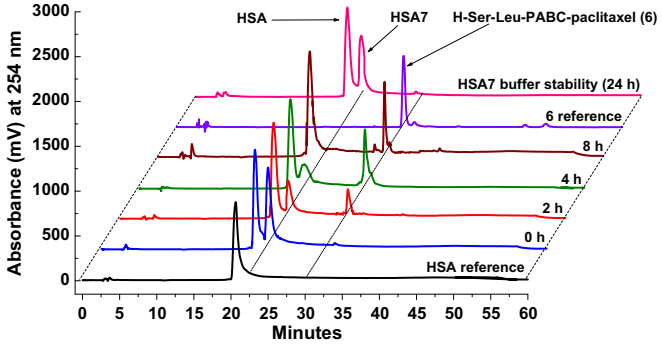


Fig. 3 – Chromatograms of incubation studies with HSA7 in the presence of enzymatically active human PSA (20 μg/mL), pH 7.8 at 37 °C. Chromatograms of HSA and 6 are shown as references. After conjugation of 7 to HSA (RT ~ 20 min), a new peak for the albumin paclitaxel conjugate HSA7 appears (RT ~ 22 min) that decreases over time due to liberation of H-Ser-Leu-PABC-paclitaxel (6) (RT ~ 30 min).

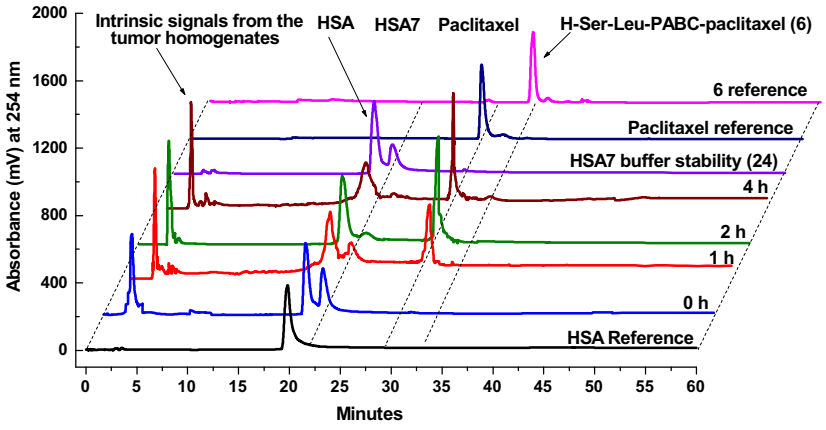
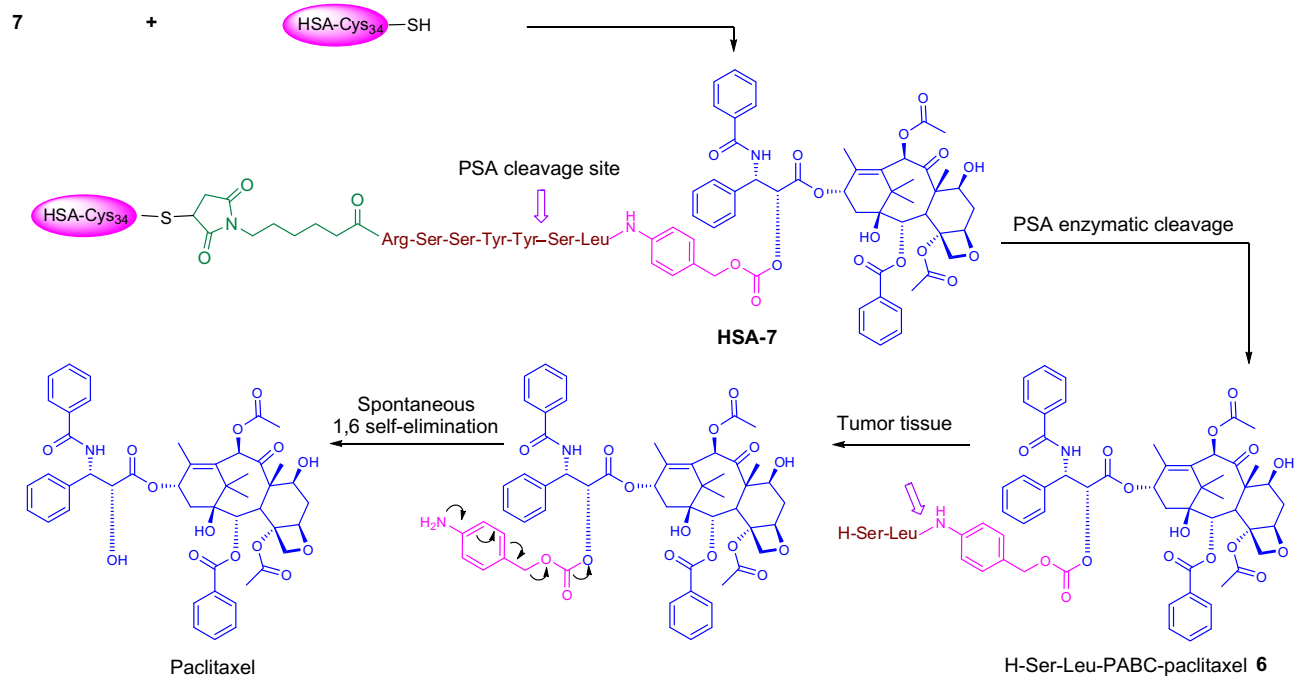


Fig. 4 – Chromatograms of incubation studies of HSA7 in the presence of LNCaP tumour tissue homogenates at 37 °C. Chromatograms of HSA, 6 and paclitaxel are introduced as references. The peak for the albumin paclitaxel conjugate HSA7 (RT ~ 21 min) decreases over time due to liberation of paclitaxel (RT ~ 28 min); of note is that H-Ser-Leu-PABC-paclitaxel (6) (RT ~ 32 min) is not observed as an intermediate suggesting a rapid degradation to paclitaxel by other proteases in the tumour homogenate. The small shift in the retention time in this experiment is due to the use of a Waters Symmetry® C18 (300 Å) column instead of the previously used Nucleosil® C18-column (100 Å) – see Fig. 3.



Scheme 2 – Schematic illustration for the albumin conjugation of 7 and the subsequent PSA enzymatic degradation of the formed albumin conjugate (HSA7) and liberation of paclitaxel by as yet unidentified proteases in LNCaP tumour homogenate followed by a spontaneous 1,6-benzylelimination to release paclitaxel.

signals, and mice without detectable tumours were excluded from the study. The take rate was around 60% underlining the importance of using high luciferase expressing LNCaP LLN cells and bioluminescence for ensuring that only tumour-bearing animals are included in the *in vivo* study.

Based on our preliminary toxicity studies, 24 mg/kg paclitaxel equivalents of **7** were compared to 12 mg/kg of free paclitaxel as a therapeutic dose (see Supplementary Data – Table 1S and Fig. 2S). Doxorubicin was also included as a comparison in this experiment at its MTD in SCID mice of 3 × 3 mg/kg. Animals were injected intravenously with the drugs once every week for a period of three subsequent weeks. The weight as well as the clinical symptoms of the animals was recorded three times weekly to monitor animal health. Animals of all four groups lost weight during the course of the experiment before treatment suggesting primary tumour burden or the metastatic spread of the tumour as a possible cause. However, after treatment control animals as well as animals treated with **7** and doxorubicin lost 15–18% of their original weight, paclitaxel-treated animals lost

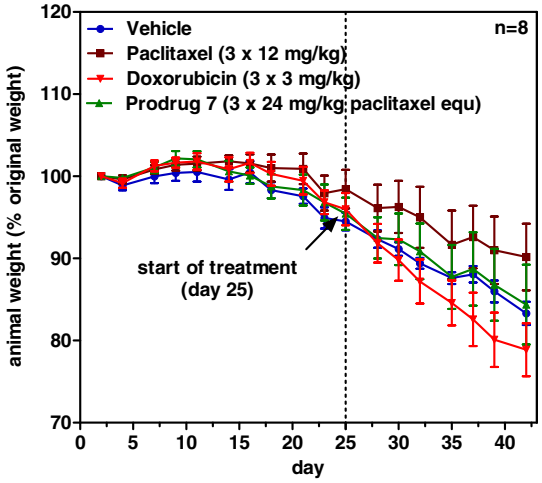


Fig. 5 – Animal weight change of mice treated with paclitaxel, doxorubicin and prodrug 7 compared to untreated mice in the orthotopic PSA-positive model (LNCaP).

slightly less weight (10%, not statistically significant to the other groups) (Fig. 5). Two animals from the control group died during the treatment period, most likely due to tumour burden. An additional animal died in the doxorubicin-treated group.

Bioluminescence measurements of the luciferase-expressing LNCaP LLN cells were used to assess the growth of the tumour once weekly. Both treatment with paclitaxel and **7** reduced the overall bioluminescence, but only **7** repressed tumour growth to a level statistically significant compared to the control group ($p < 0.05$) (Fig. 6). The doxorubicin-treated group showed no delay in tumour growth versus the control group, the apparent pro-tumour and pro-metastatic effect of doxorubicin is probably due to the fact that two animals died in the control group.

After 3 weeks of treatment (44 d after implantation) the animals were sacrificed, and tumour sizes and weights were measured. Furthermore, organs that are potential targets for LNCaP tumour metastases (lung and lumbar spine) were harvested, homogenised and assayed for luciferase activity as a measure for tumour cell infiltration of the organs. A part of the primary tumour was also homogenised and subjected to a luciferase assay. Finally, blood was taken from the animals, and the PSA levels determined as a luciferase-independent biomarker. As a confirmation of the *in vivo* bioluminescence measurements, the callipered tumour sizes indicated antitumour activity of prodrug **7** at 24 mg/kg of free paclitaxel equivalents. Tumour sizes in this group were reduced by 40% ($p = 0.1$) compared to control tumours. The *in vitro* luciferase assays confirmed the antitumour efficacy of prodrug **7** (−55% (±40%), $p = 0.058$) compared to control tumours (Fig. 7). The antitumour effect of paclitaxel was similar to that of prodrug **7** but at a lower statistical significance with $p = 0.4$ regarding the tumour size and $p = 0.11$ regarding the *in vitro* luciferase activity (Fig. 7). Serum PSA levels, as a measure of the overall tumour burden, were significantly reduced only in the group treated with prodrug **7** (−80% (±20%, $p = 0.0007$)) (see Fig. 7 and Supplementary Data – Table 2S).

In contrast, the doxorubicin-treated group showed no antitumour effect on the primary tumour at all (see Fig. 7).

Luciferase assays of potential target organs are a means to quantify metastatic tumour cells and analyse the effect of antimetastatic treatments. **7** significantly reduced luciferase activities in the lung by 75% (±17%, $p = 0.0098$) and showed some effect against LNCaP cells spread into the lumbar spine (−74% (±28%, $p = 0.13$)) when compared to control animals. Paclitaxel treatment, on the other hand, showed less pronounced effects on the lung metastases: −60% (±50%, $p = 0.083$); but showed a better effect on lumbar spine metastases: −80% (±25%, $p = 0.065$) (Fig. 8). Again, there was no noticeable effect of doxorubicin on the spread of metastases.

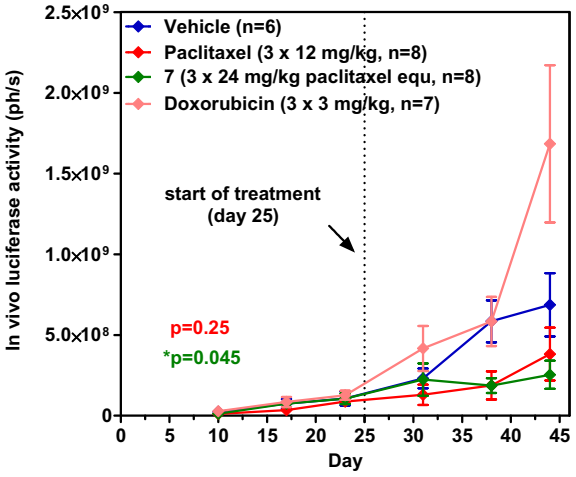


Fig. 6 – Bioluminescence activity recorded during the *in vivo* experiment of treated mice compared to untreated mice in the orthotopic PSA-positive model (LNCaP). Note the apparent pro-tumour and pro-metastatic effect of doxorubicin, which very likely reflects the loss of two animals in the control group.

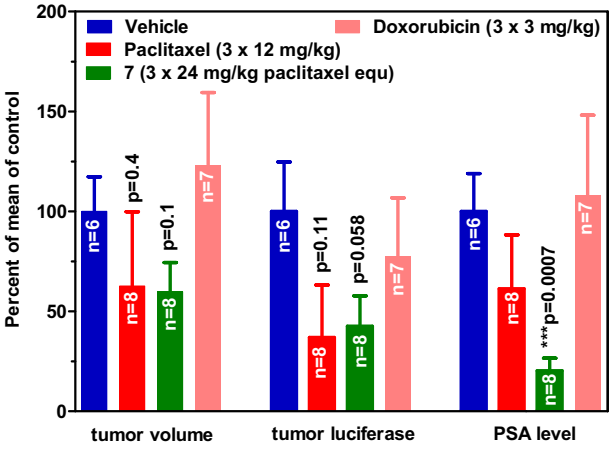


Fig. 7 – Effect of paclitaxel, doxorubicin and prodrug 7 on the primary tumours and PSA serum levels of treated mice compared to untreated mice in the orthotopic PSA-positive model (LNCaP). Note the apparent pro-tumour and pro-metastatic effect of doxorubicin, which very likely reflects the loss of two animals in the control group.

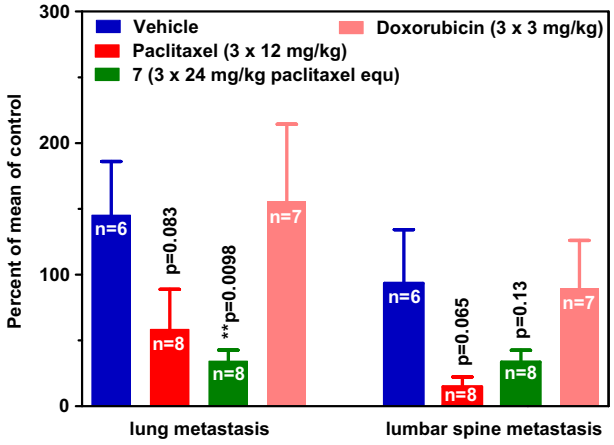


Fig. 8 – Effect of paclitaxel, doxorubicin, and prodrug 7 on lung and bone metastases of treated mice compared to untreated mice in the orthotopic PSA-positive model (LNCaP).

In conclusion, by introducing the self-eliminating spacer *p*-aminobenzyloxycarbonyl (PABC) and replacing the P2′ position of our PSA substrate by Leu, we have developed an albumin-binding PSA-cleavable paclitaxel prodrug with an advantageous cleavage profile that rapidly liberates paclitaxel as a final cleavage product. On the whole, paclitaxel and the new **7** showed promising antitumour effects in the orthotopic PSA-positive prostate cancer model that were markedly better than doxorubicin.

Considering that the primary prostate tumour grows slowly and the spread of metastases generally occurs over a long period of time, a subsequent step in our preclinical development in order to further improve the efficacy of the outlined prodrug strategy for treating prostate cancer, would be to investigate an improved long-acting dosing schedule with our paclitaxel prodrug using a parenteral route since prostate tumours in the majority of cases grow over a long time span and a three or four weekly dose scheme with conventional anticancer agents which only reaches high anticancer drug peaks in the prostate tumour for a very short period is in our opinion not an optimal way of treating slow-growing tumours. Another innovative approach that we are therefore currently investigating in our group is the development of orally applicable albumin-binding prodrugs. Administration of such agents bearing a PSA-cleavable substrate as a long term oral dosage form should ideally allow a continuous and critical concentration of the drug to be released in the prostate primary tumour as well as in metastases over a long time span thus reducing systemic toxicity and optimising the therapeutic outcome of our prodrug approach. Such an approach, although certainly ambitious, could transfer favourably to tumours that grow slowly such as prostate tumours and to achieve long term stabilisation or reduction in tumour growth. The development of orally applicable albumin-prodrugs that are cleaved by PSA is worthy of further investigation in order to obtain a critical cytotoxic concentration over a long and constant time span within the heterogenous prostate tumour characterised by tumour cells in different cell cycles which allows the patient to dose an oral prodrug according to his personal needs.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.08.018.

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