Albumin-Binding Prodrugs of Camptothecin and Doxorubicin with an Ala-Leu-Ala-Leu-Linker That Are Cleaved by Cathepsin B: Synthesis and Antitumor Efficacy

Björn Schmid,[†,§] Da-Eun Chung,[†,§] André Warnecke,[†] Iduna Fichtner,[‡] and Felix Kratz*[,†]

Tumor Biology Center, Breisacher Strasse 117, 79106 Freiburg, Germany, and Max-Delbrück Centrum, Robert-Rössle-Strasse 10, 13122 Berlin, Germany. Received September 5, 2006; Revised Manuscript Received November 20, 2006

We have recently validated a macromolecular prodrug strategy for improved cancer chemotherapy based on two features: (a) rapid and selective binding of thiol-reactive prodrugs to the cysteine-34 position of endogenous albumin and (b) acid-sensitive promoted or enzymatic release of the drug at the tumor site [Kratz, F., Warnecke, A., Scheuemann, K., Stockmar, C., Schwab, J., Lazar, P., Druckes, P., Esser, N., Drevs, J., Rognan, D., Bissantz, C., Hinderling, C., Folkers, G., Fichtner, I., and Unger, C. (2002) *J. Med. Chem. 45*, 5523−33]. In the present work, we developed water-soluble camptothecin (CPT) and doxorubicin (DOXO) prodrugs that incorporate the peptide linker Ala-Leu-Ala-Leu that serves as a substrate for the tumor-associated protease, cathepsin B, which is overexpressed in several solid tumors. Consequently, two albumin-binding prodrugs were synthesized [EMC-Arg-Arg-Ala-Leu-Ala-Leu-Ala-CPT (**1**) and EMC-Arg-Arg-Ala-Leu-Ala-Leu-DOXO (**2**) (EMC = 6-maleimidocaproic acid)]. Both prodrugs exhibited excellent water-solubility and bound rapidly and selectively to the cysteine-34 position of endogenous albumin. Further in vitro studies showed that the albumin-bound form of the prodrugs was cleaved specifically by cathepsin B as well as in human tumor homogenates. Major cleavage products were CPT−peptide derivatives and CPT for the CPT prodrug and H-Leu-Ala-Leu-DOXO, H-Leu-DOXO, and DOXO for the doxorubicin prodrug. In vivo, **1** was superior to free camptothecin in an HT-29 human colon xenograft model; the antitumor efficacy of prodrug **2** was comparable to that of free doxorubicin in the M-3366 mamma carcinoma xenograft model at equimolar doses.

INTRODUCTION

Both anticancer agents camptothecin (CPT) and doxorubicin (DOXO) are DNA-damaging drugs, which act by inhibiting the progression of the enzymes topoisomerase I (CPT) and II (DOXO), which unwind DNA for transcription. Doxorubicin is one of the most active antineoplastic agents developed to date and shows a broad spectrum of antitumor efficacy against hematological and solid cancers (*1*). Camptothecin gained clinical importance with the development of water-soluble topotecan and irinotecan that have meanwhile been approved for the therapy of colorectal, ovarian, and lung cancer (*2*, *3*).

Even though both agents show significant antitumor activity, their clinical potential is limited by severe side effects, such as myelosuppression, nausea, vomiting, and especially cardiotoxicity with DOXO and gastrointestinal disorders and stomatitis with topotecan and irinotecan. In order to circumvent these limitations and to improve the therapeutic potential of anticancer drugs, several synthetic polymers and proteins have been investigated as drug carriers (*4*−*6*).

We have recently described maleimide-bearing prodrugs of CPT and DOXO that bind in situ to the cysteine-34 position of circulating albumin after intravenous administration and release the drug at the tumor site (*7*, *8*). Albumin is a promising drug carrier because of its passive accumulation in solid tumors due to their high metabolic turnover, angiogenesis, hypervasculature, defective vascular architecture, and impaired lymphatic drainage (*9*). An acid-sensitive prodrug of doxorubicin, i.e., the 6-maleimidocaproyl hydrazone derivative (DOXO-EMCH), demonstrated superior antitumor efficacy compared to the parent compound doxorubicin in several preclinical models. In a recently completed clinical phase I study, DOXO-EMCH exhibited a good safety profile and antitumor efficacy (*10*, *11*).

In our present work, we set out to develop albumin-binding prodrugs of CPT and DOXO that incorporate the cathepsin-sensitive peptide linker Ala-Leu-Ala-Leu. This peptide sequence has been used as an inactivating moiety in several low-molecular-weight DOXO prodrugs, in particular, (CPI-0004Na) by Trouet et al. (*12*) releasing the derivative *N*-(L-leucyl)-doxorubicin that diffuses inside cells where it is cleaved to DOXO as the final product. In addition, Trouet et al. (*13*) have shown that an Ala-Leu-Ala-Leu-linker is cleaved by lysosomal enzymes in vitro. This linker has been integrated in several other conjugates, i.e., in MTX immunoconjugates by Umemoto et al. (*14*) and in human serum albumin (HSA) conjugates of MTX by Fitzpatrick et al. (*15*) to liberate the anticancer agent at the tumor site by unspecific lysosomal enzymes.

Furthermore, Struder et al. (*16*) used this linker to prepare an antibody conjugate with benzyl-EDTA that was efficiently cleaved by cathepsin B and to a lesser degree by cathepsin D. In the work by Michejda et al. (*17*), a pentapeptide Ala-Leu-Ala-Leu-Ala linker was used in a receptor-targeted drug derivative of ellipticine (an ellipticine-peptide conjugate). This linker was cleaved by the aspartate protease cathepsin D between the Ala-Leu bonds releasing Ala- and Ala-Leu-Ala derivatives of ellipticine. Both cathepsins B and D are localized in lysosomes (*18*, *19*), and overexpression in various malignant tumors has been shown, especially in breast cancer (*19*−*21*),

* To whom correspondence should be addressed. Dr. Felix Kratz, Tumor Biology Center, Department of Medical Oncology, Clinical Research, Breisacher Strasse 117, D-79106 Freiburg, Federal Republic of Germany; Tel.: +49-761-2062930; Fax.: +49-761-2062905; e-mail: felix@tumorbio.uni-freiburg.de.

† Tumor Biology Center.

‡ Max-Delbrück Centrum.

§ Both authors contributed equally to this work.

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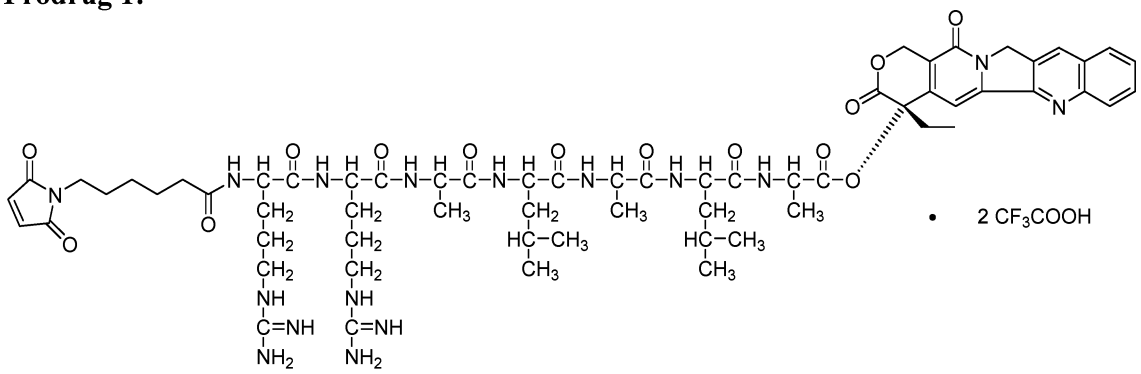
Prodrug 1:



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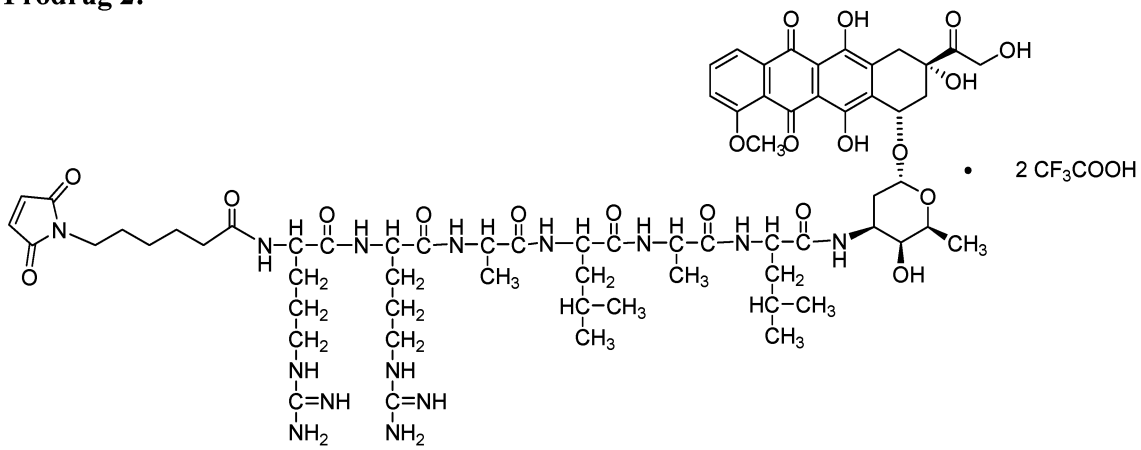


Figure 1. Structures of the albumin-binding prodrugs **1** and **2** containing a cathepsin B specific substrate.

and for cathepsin B also in melanoma (*22*), lung (*23*, *24*), and colon (*25*) carcinoma.

Inspired by this work, we developed albumin-binding prodrugs with DOXO and CPT of the general formula EMC-Arg-Arg-Ala-Leu-Ala-Leu-Ala-CPT and EMC-Arg-Arg-Ala-Leu-Ala-Leu-DOXO [EMC = 6-maleimidocaproic acid] (see Figure 1). Two arginine residues were introduced in the prodrugs to render water solubility, an important prerequisite for intravenous application and in situ coupling to endogenous albumin.

The aim of this work was to assess in detail the pH-dependent cleavage profile of the prodrugs in the presence of cathepsins B and D, as well as in biological fluids, i.e., blood plasma and tumor homogenates, and to evaluate the antitumor efficacy in vivo in comparison to free CPT and DOXO, respectively. Our cleavage studies with the Ala-Leu-Ala-Leu prodrugs showed that they exhibited a distinct cleavage pattern for cathepsin B but not for cathepsin D.

EXPERIMENTAL PROCEDURES

General. Chemicals and solvents were purchased from Sigma-Aldrich, Fluka, Merck, KMF, LGC Promochem, and Roth and were used without further purification. Camptothecin was purchased from Hande Tech Development, Inc. (U.S.A.). Doxorubicin hydrochloride was purchased from Yick-Vic Chemicals & Pharmaceuticals (HK), China. EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH was custom-made by JPT Peptide Technologies GmbH (FRG). Human serum albumin (5% solution) was purchased from Octapharma GmbH. The protein contained approximately 60% free thiol groups as assessed with the Ellmann's test. The buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius, FRG) and thoroughly degassed with ultrasound prior to use. Enzymatically active cathepsins B and D were purchased from Calbiochem (Bad Soden, FRG). Xenograft tumors were gratefully received from Dr. I. Fichtner (Experimental Pharmacology and Oncology GmbH, Berlin). Lyophilization was performed with a lyophilizator Alpha 2-4 (Christ, FRG).

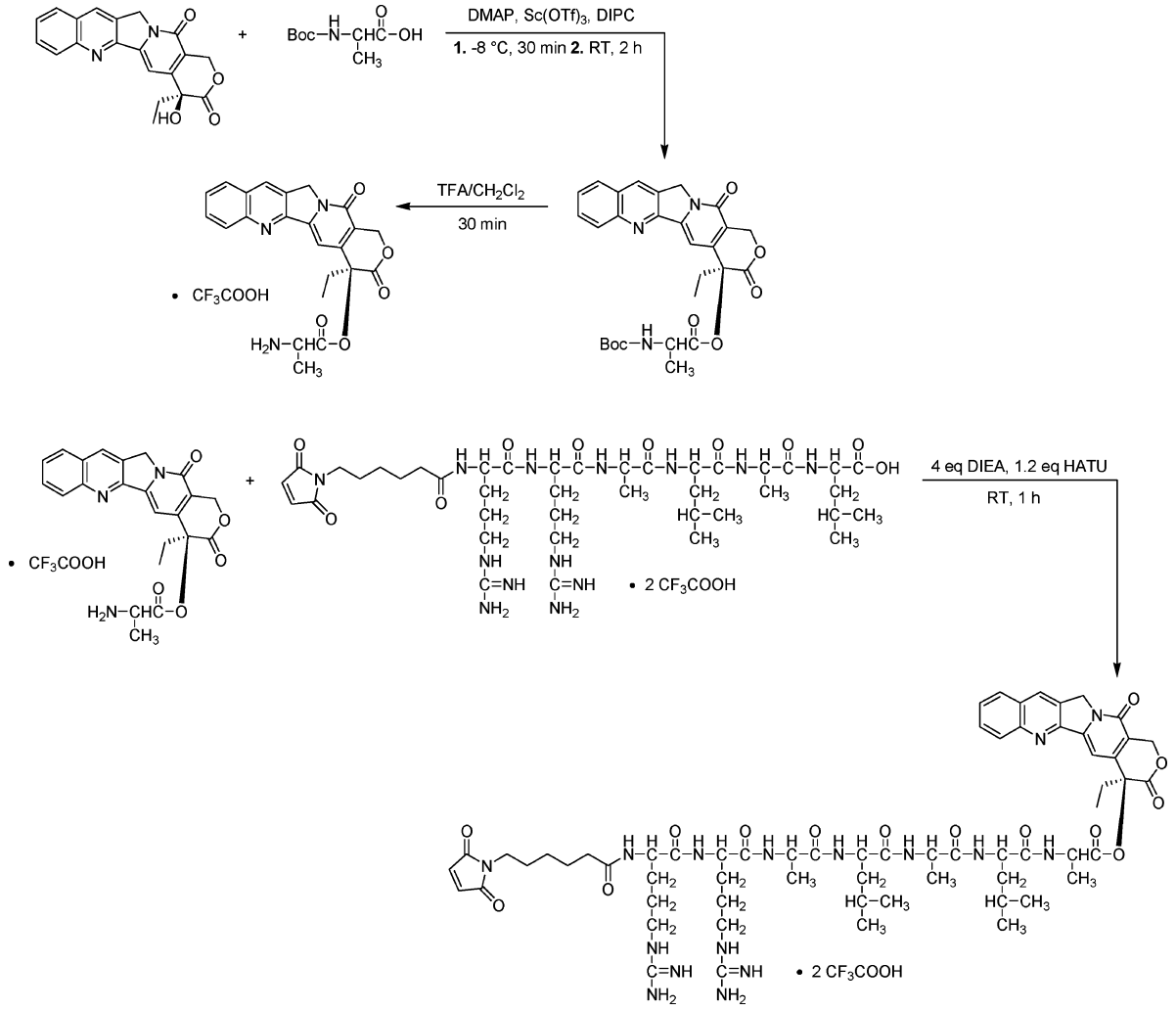
Spectral Analyses. Mass spectra were obtained on a Thermo Electron LCQ Advantage with associated MAT SS 200 data system using electron spray ionization. UV/vis spectrophotometry was carried out with a double-beam spectrophotometer U-2000 from Hitachi. LC-MS-ESI spectrometry was carried out on a Finnigan LCQ Advantage mass spectrometer (capillary temperature, 300 °C; source-induced dissociation voltage, 25 V; Thermo Electron, Dreieich, FRG) coupled to a Finnigan Surveyor HPLC system (Thermo Electron, Dreieich, FRG) with a Jupiter C_4 column (300-5, 150 × 2 mm, Phenomenex, Aschaffenburg, FRG). Chromatographic conditions: flow, 300 μL/min; mobile phase A, 1% MeCN, 99% H_2O demin, 0.06% TFA; mobile phase B, 100% MeCN, 0.06% TFA; gradient, 15−20 min increase from 10% mobile phase B to 50% mobile phase B; injection volume, 15 μL.

Chromatography. Details of the chromatographic conditions are available as Supporting Information.

Synthesis of Camptothecin-20-*O*-(L-alaninate) × TFA. The compound was synthesized according to a published procedure (*26*) and subsequent BOC cleavage with TFA/CH_2Cl_2.

Synthesis of EMC-Arg-Arg-Ala-Leu-Ala-Leu-Ala-CPT × 2 TFA (1). To a solution of camptothecin-20-*O*-(L-alaninate) trifluoroacetate (32.5 mg, 0.061 mmol), EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH × 2 TFA (75 mg, 0.067 mmol), and *N*-ethyldiisopropylamine (DIEA) (42 μL, 0.240 mmol) in 1 mL *N*,*N*-dimethylformamide (DMF) was added *O*-(azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU)

Scheme 1. Synthesis of the Albumin-Binding CPT Prodrug 1



(25.5 mg, 0.067 mmol) under a nitrogen atmosphere. The solution was stirred for 1 h at room temperature. Subsequently, the product was precipitated with diethyl ether, and the crude product was purified by preparative chromatography on a Nucleosil C18 column (100-7, 250 × 21 mm) with precolumn (100-7, 50 × 21 mm) from Macherey-Nagel. Chromatographic conditions: flow, 10.0 mL/min; mobile phase A, 30% MeCN, 70% demin H_2O, 0.1% TFA; mobile phase B, 50% MeCN, 50% demin H_2O, 0.1% TFA; gradient, 1−43 min 0% to 100% mobile phase B; 43−53 min 100% mobile phase B isocrat; 53−63 min 0% to 100% mobile phase A; injection volume, 500−1000 μL. After lyophilization, 34 mg (36%) of **1** was obtained as a bright yellow solid.

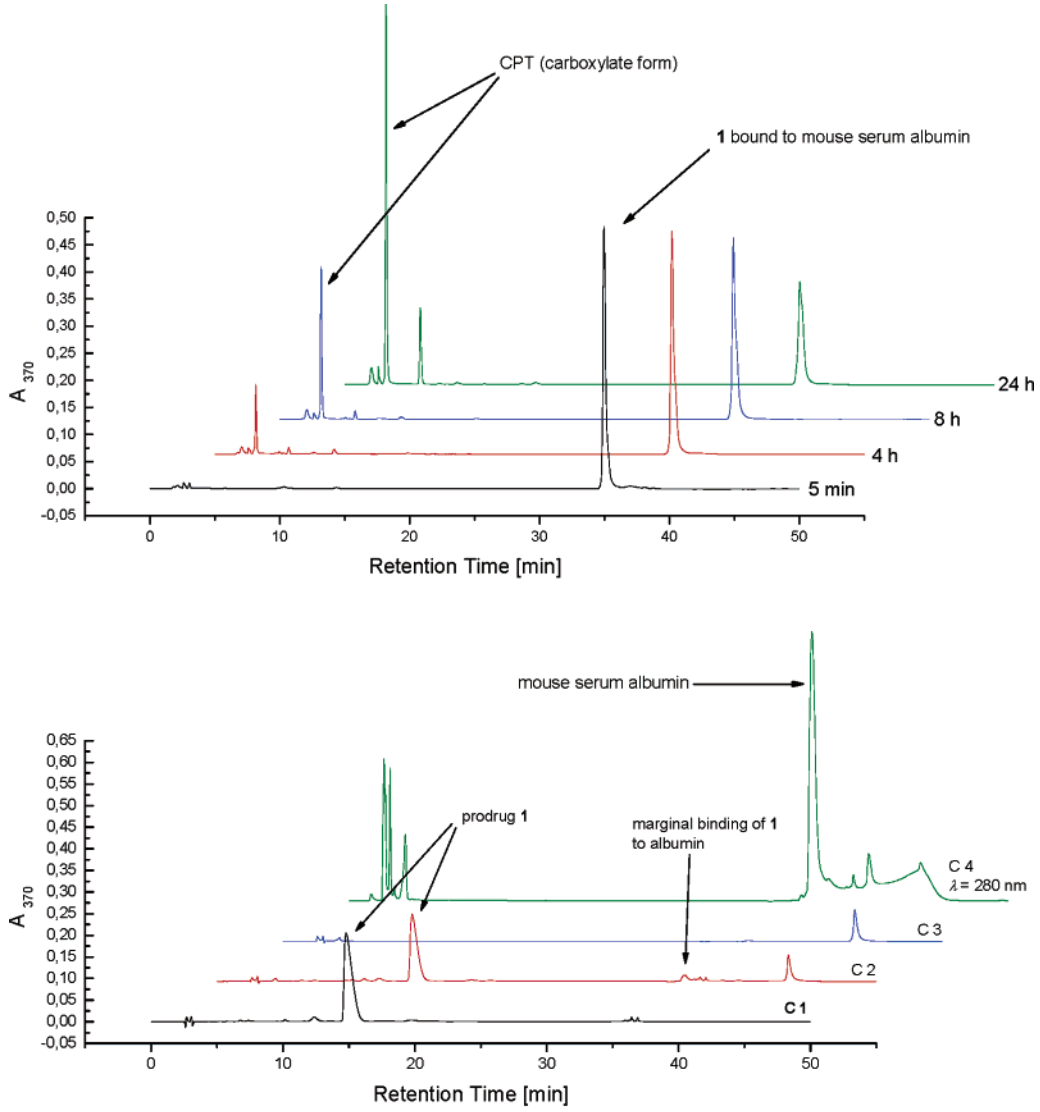
ESI-MS dir-pos (4.0 kV, 30% MeCN, 0.2% triethylammonium acetate (TEAA)): *m/z* (%) 1293.4 ($[M]^+$, 100), 1294.5 ($[M + H]^+$, 66). HPLC purity (λ = 370 nm): >95%.

Synthesis and Characterization of EMC-Arg-Arg-Ala-Leu-Ala-Leu-Doxo × 2 TFA (2). Doxorubicin hydrochloride (10.6 mg, 0.017 mmol), EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH × 2 TFA (21.6 mg, 0.019 mmol), and DIEA (10 μL, 0.060 mmol) were dissolved in 5 mL of anhydrous DMF. After stirring at room temperature for 15 min, HATU (6.5 mg, 0.017 mmol) was added, and the mixture was stirred at room temperature for 2 h. The product was precipitated with anhydrous diethyl ether, washed three times, and the solvent removed by centrifugation. The crude product was then purified by flash chromatography using reverse-phase Lichroprep RP-C18 from Merck with a particle size of 0.040−0.063 mm and acetonitrile/water 30:70 to afford 20 mg (83%) of product as a red powder after lyophilization. Mass (ESI-MS 5.0 kV, MeCN, 0.05% TFA): *m/z* (%) 1417.7 ($[M]^+$, 100), 1418.7 ($[M + H]^+$, 68). HPLC purity (495 nm): >95%.

Synthesis and Characterization of H-Leu-DOXO. Doxorubicin hydrochloride (696 mg, 1.20 mmol), Fmoc-Leu-OH (424 mg, 1.20 mmol), 1-hydroxybenzotriazole hydrate (162 mg, 1.20 mmol), and 4-methylmorpholine (264 μL, 2.40 mmol) were suspended in 20 mL of DMF. After stirring for 2 h, the solution became clear, and *N,N′*-diisopropylcarbodiimide (DIPC) (223 μL, 1.44 mmol) was added. After stirring at +5 °C for 4 days, DMF was removed in high vacuum. The doxorubicin amino acid derivative Fmoc-Leu-DOXO was purified through chromatography on silica gel using chloroform/methanol 10:1, affording 650 mg (62%) product as a red powder.

The protecting group was removed by dissolving Fmoc-Leu-DOXO (400 mg, 0.45 mmol) in 16 mL tetrahydrofuran and subsequent addition of 1,8-diazabicyclo[5.4.0]-undec-7-ene (98%) [(DBU, 400 μL)]. The blue mixture was stirred for 5 min and the product precipitated with diethyl ether. The crude solid was dried in high vacuum and then purified through chromatography on silica gel using chloroform/methanol 5:1 to afford 69 mg (23%) of H-Leu-DOXO as a red powder. Mass (ESI-MS, 4.0 kV, MeOH): *m/z* 657.1 $[M]^+$. HPLC purity (495 nm): >95%.

Synthesis of the Albumin Conjugates of 1 and 2 (HSA-1 and HSA-2). *For 1:* 1.5 mg of **1** were dissolved in 2 mL of human serum albumin (HSA) and were incubated under stirring at 37 °C for 1 h, after which no free **1** was detectable. The



C 1	**1** in glucose-phosphate buffer pH 5.8
C 2	**1** + mouse plasma blocked with EMC after 10 min
C 3	mouse plasma blocked with EMC
C 4	mouse plasma blocked with EMC at λ = 280 nm

Figure 2. Chromatograms of incubation studies of mouse plasma after 5 min, 4 h, 8 h, and 24 h with **1** at 37 °C and chromatograms of incubation studies of mouse plasma preincubated with an excess of 6-maleimidocaproic acid (EMC) for 2 h and subsequent incubation with **1** after 10 min at 37 °C. Chromatographic conditions: see Experimental Section. The peak in the blocking study with EMC at ~ 43 min results from a compound in the mouse plasma absorbing at 370 nm.

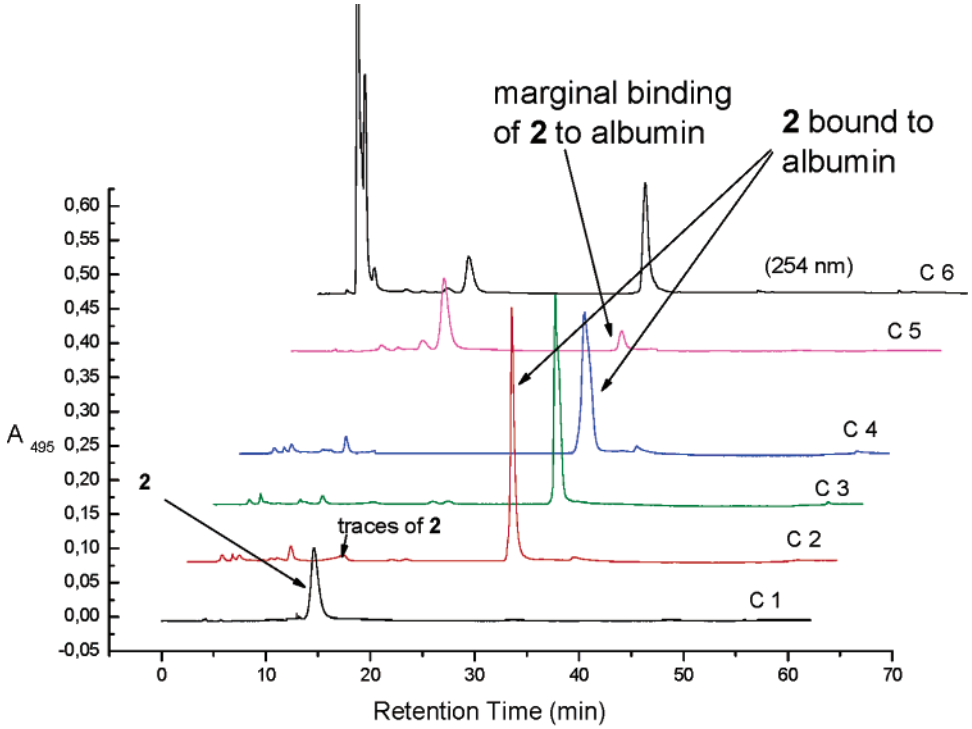
concentration was adjusted to 500 μM. The sample was kept frozen at −20 °C and thawed prior to use.

*For **2**:* 1.7 mg of **2** was dissolved in 2 mL HSA, and the solution was incubated at room temperature for 2 h. The albumin conjugate was obtained after subsequent size-exclusion chromatography (sephacryl S-100; Tris buffer: 50 mM Tris-HCl pH 7.4 or 50 mM sodium acetate buffer pH 5.0). The content of anthracycline in the sample was determined using the ϵ-value for doxorubicin [ϵ_{495} (pH 7.4) = 10 650 M^{-1} cm^{-1}]. The concentration of **2** in the conjugate was adjusted to 600 ± 50 μM by concentrating the sample with CENTRIPREP-10 concentrators (Amicon, FRG) (4 °C and 4500 rpm). Samples were kept frozen at −20 °C and thawed prior to use.

Incubation Studies of 1 and 2 with Mouse Blood Plasma. 0.75 mg of **1** or **2** was dissolved in 100 μL of sterile-filtered glucose-phosphate buffer (10 mM sodium phosphate/5% D-glucose buffer, pH 5.8). 30 μL aliquots of these solutions were added immediately to 570 μL of mouse plasma (gratefully received from Dr. N. Esser, Tumor Biology Center, Freiburg, FRG) and incubated at 37 °C. Samples were collected after 5 min, 4 h, 8 h, and 24 h and analyzed by HPLC. The decrease in the peak area of the conjugate at 370 nm for **1** and at 495 nm for **2** with time was used to determine the half-lives.

Incubation Studies of HSA-1 and HSA-2 with Cathepsin B. *For **1**:* 300 μL of the **HSA-1** stock solution (500 μM) were mixed with 290 μL of TEAA buffer (20 mM, pH 7.4 and pH 5.0) containing L-cysteine (8 mM) and 10 μL of a cathepsin B solution (71.7 μg/mL, 110 mU). The samples were incubated at 37 °C for 5 min, 2 h, and 24 h, and were analyzed by HPLC. Each 150 μL aliquot of the 2 and 24 h samples was kept frozen at −20 °C for subsequent analysis by LC-MS spectrometry.

*For **2**:* 150 μL of the **HSA-2** stock solution (600 μM) was mixed with 6 μL cathepsin B (71.7 μg/mL, 110 mU) and 204 μL of buffer (Tris buffer pH 7.4 and sodium acetate buffer pH



C 1	**2** in glucose-phosphate buffer pH 6.4
C 2	**2** + mouse plasma after 5 min
C 3	**2** + mouse plasma after 4 h
C 4	**2** + mouse plasma after 24 h
C 5/C 6	**2** + mouse plasma blocked with EMC after 10 min, **C 6**: λ = 254 nm

Figure 3. Chromatograms of incubation studies of mouse plasma after 5 min, 4 h, 8 h, and 24 h with **2** at 37 °C and chromatograms of incubation studies of mouse plasma preincubated with an excess of 6-maleimidocaproic acid (EMC) for 2 h and subsequent incubation with **2** after 10 min at 37 °C. Chromatographic conditions: see Experimental Section.

5.0) containing cysteine (8 mM). The samples at each pH (7.4 and 5.0) were incubated at 37 °C and were collected after 5 min, 1.5 h, 3 h, and 24 h. 60 μL of each sample was diluted with 40 μL of buffer to a final concentration of 150 μM and analyzed by HPLC with fluorescence detection.

Incubation Studies of HSA-1 and HSA-2 with Cathepsin D. *For **1**:* 150 μL of the **HSA-2** stock solution (500 μM) was mixed with 100 μL of cathepsin D (110.3 μg/mL, 15 U) and 50 μL of TEAA buffer pH 5 or pH 7.4. The samples were incubated at 37 °C for 5 min, 2 h, and 24 h and were analyzed by HPLC.

*For **2**:* 150 μL of the **HSA-2** stock solution (600 μM) was mixed with 150 μL cathepsin D (110.3 μg/mL, 15 U) and 150 μL of buffer (sodium acetate buffer pH 5.0) containing cysteine (8 mM). The samples were incubated at 37 °C for 5 min, 1.5 h, 3 h, and 24 h and were analyzed by HPLC.

Incubation Studies of HSA-1 and HSA-2 with Tumor Tissue Homogenates. For preparing the tumor homogenates, two protocols were used, the first at pH 5.0 that primarily liberates intracellular proteases (*27*) and the second at pH 7.4 that is used for extracting extracellular proteases (*28*). All following steps were carried out on ice. Tissues of colon HT-29 and mamma 3366 (M-3366) xenografts were cut into small pieces, and 200 mg samples were transferred into a 2 mL Eppendorf tube to which was added 800 μL of buffer (50 mM Tris−HCl buffer, pH 7.4, containing 1 mM monothioglycerol; or 50 mM sodium acetate buffer, pH 5.0, containing 100 mM sodium chloride, 4 mM EDTA Na_2, and 0.1% Brij 35). Homogenization was carried out with a micro-dissmemberator at 3000 rpm for 3 min with the aid of glass balls. Afterward, the samples were centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was aliquoted and kept frozen at −78 °C prior to use.

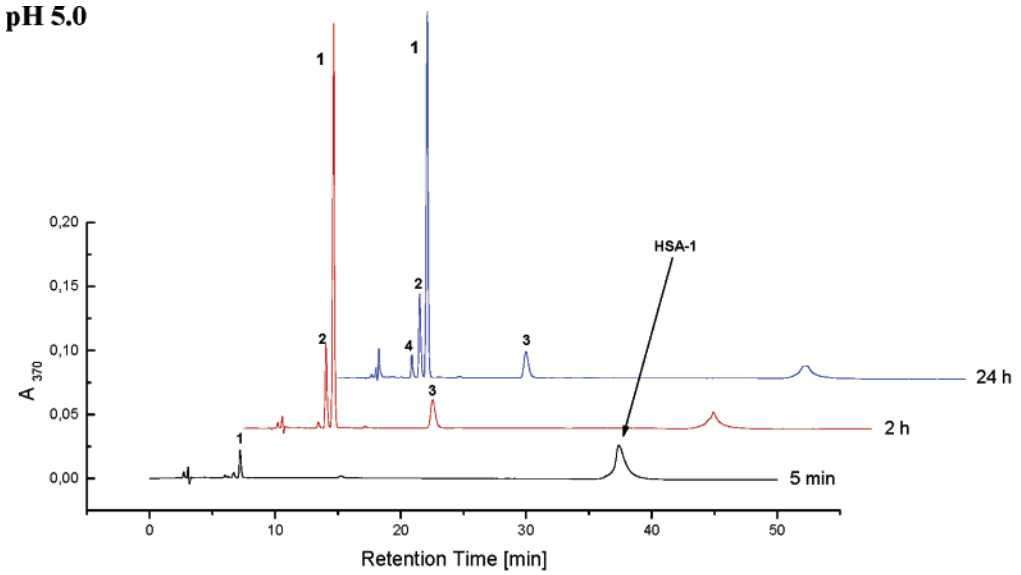
*For **1**:* 300 μL of the **HSA-1** stock solution (500 μM) was mixed with 150 μL of TEAA buffer (20 mM, pH 7.4 and pH 5.0) and 150 μL of HT-29 colon tumor tissue homogenate (pH 7.4 and pH 5.0). The samples were incubated at 37 °C for 5 min, 4 h, 8 h, and 24 h and were analyzed by HPLC. 150 μL of the 4 and 24 h samples were kept frozen at −20 °C and thawed prior to analysis by LC-MS spectrometry.

*For **2**:* 100 μL of the **HSA-2** stock solution (600 μM) was incubated with 100 μL of M-3366 tumor tissue homogenate (pH 7.4 and pH 5.0) at 37 °C for 5 min, 2 h, 5 h, and 24 h. At the mentioned times, 50 μL of each sample was diluted 1:1 to a final concentration of 150 μM and analyzed by HPLC.

Additionally, the HT-29 and M-3366 tumor homogenates were preincubated for 30 min with a 100 μM solution of the cathepsin B inhibitor CA074 (*29*) at a ratio of 10:1 (v/v), and cleavage properties were evaluated as described above.

In Vivo Efficacy of 1 and 2. For the in vivo testing of **1** and **2** in comparison with their parent compounds, female NMRI: nu/nu mice (in-house breeding) were used. The mice were held in individually ventilaged cages 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 acidified (pH 4.0) drinking water ad libitum. All animal experiments were performed under the auspices of the German Animal Protection Law.

Ten million cells of HT-29 and fragments of M-3366 tumor



Peak number	Retention time [min]	Observed fragment ions [*m/z*]	Peak assignment
1	7.20	555.1 $[M + Na]^+$	H-Leu-Ala-CPT
2	6.54	626.0 $[M + Na]^+$	H-Ala-Leu-Ala-CPT
3	15.04	739.1 $[M + Na]^+$, 717.0 $[M + 2H]^+$	H-Leu-Ala-Leu-Ala-CPT
4	5.88	349.1 $[M + H]^+$	CPT lactone

Figure 4. Chromatograms of incubation studies with **HSA-1** in the presence of cathepsin B (71.7 μg/mL, 110 mU) at pH 5.0 at 37 °C after 5 min, 2 h, and 24 h.

tissue were transplanted subcutaneously (s.c.) into the left flank region of anesthetized (40 mg/kg i.p. Radenarkon, Asta Medica, Frankfurt, Germany) mice on day zero. Mice were randomly distributed to the experimental groups (six mice per group). When the tumors were grown to a palpable size (90−130 mm^3), treatment was initiated. HT-29 xenografted mice were treated intravenously at days 6, 13, and 20 with 10 mM sodium phosphate/5% D-glucose buffer pH 5.8, camptothecin, or **1** (compound was administered as a solution in 10 mM sodium phosphate/5% D-glucose buffer pH 5.8); for doses, see corresponding tables and figures. The volume of administration was 0.23 mL/25 g body weight. M-3366 xenografted mice were treated intravenously at days 19 and 26 with 10 mM sodium phosphate/5% D-glucose buffer pH 7.0, doxorubicin, or **2** (compound was administered as a solution in 10 mM sodium phosphate/5% D-glucose buffer pH 7.0). The volume of administration was 0.2 mL/20 g body weight.

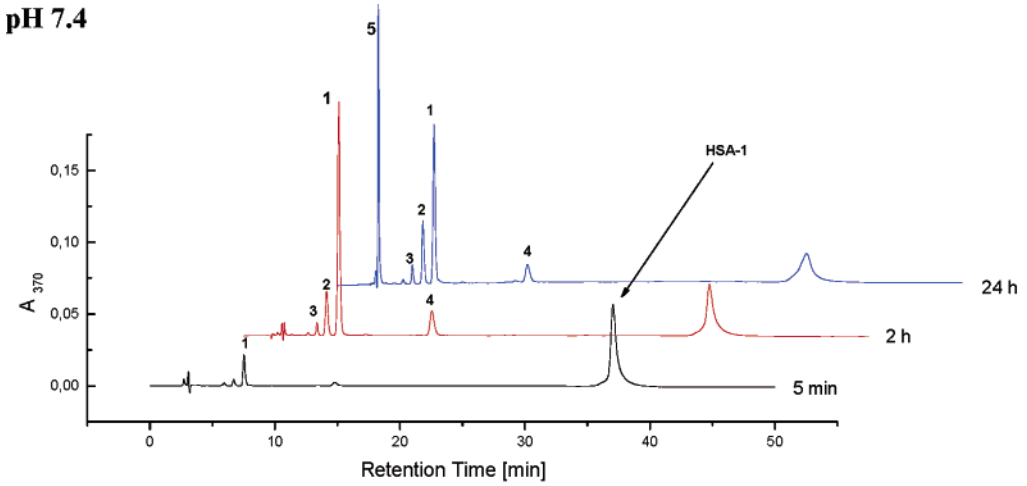
Tumor size was measured twice weekly with a caliper-like instrument in two dimensions. Individual tumor volumes (V) were calculated by the formula $V = (\text{length} + [\text{width}]^2)/2$ and related to the values on the first day of treatment (relative tumor volume, RTV). At each measurement day,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

RESULTS AND DISCUSSION

Chemistry. For the synthesis of the albumin-binding prodrug **1**, EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH was reacted with H-Ala-CPT × TFA, prepared according to a two-step procedure in which the first step is a stereoselective acylation of 20-(*S*)-camptothecin (*26*). Treatment of H-Ala-CPT × TFA with EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH × 2 TFA in the presence of HATU and DIEA afforded a quantitative reaction in a short time (Scheme 1). Purification of the crude product was achieved by preparative HPLC with a considerable loss in yield. The bright yellow solid was characterized by mass spectrometry, and purity was determined by HPLC (see Experimental Section). The incorporation of two arginines into the peptide linker of the prodrug **1** resulted in superior water solubility in buffer solution (>7 mg/mL) compared to the almost insoluble CPT (2.5 μg/mL).

For the synthesis of the albumin-binding prodrug **2**, EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH × 2TFA was coupled to the 3′-NH_2 position of doxorubicin in anhydrous DMF in the presence of HATU and DIEA as a one-step procedure. The crude product was purified through chromatography on a reverse-phase RP-C18 column. Prodrug **2** showed excellent water solubility with up to 10 mg/mL being dissolved in buffer solution in the pH range of 6.0 to 7.0.

Albumin-Binding Properties and Stability of 1 and 2. In order to show that prodrugs **1** and **2** bind rapidly and selectively to the cysteine-34 group of endogenous serum albumin, HPLC studies were performed in which mouse plasma was incubated with the prodrugs. Figures 2 and 3 show the chromatographic profiles of **1** and **2** incubated with mouse blood plasma after 5 min (λ = 370 nm for CPT derivatives, and λ = 495 nm for DOXO derivatives). While the free prodrugs elute at ∼15 min (**1**) and ∼14 min (**2**), the signals disappeared almost completely after incubation with mouse plasma, and a single peak eluting



Peak number	Retention time [min]	Observed fragment ions [*m/z*]	Peak assignment
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3	5.88	349.1 $[M + H]^+$	CPT lactone
4	15.04	739.1 $[M + Na]^+$, 717.0 $[M + 2H]^+$	H-Leu-Ala-Leu-Ala-CPT
5	3.37	349.1 $[M + H]^+$, 305.2 $[M + H - CO_2]^+$	CPT carboxylate

Figure 5. Chromatograms of incubation studies with **HSA-1** in the presence of cathepsin B (71.7 μg/mL, 110 mU) at pH 7.4 at 37 °C after 5 min, 2 h, and 24 h.

at the retention time of mouse serum albumin (MSA) (33−37 min) was observed.

In order to prove the fast binding results from a Michael addition of the sulfhydryl group of MSA with the maleimide group of the prodrugs, analogous HPLC experiments were carried out in which the cysteine-34 position of MSA was blocked with an excess of EMC prior to incubation with **1** and **2** (see Figures 2 and 3). In this case, marginal binding (**1**, ∼8%; **2**, <10%) to MSA was observed after incubating plasma with **1** and **2** for 10 min.

In order to determine the plasma stability of **1,** the respective albumin conjugate was generated by incubation of the CPT derivative with mouse blood plasma at 37 °C and subsequently analyzing samples after 3 min, 4 h, 8 h, 24 h, and 28 h through HPLC. From the decrease of the peak areas, the half-life of the conjugate of **1** was calculated as $t_{1/2} = 32$ h. Additionally, buffer stability of the albumin conjugate of **1** at pH 7.4 and pH 5.0 over 24 h was tested with the following results: degradation at pH 7.4, ∼15%; degradation at pH 5.0, <5% (chromatograms available as Supporting Information).

The stability of albumin-bound **2** in mouse blood plasma was assessed after **2** had been incubated at 37 °C with plasma for 4 and 24 h. HPLC showed that the albumin conjugate is highly stable over 24 h (∼5% degradation) (see Figure 3).

Cleavage Profiles of the Albumin Conjugate of 1. The ratio of the carboxylate form and the lactone form of released CPT is essential for the antitumor activity of the prodrug. It is known that the carboxylate form of CPT is inactive and even toxic, whereas the lactone form of CPT is the active agent. At more acidic pH, the equilibrium between the carboxylate and lactone forms generally shifts to the lactone form of CPT.

The cleavage properties of compound **1** were evaluated using enzymatically active cathepsin B, cathepsin D, and HT-29 colon tumor tissue homogenates at both pH 7.4 and pH 5.0. After incubation with cathepsin D, no cleavage of **HSA-1** could be detected (data not shown). In contrast, incubation with cathepsin B and HT-29 colon tissue homogenate resulted in a fast and almost complete cleavage over a period of 24 h, especially at pH 5.0 (see Figures 4, 5, 7, and 8). The different cleavage products were identified by LC-MS and by the use of CPT and H-Ala-CPT standards. Unfortunately, the HPLC method used for incubation studies could not be transferred one-to-one to the LC-MS investigations. Therefore, the cleavage products could not be assigned by retention times but by their peak areas and by the use of the reference substances (mass spectra of the individual peaks are available as Supporting Information). For cleavage with cathepsin B (see Figures 4 and 5), the following products were identified: H-Leu-Ala-Leu-Ala-CPT, H-Ala-Leu-Ala-CPT, H-Leu-Ala-CPT as the major product, CPT (lactone form), and additionally at pH 7.4, CPT in the carboxylate form. Cleavage in HT-29 colon tumor homogenate (see Figures 7 and 8) at pH 5.0 resulted in a more pronounced degradation of the albumin conjugate over 24 h than that observed at pH 7.4. Interestingly, the cleavage profile with tumor homogenate additionally showed the formation of H-Ala-CPT, which could not be observed after cleavage with cathepsin B. The identified cleavage products at pH 5.0 were H-Ala-Leu-Ala-CPT, H-Leu-Ala-CPT, H-Ala-CPT, and CPT (lactone form). After 4 h of incubation, the main products were H-Leu-Ala-CPT and H-Ala-CPT, changing after 24 h of incubation to H-Ala-CPT and CPT in the carboxylate form. At pH 7.4, H-Leu-Ala-CPT, H-Ala-CPT, CPT (lactone form), and CPT in the carboxylate form were found. Cleavage at pH 7.4 to CPT in the carboxylate form is also attributed to unspecific hydrolysis, although cleavage in pH 7.4 buffer solution after 24 h was not as

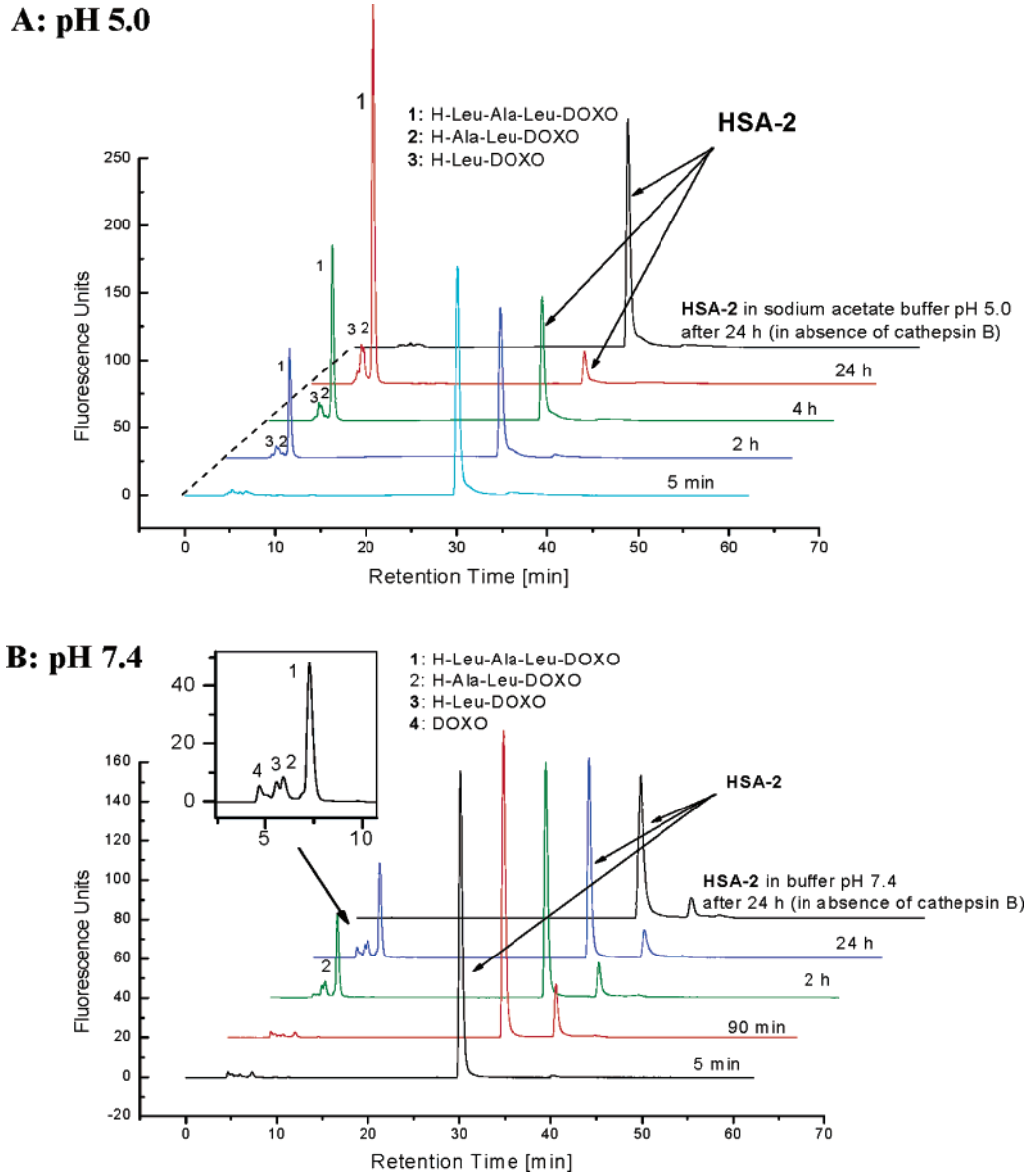


Figure 6. Chromatograms of incubation studies with **HSA-2** in the presence of cathepsin B (71.7 μg/mL, 110 mU) at pH 5.0 (A) and pH 7.4 (B) at 37 °C after 5 min, 2 h, 24 h and 5 min, 90 min, 2 h, 24 h respectively.

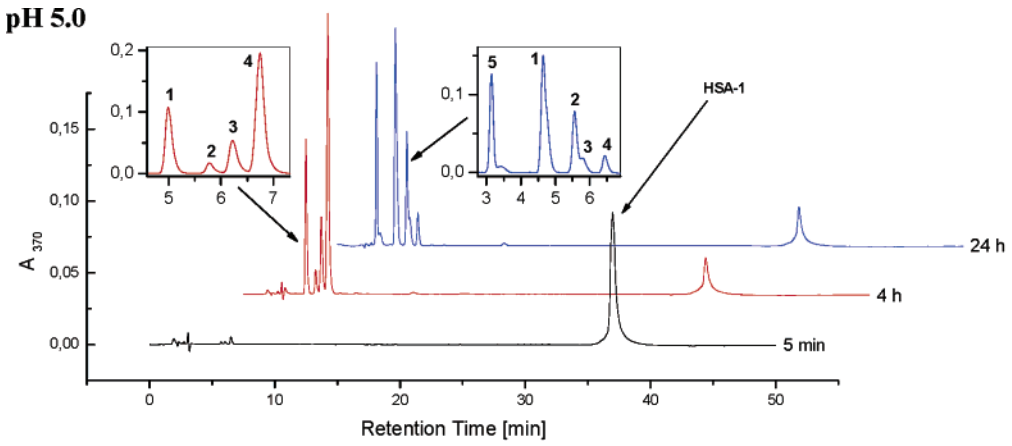
pronounced as with tumor tissue homogenate or cathepsin B. Additionally, the cleavage properties of the HT-29 tumor homogenate preincubated with the cathepsin B inhibitor CA074 (*29*) at pH 5.0 were investigated to determine if cathepsin B is the major protease responsible for the cleavage of the peptide sequence. The corresponding chromatograms (see Figure 9) recorded after 5 min, 4 h, and 24 h show that the cleavage of prodrug **1** was notably inhibited with respect to the cleavage products H-Leu-Ala-CPT, H-Ala-Leu-Ala-CPT, and CPT (carboxylate form).

The appearance of the carboxylate form of CPT at pH 7.4 in several studies, especially in the mouse plasma stability study (Figure 2) and the study with tumor homogenate (Figure 8), might be related to the fact that proteins, primarily albumin, switch the equilibrium to the carboxylate form due to interaction with the binding domain IIA (*30*−*35*).

The incubation study at pH 5 with **HSA-1** in the presence of cathepsin B (Figure 4) showed no generation of CPT in the carboxylate form, in contrast to the incubation study with HT-29 tumor homogenate at the same pH value (Figure 7). Two reasons could explain this difference: (a) preferential binding of the carboxylate form to proteins in the tumor tissue homogenate, and (b) in the presence of cathepsin B, no H-Ala-CPT was generated, which readily releases free CPT; di-, tri-, and tetrapeptide derivatives of CPT are reported to be more stable than amino acid derivatives of CPT (*36*).

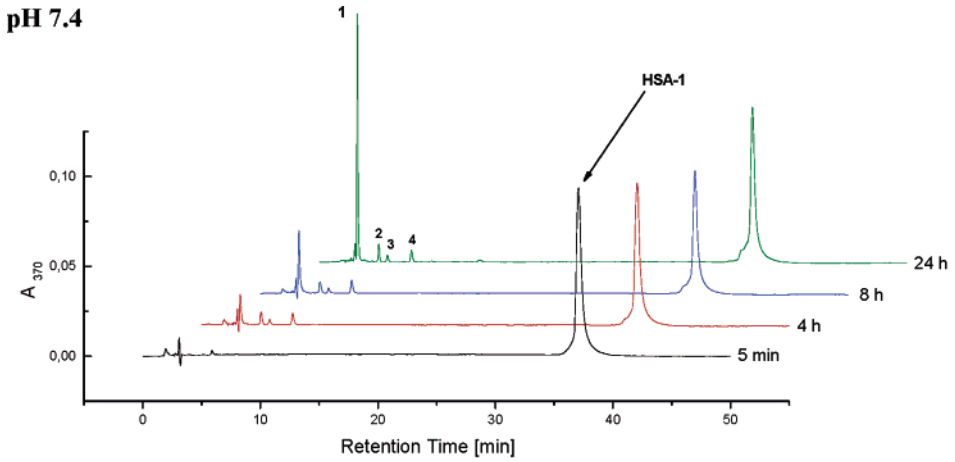
Cleavage Profiles of the Albumin Conjugate of 2. In order to investigate whether and how fast **HSA-2** is cleaved by cathepsin B at pH 5.0 and pH 7.4 as well as by cathepsin D at pH 5.0, **HSA-2** was incubated with the enzymes and chromatograms recorded with fluorescence spectrophotometer using reverse-phase HPLC.

Rapid cleavage to a main product eluting at 6.3 min (see Figure 6A) was observed after incubating **HSA-2** with cathepsin B at pH 5.0. After 24 h of incubation, cleavage was almost complete. In order to characterize this cleavage product, 1000 μL [1100 μM] **HSA-2** and 300 μL of cathepsin B were incubated, and the cleavage product was isolated through size exclusion chromatography (sephadex G-25) using demineralized water. Mass spectrometry of the lyophilized sample showed a distinct mass peak at 840.8 $[M]^+$ and its Na^+ adduct at 863.1 $[M + Na]^+$ corresponding to [H-Leu-Ala-Leu-DOXO] (mass spectrum available as Supporting Information). Small amounts



Peak number	Retention time [min]	Observed fragment ions [*m/z*]	Peak assignment
1	5.05	331.2 [– alanine]$^+$, 303.2 [– alanine – CO]$^+$	H-Ala-CPT
2	5.71	349.1 [M + H]$^+$	CPT lactone
3	6.21	626.1 [M + Na]$^+$	H-Ala-Leu-Ala-CPT
4	6.75	555.0 [M + Na]$^+$	H-Leu-Ala-CPT
5	3.20	349.1 [M + H]$^+$, 305.2 [M + H – CO_2]$^+$	CPT carboxylate

Figure 7. Chromatograms of incubation studies with **HSA-1** and HT-29 tissue homogenate after 5 min, 4 h, (8 h), and 24 h at pH 5.0 at 37 °C.



Peak number	Retention time [min]	Observed fragment ions [*m/z*]	Peak assignment
1	3.22	349.1 [M + H]$^+$, 305.2 [M + H – CO_2]$^+$	CPT carboxylate
2	5.05	331.2 [– alanine]$^+$, 303.2 [– alanine – CO]$^+$	H-Ala-CPT
3	5.88	349.1 [M + H]$^+$	CPT lactone
4	7.88	555.0 [M + Na]$^+$	H-Leu-Ala-CPT

Figure 8. Chromatograms of incubation studies with **HSA-1** and HT-29 tissue homogenate after 5 min, 4 h, (8 h), and 24 h at pH 7.4 at 37 °C.

of the cleavage products H-Leu-DOXO and H-Ala-Leu-DOXO were also observed, which were identified by an additional LC-MS experiment (mass spectra available as Supporting Information).

The cleavage of **HSA-2** by cathepsin B at pH 7.4 showed the same cleavage products, especially [H-Leu-Ala-Leu-DOXO] at pH 5.0, but cleavage at pH 7.4 proved to be less effective and much slower; therefore, the cathepsin B concentration for the incubation study at pH 7.4 was increased (see Figure 6B). Incubation studies at pH 7.4 with **HSA-2** also show a sparse degradation to an unidentified product eluting at ~37 min only visible with fluorescence detection.

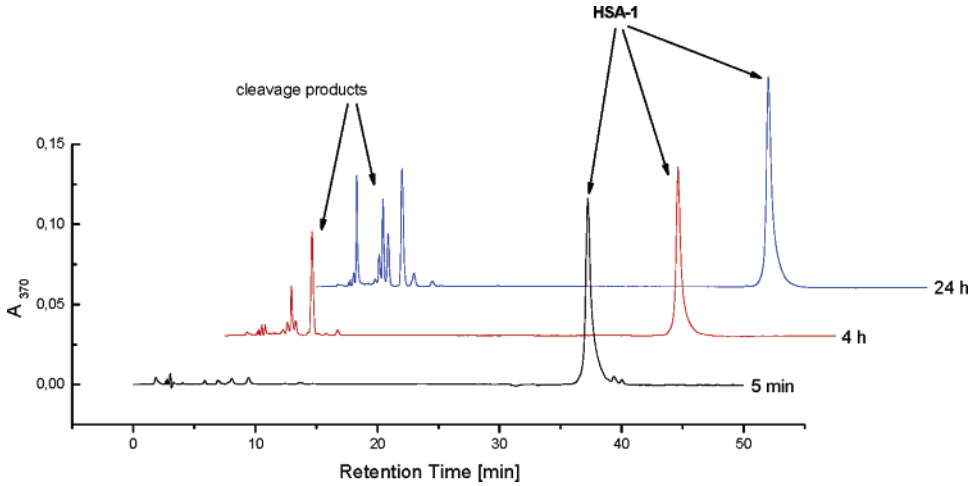


Figure 9. Chromatograms of incubation studies with **HSA-1** and HT-29 tissue homogenate preincubated with the cathepsin B inhibitor CA-074 after 5 min, 4 h, and 24 h at pH 5.0 and 37 °C. Chromatographic conditions: see Experimental Section.

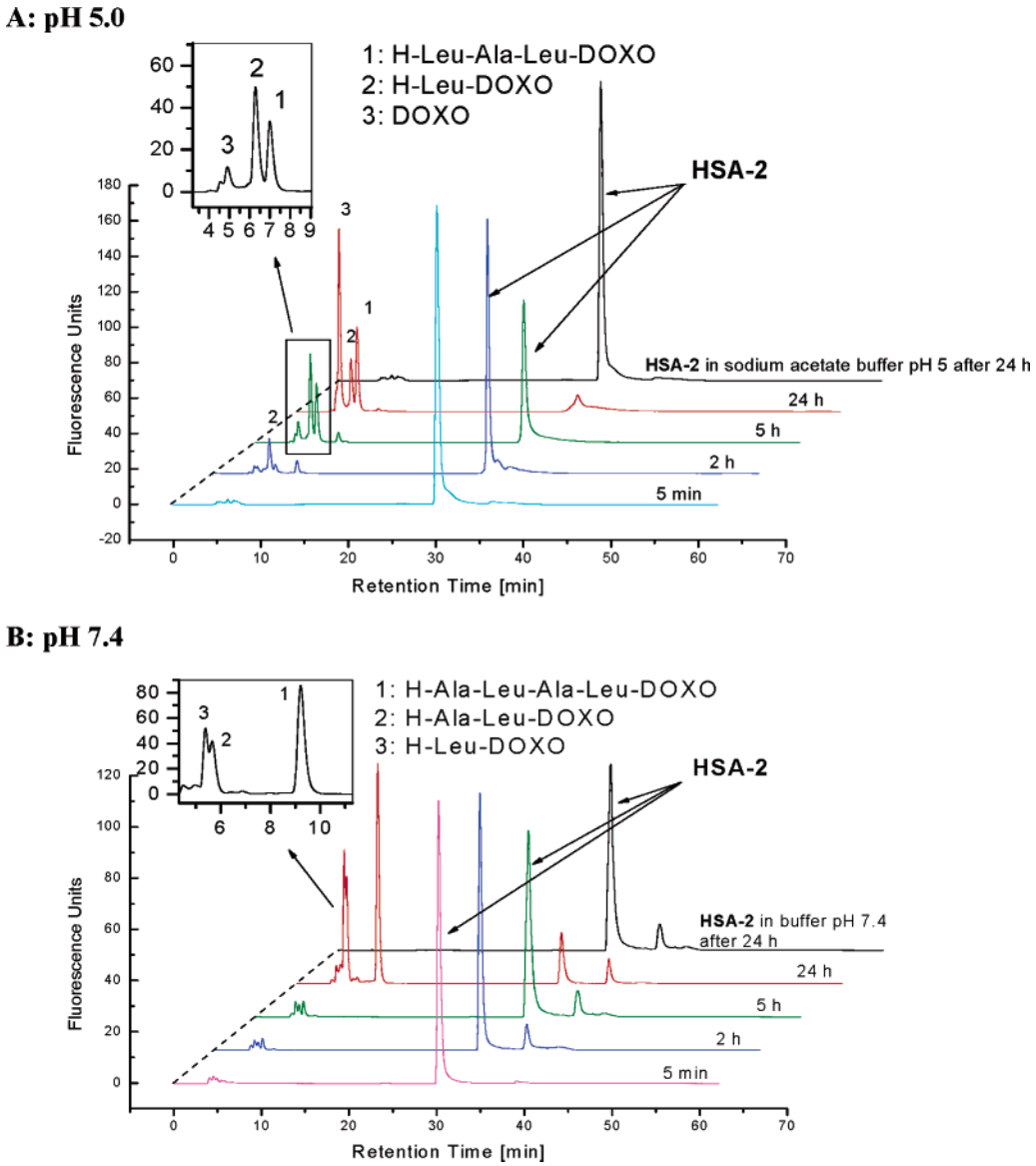


Figure 10. Chromatograms of incubation studies with **HSA-2** and M-3366 tissue homogenate after 5 min, 2 h, and 5 h at pH 5.0 (A) and pH 7.4 (B) at 37 °C.

Incubation studies with **HSA-2** and cathepsin D showed only marginal cleavage (data not shown).

In order to obtain a picture of the cleavage profile in biological samples, we incubated both **HSA-2** and the cleaved

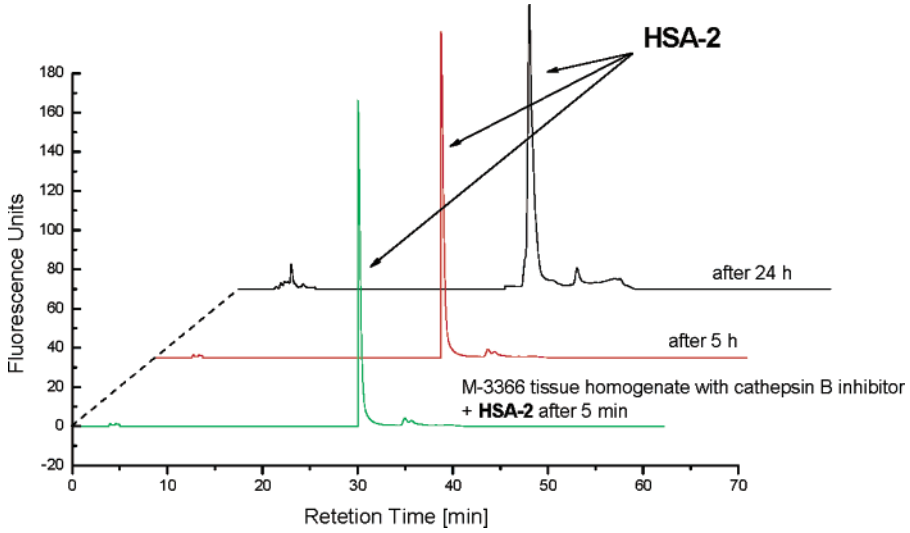


Figure 11. Chromatograms of incubation studies with **HSA-2** and M-3366 tissue homogenate preincubated with the cathepsin B inhibitor CA-074 after 5 min, 5 h, and 24 h at pH 5.0 and 37 °C.

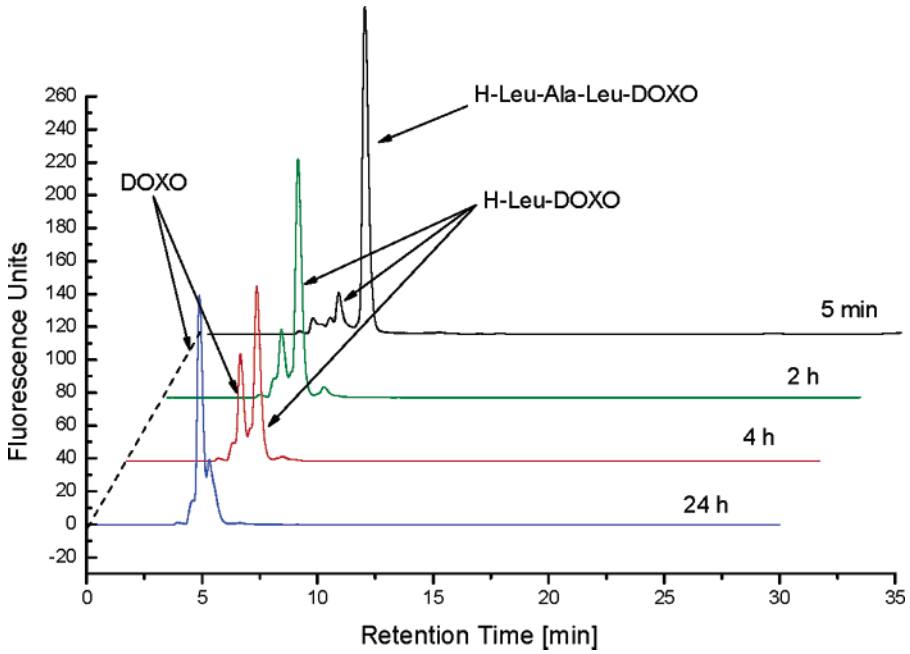


Figure 12. Chromatograms of incubation studies with the cleavage product [H-Leu-Ala-Leu-DOXO] and M-3366 tissue homogenate after 5 min, 2 h, 4 h, and 24 h at 37 °C. Concentration of [H-Leu-Ala-Leu-DOXO] was 125 μM. Chromatographic conditions: see Experimental Section.

Table 1. Antitumor Activity of CPT Prodrug 1 against Human Colorectal Xenografts (HT-29) in Vivo

compound	dose[a] [mg/kg]	total mortality	body weight change [%] (day 6−9)	*T/C* [%] maximal
CPT	3 × 12.5	0/6	−2	40[b] (day 22)
1	3 × 12.5	1/6 (on day 20)	−1	17[b,c] (day 29)

[a] Dose refers to CPT equivalents. [b] Significant to buffer solution. [c] Significant to camptothecin.

doxorubicin tripeptide [H-Leu-Ala-Leu-DOXO] with M-3366 tissue homogenates and analyzed the cleavage products over 24 h (see Figures 10and 12). The cleavage products were identified by "peak spiking" with H-Leu-DOXO, H-Leu-Ala-Leu-DOXO, and DOXO references, which were added to the incubated samples. An increase of the peak area at the corresponding retention time confirmed the identification of the respective cleavage product.

Incubation with **HSA-2** at pH 5.0 showed two cleavage products, H-Leu-Ala-Leu-DOXO and H-Leu-DOXO after 2 h. After 5 h, the main cleavage product was H-Leu-DOXO and small amounts of DOXO were observed, which increased over 24 h (see Figure 10A). Incubation with **HSA-2** at pH 7.4 showed a different cleavage profile than at pH 5.0. Only after 24 h were the cleavage products H-Leu-DOXO, H-Ala-Leu-DOXO, and the tetrapeptide derivative H-Ala-Leu-Ala-Leu-DOXO eluting at 9 min observed that were obviously produced by enzymes in M-3366 tissue homogenate other than cathepsin B (see Figure 10B). Identification of these cleavage products was performed by LC-MS (mass spectra of the individual peaks are available as Supporting Information).

In a further experiment, we preincubated the M-3366 tissue homogenate with the cathepsin B inhibitor CA-074 at pH 5.0 in order to elucidate whether cathepsin B is responsible for cleaving the peptide sequence to the cleavage products H-Leu-Ala-Leu-DOXO and H-Leu-DOXO. As shown in Figure 11, no cleavage was observed after 5 and 24 h, confirming that

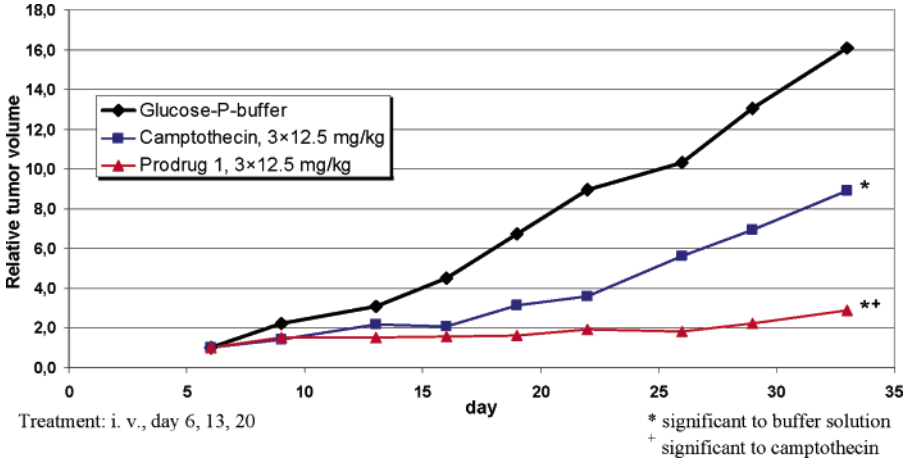


Figure 13. Curves depicting tumor growth inhibition of subcutaneously HT-29 xenografts under therapy with CPT and prodrug **1**.

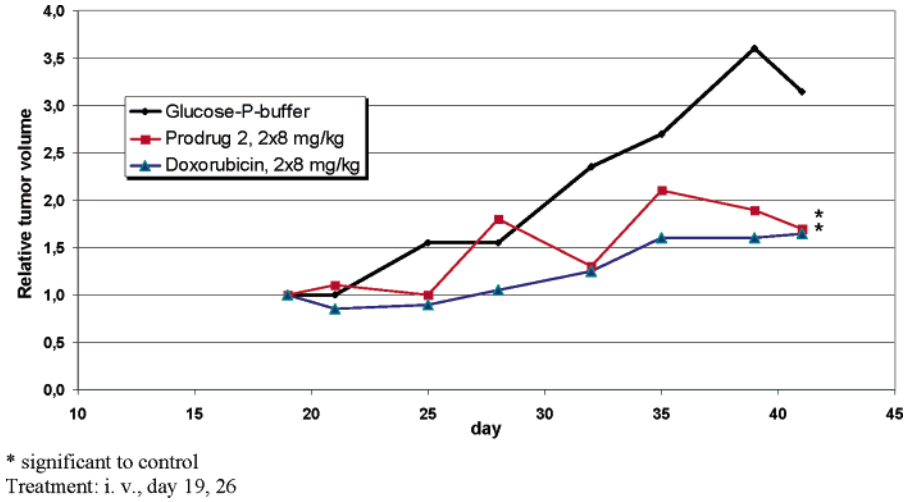


Figure 14. Curves depicting tumor growth inhibition of subcutaneous M-3366 xenografts under therapy with doxorubicin and prodrug **2**.

cathepsin B is the major enzyme responsible for the cleavage in M-3366 tissue homogenate at pH 5.0.

H-Leu-Ala-Leu-DOXO was cleaved primarily to H-Leu-DOXO in M-3366 tissue homogenate after 2 h, and almost complete degradation to DOXO over 24 h was observed (see Figure 12).

H-Leu-DOXO is known to have activity against several human tumor cell lines, e.g., MCF-7 (breast carcinoma) (*37*, *38*), HT-1080 (fibrosarcoma) (*39*), and LNCaP (prostate carcinoma) (*40*−*46*); it was also shown to have less cardiac toxicity than DOXO in animal models (*40*, *41*).

In Vivo Activity of 1 and 2 Compared to the Free Drug. The in vivo efficacy of **1** and CPT was assessed in xenografted nude mice using a human HT-29 colorectal cell line. CPT was evaluated at a dose of 3 × 12.5 mg/kg (i.v.), which was reported to be the maximum tolerated dose (MTD) for camptothecin in nude mice (*47*). Prodrug **1** was tested at the same dose of 3 × 12.5 mg/kg (i.v.) CPT equivalents. Table 1 and Figure 13 show the results of this experiment. In general, although one animal died, therapy with **1** produced no significant body weight loss (see Table 1 and curves depicting body weight change, available as Supporting Information). Whereas CPT (3 × 12.5 mg/kg) was moderately active (*T*/*C* max: 40%), **1** exhibited superior antitumor activity at the same dose, and mice treated with **1** showed a good antitumor response (*T*/*C* max: 17%).

The antitumor efficacy of **2** was evaluated in nude mice using a breast carcinoma 3366 (M-3366) xenograft in a strict comparison to free doxorubicin. A preliminary toxicity study in two healthy mice showed that the MTD of **2** was 1 × 24 mg/kg doxorubicin equivalents and thus ∼3-fold higher than for free doxorubicin. Consequently, **2** was compared with doxorubicin at the following doses: doxorubicin at a dose of 2 × 8 mg/kg corresponding to the MTD of doxorubicin in nude mice and **2** at doses of 2 × 8 mg/kg doxorubicin equivalents and 1 × 24 mg/kg doxorubicin equivalents. The results of these in vivo experiments are shown in Table 2 and Figure 14.

Table 2. Antitumor Activity of DOXO Prodrug 2 Against Human Mamma Carcinoma Xenografts (M-3366) in Vivo

compound	dose[a] [mg/kg]	total mortality	body weight change [%] (day 6−9)	*T*/*C* [%] maximal
DOXO	2 × 8	0/6	−9	44[b] (day 39)
2	2 × 8	1/6 (on day 20)	0	53[b] (day 39)
2	1 × 24	5/6 (on day 20)	−14	n.e.[c]

[a] Dose refers to DOXO equivalents. [b] Significant to buffer solution [c] n.e.: not evaluatable.

Table 2 and Figure 14.

Doxorubicin at its optimal dose of 2 × 8 mg/kg produced a moderate inhibition in tumor growth with a body weight loss of up to −9% with a slow recovery thereafter. The efficacy of **2** at 2 × 8 mg/kg was comparable with the therapeutic results of doxorubicin at 2 × 8 mg/kg, but no body weight loss was observed (see curves depicting body weight change, available as Supporting Information). After the first application of **2** at the dose of 2 × 8 mg/kg, one animal died due to an acute reaction. After application, all mice showed a mild ataxia and red flushing prolonging for 20 min. The in vivo testing of **2** at the dose of 1 × 24 mg/kg was aborted after the first application

due to acute toxicity (5 of 6 mice died), an observation that was not made in preliminary toxicity studies in healthy nude mice.

CONCLUSIONS

In the past 30 years, cysteine proteases have been known to be involved in a number of steps in tumor progression, such as tumor growth, invasion, migration, and the formation of metastasis (*48*). Especially, the cysteine proteases of the papain family such as cathepsins B, L, H, and S have been investigated intensively in the past. Cathepsins are known to digest and cleave a number of tissues such as collagen and proteoglycane (*49*). The cysteine protease cathepsin B is mostly localized in lysosomes (*18*), and overexpression of cathepsin B has been found in various malignant tumors, especially in melanoma (*22*), breast (*20*), lung (*23*, *24*), and colon (*25*) carcinoma. The overexpressed levels of cathepsin B in tumors as well as in some extracellular fluids are associated with tumor progression and with disease-free and overall survival and may therefore serve as a prognostic factor for cancer patients.

Thus, we developed two albumin-binding prodrugs incorporating a peptide sequence which can be specifically cleaved by cathepsin B, releasing an amino acid derivative of doxorubicin or camptothecin, which subsequently is cleaved to free doxorubicin and camptothecin as the final product.

In the past few years, several low- and high-molecular-weight prodrugs of doxorubicin have been developed that were designed to be activated by cathepsin B. The HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate PK1 incorporating a cathepsin B cleavable linker has been evaluated clinically (*50*). Furthermore, two immunoconjugates of doxorubicin, in which DOXO is linked to the chimeric BR96 antibody through lysosomally cleavable dipeptides Phe-Lys or Val-Cit (BR96-Phe-Lys-PABC-DOXO and BR96-Val-Cit-PABC-DOXO; PABC = *para*-aminobenzyloxycarbonyl) (*51*) have been developed. Both conjugates showed rapid and nearly quantitative drug release with cathepsin B.

To date, three polymeric derivatives of camptothecin have entered clinical trials: two HPMA copolymer-bound camptothecin drugs (*47*, *52*) and Prothecan, a PEG-based CPT-Ala prodrug (*53*). In all cases, binding CPT to polymers leads to highly water-soluble formulations which release the drug in a controlled fashion due to the incorporation of a hydrolyzable or enzymatically degradable amino acid spacer. Especially, cleavage by cathepsin B was described for the following CPT prodrugs: a camptothecin analogue-carboxymethyl dextran conjugate bearing a Gly-Gly-Gly linker (*54*), an immunoconjugate of CPT containing a Phe-Lys linker (*55*), a poly(ethylene glycol) conjugate of 10-amino-7-ethylcamptothecin (*56*), and recently a folate receptor-targeted PEG-peptide conjugate of CPT (*57*).

In the present work, we could show that cathepsin B cleaves the Ala-Leu-Ala-Leu linker in the albumin-bound form of the CPT prodrug **1** and the DOXO prodrug **2** in all of the peptide bonds adjacent to the EMC-Arg-Arg-Ala residue. Cleavage was more efficient at pH 5.0 than at pH 7.4. In tumor homogenates, the cleavage pattern was similar, but a pronounced cleavage to CPT (and the CPT carboxylate form) and to DOXO was observed. Although the extent of cleavage for both prodrugs was similar in the HT-29 and M-3366 tumor xenografts, **1** was significantly more active than CPT in contrast to **2**, which was as effective as doxorubicin. A possible explanation for this difference could be differences in the EPR (enhanced permeation and retention) effect for these tumors.

In summary, we have prepared albumin-binding prodrugs with the anticancer drug camptothecin and doxorubicin that are water-soluble, bind rapidly and selectively to the cysteine-34 position of endogenous serum albumin, and are cleaved specifically by cathepsin B. Further studies of **1** in a larger panel of human tumor xenografts should reveal the potential of this CPT prodrug.

ACKNOWLEDGMENT

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Supporting Information Available: Chromatography; curves depicting body weight change; chromatograms of the albumin conjugate of prodrug **1** (250 μM) depicting the buffer stability; mass spectra of the cleavage products of **1** and mass spectra of the cleavage products of **2**. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

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