Full Paper

Synthesis, Cleavage Profile, and Antitumor Efficacy of an Albumin-Binding Prodrug of Methotrexate that is Cleaved by Plasmin and Cathepsin B

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Cathepsin B and plasmin are intra- or extracellular proteases that are overexpressed by several solid tumors. In order to exploit both proteases as molecular targets for tumor-specific cleavage of prodrugs, an albumin-binding formulation of methotrexate was developed that incorporated the peptide sequence D-Ala-Phe-Lys as the protease substrate. Albumin is a suitable carrier for cytostatic agents due to passive accumulation in solid tumors. Synthesis was performed by coupling the peptide linker EMC-D-Ala-Phe-Lys(Boc)-Lys-OH (EMC = ε-maleimidocaproic acid) to the γ-COOH group of α-*tert*-butyl protected methotrexate. After cleavage of the protective groups and purification on reverse phase HPLC, a highly water-soluble methotrexate-peptide derivative was obtained that binds rapidly and selectively to human serum albumin. The albumin-bound form of the prodrug was shown to be efficiently cleaved by cathepsin B and plasmin as well as in an ovarian carcinoma homogenate (OVCAR-3) liberating a methotrexate-lysine derivative. In an OVCAR-3 xenograft model, the prodrug at a dose of 4×15 mg/kg methotrexate equivalents demonstrated distinctly superior antitumor efficacy compared to free methotrexate at a dose of 4×100 mg/kg [T/C(%) for MTX = 69; T/C(%) for MTX prodrug = 29]. The data provide a further proof of concept for the development of albumin-binding, enzymatically cleavable prodrugs of anticancer drugs.

Keywords: Cathepsin B / Human serum albumin / Macromolecular prodrug / Methotrexate / Plasmin

Received: February 12, 2007; accepted: May 4, 2007

DOI 10.1002/ardp.200700025

Introduction

Therapy of cancer diseases with cytostatic drugs is mainly restricted by adverse effects that range from cosmetic problems to severe and – consequently – dose-limiting disorders. When administered, most low-molecular weight anticancer agents are evenly distributed over the organism thus damaging all proliferating cells including healthy ones. Therefore, therapeutic strategies to overcome these drawbacks and to make anticancer drugs act more tumor-specifically should concentrate on inherent differences between normal and malignant tissue. A promising approach is to employ macromolecules as vehicles for anticancer drugs [1]. One to several low-molecular weight drugs are covalently linked to a macromolecular carrier, *e.g.* synthetic polymers or serum proteins. This macromolecular prodrug concept exploits the differences between tumor tissue and healthy tissue on two levels: First, a passive accumulation of macromolecules in tumor tissue is achieved due to vascular anoma-

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Abbreviations: Boc: *tert*-butoxycarbonyl; **BOP:** (benzotriazol-1-yloxy)-tris-(dimethylamino)phosphonium hexafluorophosphate; **DIEA:** diisopropylethylamine; **DMF:** *N,N*-dimethylformamide; **HATU:** *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; **HSA:** human serum albumin; **MTD**: maximum tolerated dose; **MTX:** methotrexate

lies and an impaired lymphatic drainage (EPR, enhanced permeability and retention) [2]. Second, enzymes that are overexpressed in malignant tissue can be exploited for tumor-specific release of the carrier-bound drugs.

The overexpression of a variety of proteases is well documented for a number of different tumors, and these enzymes are known to play a decisive role in metastasis and tumor progression [3–5]. Elevated levels were reported for cathepsins [6], matrix metalloproteases [7], urokinase-type plasminogen activator (uPA) [8], prostate-specific antigen (PSA) [9], and plasmin [8]. Short peptide sequences that are cleaved by these proteases can be employed as linkers acting as a molecular bridge between the drug and the carrier. Ideally, such linkers are non-toxic and non-immunogenic, plasma stable, and rapidly and selectively cleaved in malignant tissue to release the drug in its active form.

Previously, we developed a number of albumin-binding prodrugs of doxorubicin incorporating peptide sequences that were cleaved by uPA [10], PSA [11], cathepsin B [12], and the matrix metalloproteases 2 and 9 [13, 14]. These prodrugs were shown to bind rapidly and selectively to circulating serum albumin after intravenous administration, and the respective drug-albumin conjugates were selectively cleaved by the target enzymes or after incubation with native tumor material (tissue homogenate). In this work, we report on the first albumin-binding formulation of the anticancer drug methotrexate (MTX). The prodrug incorporates the peptide sequence D-Ala-Phe-Lys which is known to be cleaved by either the extracellular enzyme plasmin [15] or the lysosomal protease cathepsin B [16]. For both proteases, an overexpression in various solid tumors is documented [6, 8]. Synthesis of the prodrug, HPLC studies on stability in plasma and its cleavage properties in the presence of the target enzymes as well as an *in vivo* experiment in xenografted mice are described.

Results and discussion

Results

We designed a prodrug of MTX that consists of the drug, a lysine spacer, and an enzymatically cleavable peptide linker bearing the albumin-binding maleimide group. The MTX's glutamic acid moiety possesses two carboxylate groups that can serve as functional groups for attaching the drug to carrier molecules. However, it has been shown that a free α-carboxylate group is essential for retaining the drug's efficacy whereas chemical modifications of the γ-carboxylate group are much better tolerated [17]. Therefore, our synthesis of the prodrug started with the preparation of α-protected MTX (**2**). Compound **2** was obtained by condensing the MTX precursor 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) with H-Glu(OMe)-Ot-Bu using BOP as the coupling agent (see Scheme 1). Subsequent hydrolysis of the methyl ester **1** afforded the MTX α-*tert*-butyl ester **2** that was further reacted with the maleimido peptide EMC-D-Ala-Phe-Lys(Boc)-Lys-OH. The MTX prodrug **3** was obtained after removal of the protective groups, chromatography on RP silica gel and lyophilization. Compound **3** displays an excellent water-solubility (>30 mg/mL in water) and is conveniently administered in buffer solution.

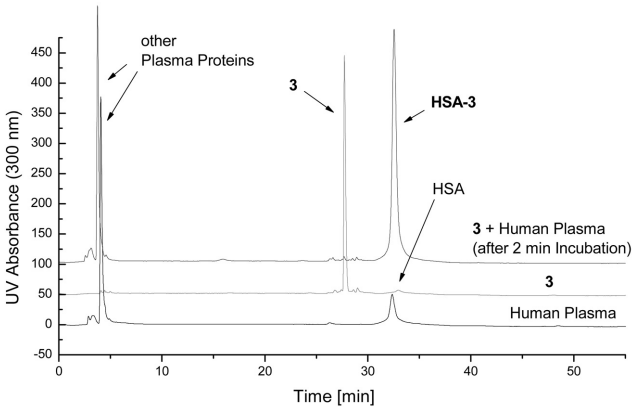
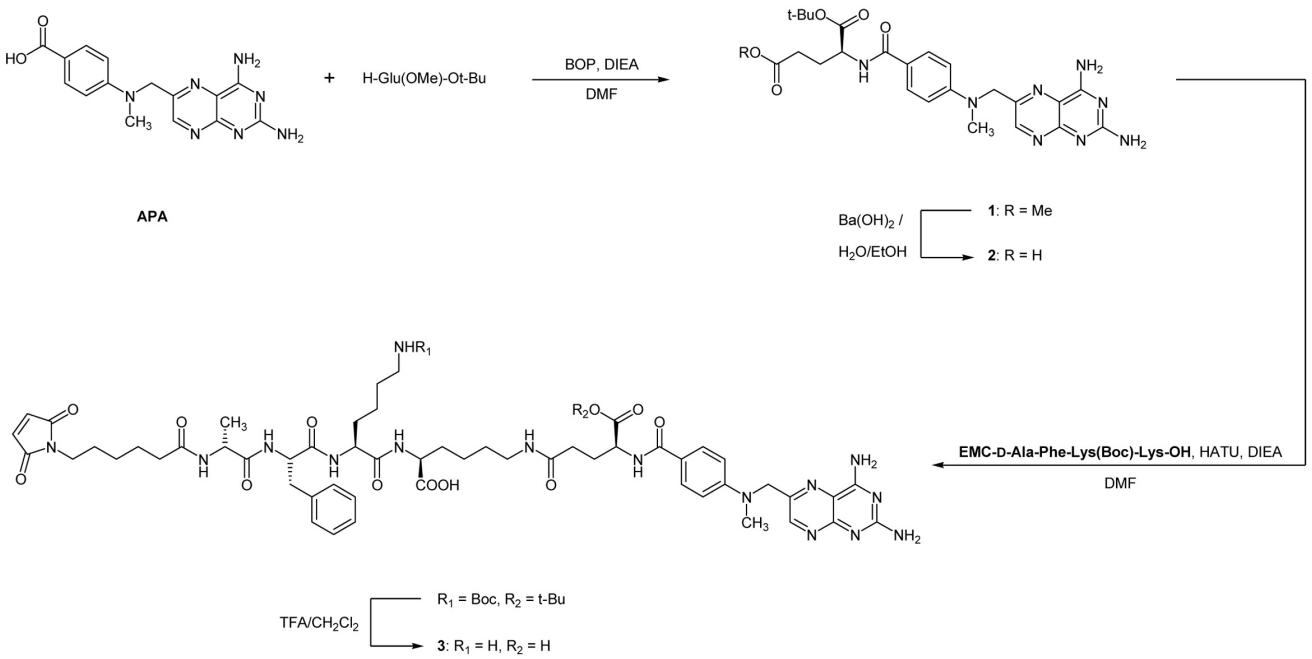


Figure 1. Chromatogram of human plasma, **3**, and plasma incubated with **3**.

In order to show that prodrug **3** binds rapidly and selectively to endogenous HSA, HPLC-studies with human plasma were performed at λ = 300 nm (see Fig. 1). A chromatogram of pure human plasma recorded at this wavelength shows two main signals: a sharp peak eluting at 4 min as well as a smaller broad peak at ~32 min, the latter can be assigned to HSA. Figure 1 depicts the chromatographic profile on a reversed-phase system of **3** incubated with human blood plasma for 2 min. While the unbound prodrug elutes at ~27 min, this signal disappears almost completely after incubation with human plasma, and a single peak eluting at the retention time of HSA (~32 min) is observed. In our previous work on albumin-binding prodrugs with doxorubicin, camptothecin, and platinum derivatives, we have shown that this fast reaction results from a Michael addition between the maleimide group and the sulfhydryl group of the cysteine-34 position of serum albumin [10, 11, 18–22].

The plasma stability of the albumin conjugate of **3** was evaluated by incubation of **3** with human blood plasma and subsequently analyzing samples after 2 min and 24 h through HPLC. From the decrease of peak area the loss of MTX after 24 h was determined to be <10%.



Scheme 1. Synthesis route of **3**.

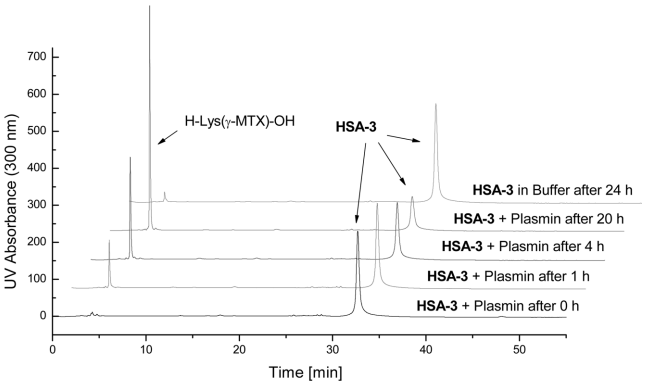


Figure 2. Chromatograms of incubation studies with **HSA-3** in the presence of plasmin at pH 7.4 and 37°C after 0 h, 1 h, 4 h, and 20 h.

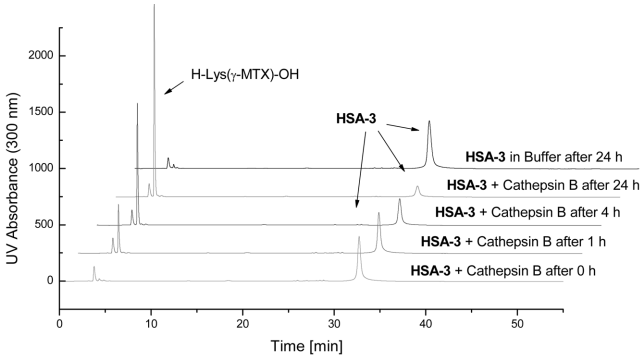


Figure 3. Chromatograms of incubation studies with **HSA-3** in the presence of cathepsin B at pH 5.0 and 37°C after 0 h, 1 h, 4 h, and 24 h.

For assessing the cleavage properties of the albumin-bound form of **3**, the HSA conjugate (**HSA-3**) of the prodrug was prepared by incubating with an excess of **3** with HSA. A solution of **HSA-3** was then incubated with either human plasmin or cathepsin B, and samples were analyzed by HPLC over a period of 20 – 24 h. In both cases, a time-dependent cleavage of the D-Ala-Phe-Lys linker was observed. The chromatograms in Figs. 2 and 3 illustrate the decrease in peak area of **HSA-3** and the formation of a single cleavage product eluting at ~4 min. (Due to the use of buffer containing D-cysteine in the experiments with cathepsin B, an additional small peak of constant height eluting at 3.5 min can be observed.) By comparison with a reference substance, the structure of the liberated MTX derivative was confirmed to be H-Lys(γ-MTX)-OH which results from a proteolytic cleavage of the Lys-Lys bond.

In order to show that cleavage of the macromolecular prodrugs also occurs in the presence of native tumor tissue, **HSA-3** was incubated with homogenized OVCAR-3 material (ovarian carcinoma xenograft that was used in the *in vivo* experiment, see below Section 3.4). The samples were homogenized according to two different protocols [23]: while homogenization at pH 5.0 should retain activity of lysosomal proteases, such as cathepsins, a work-up at pH 7.4 was performed to preserve the extracel-

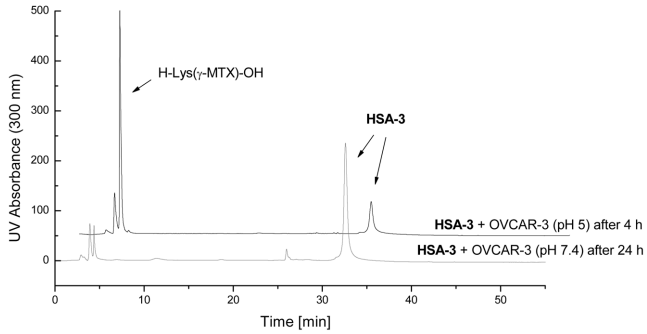
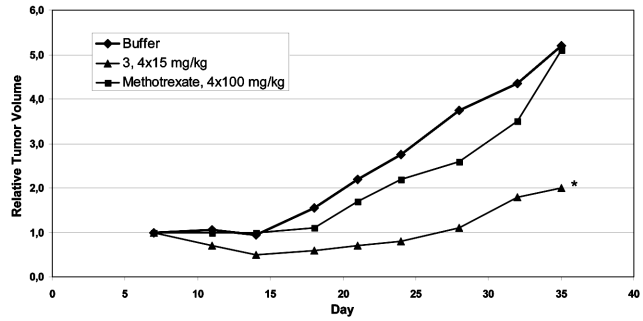


Figure 4. Chromatograms of incubation studies with **HSA-3** in the presence of OVCAR-3 homogenate at pH 5.0 at 37°C after 4 h and at pH 7.4 at 37°C after 24 h.



Compound	Dose (mg/kg)	BWC† (%)	Optimum T/C (%) (on Day)
MTX	4 × 100	+19	69 (28)
3	4 × 15	+12	29§(24)

† BWC Body weight change; § significant to control

Figure 5. Curves depicting tumor growth inhibition of subcutaneously OVCAR-3 xenografts under therapy with methotrexate and prodrug **3**.

lular enzymes (including plasmin) in their active forms. As shown in Fig. 4, incubation with OVCAR-3 homogenate at pH 7.4 resulted in very little cleavage even after a 24 h incubation whereas the conjugate was readily cleaved in homogenate at pH 5.0 (approximately 90% after 4 h of incubation). In all cases, only one cleavage product (H-Lys(γ-MTX)-OH) was observed (other visible peaks are caused by the homogenized material). Also in tumor homogenate, a further (enzymatic) degradation to free MTX was not detected.

Subsequently, the *in vivo* antitumor efficacy of the MTX prodrug **3** was evaluated in xenografted nude mice (human ovarian carcinoma OVCAR-3) in comparison to methotrexate. Compound **3** was assessed at a dose of 4 × 15 mg/kg (i.v.) MTX equivalents. This dose was chosen after an orientating toxicity study in healthy mice in which a dose of 8 × 15 mg/kg was well-tolerated producing only a slight decrease in body weight (-5% from day 1 to day 28). The dose of MTX was chosen according to previous *in vivo* investigations which showed that 4 × 100 mg/kg of MTX is the approximate MTD in nude mice [24]. The results of the xenograft experiment are summarized in Fig. 5. Treatment with the prodrug **3** resulted in a clear response (T/C max. 29%) whereas mice treated with methotrexate showed no antitumor effects at all. Both the prodrug and MTX were well-tolerated and no decrease in body weight could be observed in either case.

Discussion

Coupling anticancer agents to macromolecular carriers is a promising strategy for passively targeting solid tumors. Among macromolecular prodrugs of the folic acid antagonist MTX, only the albumin conjugate MTX-HSA gained clinical importance and was assessed in various clinical trials [25–27]. The conjugate is obtained from directly coupling the drug to lysine residues of human serum albumin and is preferably adjusted to a loading rate of close to one equivalent MTX per molecule of albumin [28, 29]. Since this condensing reaction is not regioselective, MTX-HSA represents a complex mixture of α- and γ-amides with the drug bound to different lysine positions of the protein. In addition, preactivation of MTX with carbodiimides results in a considerable degree of racemization producing a mix of D,L-MTX derivatives [24]. Although there are some indications that MTX-HSA acts as a prodrug by being lysosomally digested, the release mechanism of the drug or respective drug-peptide derivatives still remains unclear [30].

Our approach focused on designing a chemically well-defined MTX-HSA conjugate **3** with distinct cleavage properties. For this purpose, we chose the short peptide sequence D-Ala-Phe-Lys (which is known to be efficiently cleaved at its C-terminus by the proteases plasmin and cathepsin B) as a crosslinker to link the drug with an albumin-binding maleimide group. This anchor ensures a rapid and regioselective coupling to the cysteine-34 position of HSA and thus can be successfully employed for the *in situ* generation of drug-albumin conjugates as demonstrated in our previous work [18]. Due to the fact that the peptide sequence must be linked to MTX via its C-terminus, we incorporated the amino acid lysine as a diamine spacer. Upon cleavage of the Lys-Lys bond we expected the MTX derivative H-Lys(γ-MTX)-OH to be released as the active species. H-Lys(γ-MTX)-OH in which the ε-amino group of lysine is bound to the γ-position of the drug was shown to inhibit the dihydrofolate reductase nearly as efficiently as the drug itself [31].

HPLC studies performed with **3** or the HSA conjugate of **3** (**HSA-3**) confirmed the expected binding and cleavage properties: (1) after incubation with human plasma, the major amount of **3** binds to HSA within 2 min; (2) the

conjugate **HSA-3** is sufficiently plasma-stable; (3) efficient cleavage of the conjugate can be observed in the presence of either human plasmin or cathepsin B; (4) cleavage results in the formation of H-Lys(γ-MTX)-OH as the only low-molecular weight cleavage product. Since **HSA-3** is cleaved by either intra- or extracellular proteases, we also expected a release of H-Lys(γ-MTX)-OH in homogenized native tumor material. For this purpose, we prepared two samples of OVCAR-3 material at different pH. The first one, worked up at pH 5, should preserve lysosomal proteolytic activity whereas the second one, prepared at neutral pH, was assumed to maintain extracellular enzyme activity. Interestingly, when incubating **HSA-3** with the respected tumor homogenates, a clear difference in the cleavage rate was observed. The conjugate was degraded much more effectively in the homogenate at pH 5. At pH 7.4 we expected to see plasmin activity but almost no cleavage occurred. A reason for this can be that OVCAR-3 lacks a high plasmin expression, likewise it cannot be excluded that plasmin activity was lost during the homogenization process (*e.g.* by deactivation with α_2-antiplasmin or the degradation by other proteases).

The finding that H-Lys(γ-MTX)-OH is not further degraded to MTX and lysine is in accordance with previous work by Fitzpatrick and Garnett who showed that H-Lys(γ-MTX)-OH withstands exposure to harsh proteolytic conditions as found in liver tritosomes [32]. However, the results of the *in vivo* experiment suggest that the released MTX-lysine derivative is highly active *per se*. Compared to methotrexate, **3** exhibited enhanced efficacy at a much lower dose. Therefore, it seems that further degradation to MTX is not necessary although it would be interesting to compare **3** with an analogous prodrug incorporating a more sophisticated (self-immolative) spacer that enables the release of pure MTX.

In summary, we developed the first albumin-binding prodrug of methotrexate **3** that is cleaved by either intra- or extracellular proteases and demonstrated superior *in-vivo* efficacy over MTX thus warranting further preclinical assessment.

Experimental

Chemicals, materials, and spectroscopy

4-Amino-4-deoxy-N^{10}-methylpteroic acid (APA) was a gift from Klinge Pharma (Munich, Germany); EMC-D-Ala-Phe-Lys(Boc)-Lys-OH was custom-made by JPT (Berlin, Germany); organic solvents: HPLC grade (Merck, Darmstadt, Germany). All other chemicals used were at least reagent grade and obtained from Sigma-Aldrich, Fluka (Buchs, Switzerland), or Merck and used without further purification. Human serum albumin (5% solution) was purchased from Octapharma GmbH (Langenfeld, Germany). 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, Germany) and thoroughly degassed with ultrasound prior to use. Enzymatically active cathepsin B and plasmin were purchased from Calbiochem (Bad Soden, Germany). OVCAR-3 xenograft material was gratefully received from Dr. I. Fichtner (EPO Exp. Pharmakol. & Onkol. GmbH, Berlin, Germany). Lyophilization was performed with a lyophilizator Alpha 2-4 (Christ, Germany). ESI-MS spectra were obtained on a Finnigan MAT 312 with associated MAT SS 200 data system (Thermo Electron Corporation, Bremen, Germany).

HPLC method

HPLC for binding and cleavage studies with **3** and its albumin conjugate **HSA-3** was performed using a Gilson 321 pump (Gilson Middleton, MI, USA), a Pharmacia LKB Uvicord VW2251 UV detector (λ = 300 nm; Pharmacia-LKB, Uppsala, Sweden), and KromaSystem Software (Northstar Scientific, UK); column: Waters 300 Å (Waters Corporation, Milford, MA, USA), Symmetry C18 (4.6 × 250 mm) with pre-column; chromatographic conditions: flow: 1.2 mL/min, mobile phase A: 15% CH_3CN, 85% 20 mM potassium phosphate (pH 7.0), mobile phase B: 70% CH_3CN, 30% 20 mM potassium phosphate (pH 7.0); gradient: 0 – 20 min 100% mobile phase A; 20 – 45 min increase to 100% mobile phase B; 45 – 50 min 100% mobile phase B; 50 – 56 min decrease to 100% mobile phase A; injection volume: 50 μL.

Chemistry

MTX-α-Ot-Bu-γ-OMe ***(1)***

To a suspension of 4-amino-4-deoxy-N10-methylpteroic acid (APA) (3.15 g, 8.71 mmol, 90% purity) in 200 mL of anhydrous DMF was added 6 mL of triethylamine and BOP (5.23 g, 11.8 mmol). After stirring for 30 min at room temperature, H-Glu(OMe)-Ot-Bu.HCl (2.38 g, 9.40 mmol) was added, and stirring was continued for 16 h. The dark-orange mixture was filtered over celite, and the filtrate was concentrated *in vacuo* to a final volume of 100 mL. Subsequently, the crude product was precipitated by pouring the solution slowly into vigorously stirred diethyl ether (2000 mL). The suspension was left at 4°C for 3 h after which precipitation was complete. The solids were collected by filtration and washed twice with cold diethyl ether. The crude product was recrystallized from 1000 mL of ethanol to yield 4.08 g (89%) of crude diester **1** which was used in the next step without further purification.

MTX-α-Ot-Bu ***(2)***

Compound **1** (4.05 g, 7.72 mmol) was suspended in 150 mL of ethanol/water 1 : 1 in an unstoppered Erlenmeyer flask. $Ba(OH)_2 \times 8\ H_2O$ (2.02 g, 6.40 mmol) was added, and, after stirring, the initially cloudy mixture cleared off within 10 min. Stirring was continued at room temperature for at least 16 h, and progress of the reaction was monitored using TLC ($CHCl_3$/MeOH, 5 : 1, +1% AcOH). In some cases it was found to be necessary to continue stirring until conversion was complete (up to three days). Subsequently, the solution was filtered over celite, and a saturated solution of Na_2SO_4 (equimolar to $Ba(OH)_2$) was added. The mixture was left at 4°C for 5 h after which precipitation of $BaSO_4$ was complete, and was then filtered over celite. Remaining ethanol was removed under reduced pressure, and the solution was washed with 3 × 50 mL of chloroform. After acidifying

the aqueous phase with 0.5 M HCl to pH 3, a yellow solid precipitated which was filtered off, washed with water, 2-propanol, diethyl ether, and pentane, and dried in high vacuum to afford 2.68 g (68%) of pure monoester **2**. ^{1}H-NMR (DMSO-d_6): δ = 1.38 (s, 9H, Ot-Bu), 1.82–1.96 (m, 2H, β-CH_2), 2.03–2.22 (m, 2H, γ-CH_2), 3.18 (s, 3H, NCH_3), 4.06 (m, 1H, α-CH), 4.76 (s, 2H, 9-CH_2), 6.60 (bs, 2H, 4-NH_2), 6.81 (d, 2H, *J* = 8.7 Hz, H-3', H-5'), 7.40 (bs, 1H, 2-NH_2), 7.68 (bs, 1H, 2-NH_2), 7.75 (d, 2H, *J* = 8.7 Hz, H-2', H-6'), 8.56 (s, 1H, H-7), 10.10 (bs, 1H, NH). ^{13}C-NMR (DMSO-d_6): δ = 26.51 (C-β), 27.63 (OC(CH_3)$_3$), 31.10 (C-γ), 39.01 (C-11), 54.39 (C-α), 54.74 (C-9), 79.53 (O*C*(CH_3)$_3$), 111.00 (C-3', C-5'), 121.31 (C-1'), 121.50 (C-4a), 128.69 (C-2', C-6k), 145.88 (C-6), 149.02 (C-7), 150.63 (C-4'), 155.08 (C-8a), 162.58 (C-4), 162.74 (C-2), 165.70 (C-7'), 171.65 (*C*OOt-Bu), 176.57 (COOH). MS (APCI) *m/z* = 525.4 $[M+H]^+$, (100).

EMC-D-Ala-Phe-Lys-Lys(γ-MTX)-OH ***(3)***

To a solution of **2** (125 mg, 244 μmol) in 0.7 mL DMF was added HATU (93 mg, 244 μmol) and DIEA (186 μL, 1.10 mmol). The mixture was stirred vigorously for 1 min and then transferred to a solution of EMC-D-Ala-Phe-Lys(Boc)-Lys-OH · 2 TFA (200 mg, 222 μmol) and DIEA (186 μL, 1.10 mmol) in 3.3 mL DMF. After 1 h stirring at room temperature, the reaction mixture was poured slowly into 300 mL of vigorously stirred diethyl ether. The solids were collected by filtration, washed twice with diethyl ether and dried *in vacuo*. Subsequently, both Boc and *tert*-butyl protective groups were removed by treating the product with 4 mL of TFA/CH_2Cl_2 (1 : 1) over 1 h at room temperature. The product was precipitated by pouring the solution slowly into vigorously stirred diethyl ether (300 mL), the solids were collected by filtration, and purification of the crude product was carried out by column chromatography (C_{18}-RP silica gel, water/MeCN, 70 : 30, +0.1% TFA). After lyophilization, 148 mg (54%) of **3** were obtained as a yellow solid. Purity determined by HPLC (λ = 370 nm): >99%. MS (ESI) *m/z* = 1122.3 $[M+H]^+$ (100), 1144.4 $[M+Na]^+$ (73).

HSA conjugate of ***3*** ***(HSA-3)***

To 5.16 mg of **3** were added 10.3 mL of HSA solution (Octapharma, 5%). The mixture was shaken for 2 h at room temperature and concentrated with the aid of Centripreps® to a final volume of approximately 3 mL. The concentration of **HSA-3** was determined photometrically at 370 nm (ε_{370} = 7420 $M^{-1}cm^{-1}$) to be 700 μM.

In-vitro **testing**

Incubation studies of ***3*** *with human blood plasma*

Compound **3** (1.00 mg) were dissolved in 200 μL of sterile-filtered glucose-phosphate buffer (10 mM sodium phosphate/5% D-glucose buffer, pH 6.4). 20 μL of this solution were added to 380 μL of human plasma and incubated at 37°C. Samples were collected after 2 min, 1 h, and 20 h and were analyzed by HPLC.

Incubation studies of ***HSA-3*** *with cathepsin B*

180 μL of the **HSA-3** stock solution (700 μM) were mixed with 270 μL of acetate buffer (50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA-Na_2, pH 5.0) containing L-cysteine (8 mM) and 90 μL of a cathepsin B solution (71.7 μg/mL, 110 mU) and incubated at 37°C. Samples were collected after 2 min, 1 h, 4 h, and 24 h and were analyzed by HPLC.

Incubation studies of ***HSA-3*** *with plasmin*

100 μL of the **HSA-3** stock solution (700 μM) were mixed with 500 μL of phosphate buffer (4 mM sodium phosphate, 150 mM NaCl, pH 7.4) and 20 μL of a plasmin solution (1.87 mg/mL, 18.7 units/mL) and incubated at 37°C. Samples were collected after 2 min, 1 h, 4 h, and 20 h and were analyzed by HPLC.

Preparation of OVCAR-3 tissue homogenates

For preparing the tumor homogenates two protocols were used: the first at pH 5.0 that primarily liberates lysosomal proteases, the second at pH 7.4 that is used for extracting extracellular proteases. All following steps were carried out on ice. Tissues of OVCAR-3 xenografts were cut into small pieces, and 200 mg samples were transferred in 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 microdismemberator at 3000 rpm for 3 min with the aid of glass balls. Subsequently, 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.

Incubation studies of ***HSA-3*** *with OVCAR-3 tissue homogenates*

100 μL of the **HSA-3** stock solution (700 μM) were either mixed with OVCAR-3 tumor homogenate at pH 7.4 or pH 5.0 and incubated at 37°C. Samples were collected over 24 h and were analyzed by HPLC.

In vivo **testing**

Orientating toxicity studies

The experiment was carried out using 6–8 weeks old female DAB/1 mice (Charles River, Sulzfeld, Germany) that were treated i.v. on days 1, 4, 8, 11, 14, 18, 22, and 25 with **3** at a dose of 15 mg/kg MTX equivalents freshly dissolved in glucose-phosphate buffer (10 mM sodium phosphate, 5% D-(+)-glucose, pH 6.4, 5 mg/mL). Animal weight, abnormal animal behavior, and potential signs of sickness were documented. Experimental protocols had been approved by the Ethics Committee for Animal Experimentation according to the United Kingdom Coordination Committee on Cancer Research Guidelines and the Ethics Committee of the University Freiburg.

In vivo efficacy

For the *in-vivo* testing of **3** in comparison with MTX female NMRI nu/nu mice (M&B A/S, Ry, Denmark) were used. The mice were held in laminar flow shelves under sterile and standardized environmental conditions (25 ± 2°C room temperature, 50 ± 10% relative humidity, 12-hour 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. A number of 10^7 cells of human ovarian cancer cells OVCAR-3 from *in vitro* culture were transplanted subcutaneously (s.c.) into the left flank region of anaesthetized (40 mg/kg i.p.; Radenarkon, Asta Medica, Frankfurt, Germany) mice on day zero. Mice were randomly distributed to the experimental groups (seven mice per group). When the tumors were grown to a palpable size, treatment was initiated (see Fig. 5).

Mice were treated intravenously at day 7, 14, 21, and 28 with either glucose-phosphate buffer (10 mM sodium phosphate, 5% D-(+)-glucose, pH 6.4, 5 mg/mL), MTX (100 mg/kg), or **3** (15 mg/kg MTX equivalents) freshly dissolved in glucose-phosphate buffer. The injection volume was 0.2 mL/20 g body weight.

Tumor size was measured twice weekly with a calliper-like instrument in two dimensions. Individual tumor volumes *(V)* were calculated by the formula V = (length + [width]^2)/2 and related to the values on the first day of treatment (relative tumor volume, RTV). Statistical analysis was performed with the U-test (Mann and Whitney) with $p < 0.05$. The body weight of mice was determined every 3 to 4 days.

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