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Development of Novel Bisphosphonate Prodrugs of Doxorubicin for Targeting Bone Metastases That Are Cleaved pH Dependently or by Cathepsin B: Synthesis, Cleavage Properties, and Binding Properties to Hydroxyapatite As Well As Bone Matrix

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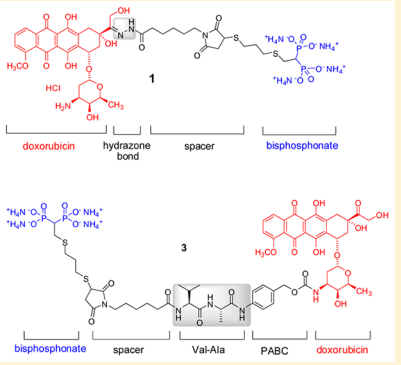
Supporting Information

ABSTRACT: Bone metastases are a frequent cause of morbidity in cancer patients. The present palliative therapeutic options are chemotherapy, hormone therapy, and the administration of bisphosphonates. The affinity between bisphosphonates and the apatite structure of bone metastases is strong. Thus, we designed two low-molecular-weight and water-soluble prodrugs which incorporate a bisphosphonate group as a bone targeting ligand, doxorubicin as the anticancer agent, and either an acid-sensitive bond (**1**) or a cathepsin B cleavable bond (**3**) for ensuring an effective release of doxorubicin at the site of action. Cleavage studies of both prodrugs showed a fast release of doxorubicin but sufficient stability over several hours in human plasma. Effective binding of prodrug **1** and **3** was demonstrated with hydroxyapatite and with native bone. In orientating toxicity studies in nude mice, the MTD of **1** was 3-fold higher compared to conventional doxorubicin, whereas **3** showed essentially the same MTD as doxorubicin.



INTRODUCTION

Bone cancer is manifested as: (a) primary bone cancer which directly develops from bone and bone tissue (e.g., cartilage, osteoblasts, or osteoclasts) and (b) secondary bone tumors which originate in other sites and form metastases in the skeleton. The latter occur far more often than primary bone cancer. The annual incidence of cancer in the United Stated in 2011 was estimated to be 1.6 million cases, with approximately 3000 new cases of primary bone cancer. In striking contrast, secondary bone cancer will occur in up to 50% of patients.[1] Bone metastases are a frequent cause of morbidity in cancer patients and are usually incurable.[2] Bone metastases are predominant in breast (65−75%), prostate (65−75%), thyroid (60%), lung (30−40%), and kidney cancer (20−25%).[3] In the majority of cases, bone metastases cause pathologic fractures, severe pain, and life-threatening hypercalcaemia. Patients who are diagnosed with bone metastases generally cannot be treated curatively, e.g., only 20% of patients with breast cancer are still alive five years after the diagnosis of bone metastases.[4]

The present options for treating bone metastases are surgery, radiotherapy, chemotherapy, hormone therapy, and the administration of bisphosphonates. These therapeutic options can improve the quality of life in some cases but without prolonging survival. Bisphosphonates possess a high accumulation in bone and bone metastases and are presently the most active inhibitors of bone degradation. Their chemical structure is derived from pyrophosphate (Figure 1) in which the ester group is replaced by an enzymatically stable CR_2 group.

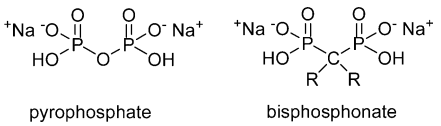


Figure 1. Structure of pyrophosphate and bisphosphonate.

To date, eight bisphosphonates (Figure 2) are clinically established which vary in their side groups and binding constants to calcium hydroxyapatite.

Although bisphosphonates are one of the best options to date for alleviating bone pain, reducing bone destruction, and inhibiting tumor growth, new therapeutic approaches are urgently required in order to improve the outcome of treating bone metastases that are at present merely palliative.

As bone-seeking agents, bisphosphonates demonstrate an uptake in the bone of 20−80% of the administered dose.[5] After intravenous administration of a bisphosphonate, approximately 50−75% of the injected dose binds to exposed bone mineral, with half-lives in the blood circulation in the range of 0.5−6 h in humans depending on the bisphosphonate and administered dose.[5−7] An important feature of bisphosphonates is that in general the uptake in bone metastases is 10−20-fold higher than in healthy bone tissue.[8−10]

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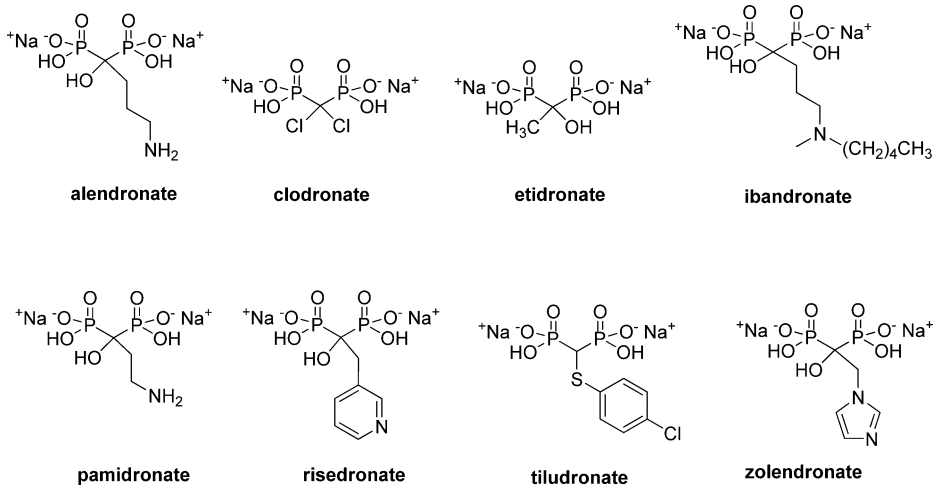

Figure 2. Structure of clinically established bisphosphonates.

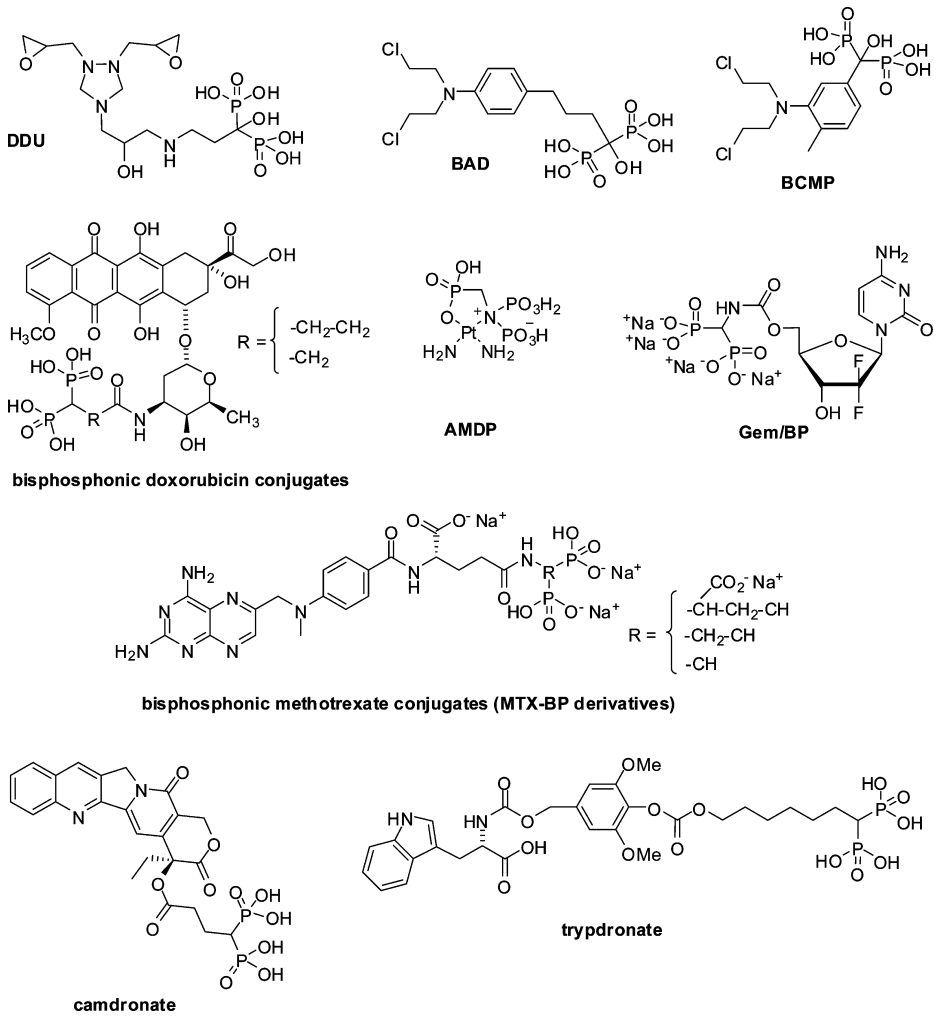

Figure 3. Structures of bisphosphonate drug conjugates.

Surprisingly, only studies to a limited extent have exploited the bone-seeking properties of low-molecular-weight bisphosphonates by conjugating them with an anticancer agent. Figure 3 depicts synthetically conjugates or complexes of bisphosphonates.

Diglycidyl-[3-(3,3-diphosphonate-3-hydroxy-propylamino)-2-hydroxy-propyl]-urazol (DDU), a conjugate of pamidronate and 1,2,4-triglycidylurazol, was first reported in 1986 by Wingen et al..[11] This compound demonstrated a reduction of tumor osteolysis but was inferior to pamidronate.[11] Conjugation of pamidronate with melphalan yielded 4-[4-[bis(2-chloroethyl)amino]phenyl]-1-hydroxybutane-1,1-bisphosphonic acid (BAD) with an improved survival time in a bone metastasis model Walker 256 carcinosarcoma. However, the

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cytostatic efficacy of BAD was similar compared to melphalan.[12] Although it showed a higher anticancer activity than monotherapy with either melphalan or pamidronate in *N*-methyl-*N*-nitrosourea induced mammary carcinomas in Sprague–Dawley rats, a combination therapy of pamidronate and melphalan showed the best therapeutic efficacy in this tumor model.[13] 3-[Bis-(2-chloroethyl)-amino]-4-methylphenyl-hydroxymethane-1,1-bisphosphonic acid (BCMP), a derivative of BAD, had a lower cytostatic activity than BAD alone against rat osteosarcoma, and as a result it was not further investigated.[14]

Keppler et al. prepared a bisphosphonate cisplatin-analogue in which a phosphonate is complexed with diaminoplatinum (*cis*-diammine[nitrilotris(methylphosphonato)(2)-O^1,N^1]-platinum(II), AMDP).[15] The therapeutic efficacy of this complex was better than pamidronate but not superior to conventional cisplatin in a transplantable osteosarcoma model of the rat, even at a 28-fold higher dose.[16]

A bisphosphonate derivative of methotrexate (MTX-BP) demonstrated a higher therapeutic activity than conventional methotrexate against human osteosarcoma implanted in nude mice.[17,18] A biodistribution study of a $^{99\,m}$Tc-labeled MTX-BP conjugate was comparable to $^{99\,m}$Tc-labeled bisphosphonate, demonstrating an accumulation of ∼20% of the administered dose in skeletal tissue of mice.[19]

No antitumor effect at all was obtained when using a conjugate incorporating a bisphosphonate bound to the amino group of doxorubicin when evaluated against human tumor xenografts.[20]

El-Mabhouh et al. prepared a compound in which a bisphosphonate was conjugated to the anticancer agent gemcitabine (Gem/BP, see Figure 3). This conjugate was examined in in vitro and in vivo studies, and approximately ∼67% of retained whole-body activity was bound to the bone at 8 h after administration of labeled ^{188}Re-Gem/BP. In addition, in a nude mice model using the human breast cancer cell line MDA-MB-231BO, unlabeled Gem/BP reduced the number and size of bone metastases relative to gemcitabine-treated and untreated control groups. Histological examinations confirmed the superior therapeutic efficacy of Gem/BP in comparison with commercial gemcitabine.[21−23]

Recently, Shabat et al. developed bisphosphonate conjugates with the anticancer drug camptothecin in which the hydroxyl group of camptothecin was bound through an ester bond to tetraethyl bisphosphonate carboxylic acid (camdronate) and tryptophan as a model compound in which the amino group was bound through a carbonate bond to a bisphosphonate (trypdronate, see Figure 3). Both bisphosphonate conjugates were shown to bind to hydroxyapatite in vitro.[24] Besides low-molecular-weight bisphosphonate compounds, HPMA copolymer drug conjugates with alendronate and either paclitaxel or the antiangiogenic drug TNP-470 have recently been described, showing antiangiogenic activity in bone metastases models in vivo.[25−28]

However, proof-of-concepts for straightforward low-molecular-weight anticancer bisphosphonate drug conjugates have, on the whole, been disappointing. Although a bone targeting effect for most bisphosphonate drug conjugates was obtained, the major drawback in the design of these conjugates is to our mind a lack of a predetermined breaking point that allows the bound drug to be specifically released in bone metastases. Surprisingly, bisphosphonate prodrugs have not been reported in the literature. Two physiological and biochemical properties of bone metabolism that are especially predominant in the formation and growth of bone metastases are: (a) the acidic environment in the lacunae that is necessary for bone desorption[29−34] and (b) the expression of several proteases such as matrix metalloproteinase, urokinase plasminogen activator, and cathepsins (cathepsins K, L, and B)[35−40] that are responsible for osteolysis, bone remodeling, and the growth (osteoblasts) or degradation (osteoclasts) of the bone matrix and bone metastases. Thus we set out to develop novel bisphosphonate prodrugs that exploit the profound bone-seeking properties of the bisphosphonate moiety as well as the microenvironment of bone metastases to ensure a selective release and accumulation of anticancer agent at the desired site of action.

In this work, we describe the synthesis of novel bisphosphonate doxorubicin prodrugs that are effectively cleaved in a pH-dependent manner or by cathepsin B and demonstrate pronounced binding properties to hydroxyapatite and native bone matrix.

RESULTS AND DISCUSSION

Rationale and Chemistry. The aim of this work was to develop prodrugs with bone targeting properties that selectively release an anticancer agent in the skeletal metastases. The general design of our new bisphosphonate prodrugs is shown in Figure 4.

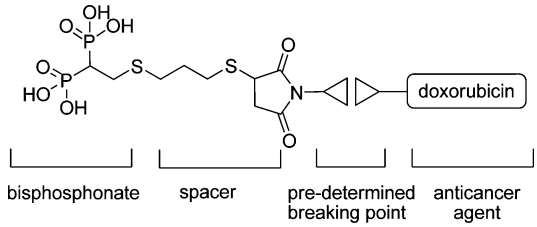

Figure 4. General design of our new bisphosphonate prodrugs.

The prodrugs comprise a thiol-bearing bisphosphonate that has reacted with a maleimide-bearing doxorubicin prodrug. This design is based on our long-term experience in the development of thiol-binding doxorubicin prodrugs.[41−46] Such maleimide-based prodrugs were initially developed to bind rapidly and selectively to the cysteine-34 position of circulating albumin due to tumor uptake of albumin uptake in malignant tissue.[47] In situ binding of prodrugs to endogenous albumin is meanwhile a preclinically and clinically validated technology of increasing the therapeutic index of anticancer agents. An acid-sensitive albumin-binding prodrug of doxorubicin **9**, the (6-maleimidohexanoyl)hydrazone derivative of doxorubicin (INNO-206, previously known as DOXO-EMCH) developed in our group[42] has been studied clinically[46] and is undergoing a phase II study in soft tissue sarcoma and pancreatic cancer (see http://www.cytrx.com).

Furthermore, a series of albumin-binding prodrugs with enzymatically cleavable linkers have been developed in the past 10 years demonstrating superior efficacy and tolerability in preclinical tumor-bearing mice models.[47] Maleimide-bearing prodrugs were therefore an obvious choice for developing bisphosphonate prodrugs.

For developing bone-seeking prodrugs, we chose doxorubicin for the following reasons: (a) doxorubicin is commonly used in those cancer indications where bone metastases frequently

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occur such as breast, prostate, and thyroid cancer as well as against osteosarcoma,[48] (b) in the past, we have developed various thiol-binding prodrugs of doxorubicin with acid-sensitive or enzymatically cleavable linkers.[47,49,50]

A suitable thiol-bearing bisphosphonate is 2-(3-mercaptopropylthio)ethane-1,1-diyl-diphosphonic acid that has been described in the literature.[51] The rationale for selecting an appropriate predetermined breaking point when designing bisphosphonate prodrugs was based on the characteristic biochemical and physiological properties of bone metastases. Osteoclasitc and osteoblastic bone metastases exhibit an acidic environment in order to degrade the apatite matrix during bone remodelling and the formation of bone metastases. Bone metastases are characterized by a high expression of several proteases including cathepsins, matrix metalloproteases, plasmin, and uPA.[35,37−40,52,53] Key proteases which are overexpressed in bone metastasis are cathepsin K and B.[38−40]

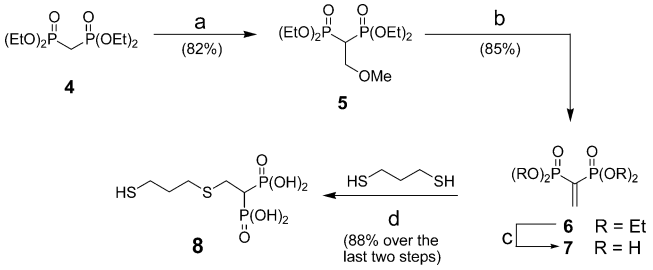
Cathepsin B is a cysteine proteases which is activated in an acidic microenvironment and can participate in the vicious cycle of bone metastases.[53] For example, it is expressed at low levels in primary prostate tumors; however, bone metastatic lesions express high levels of activated cathepsin B, suggesting that protease activity is modulated by interactions between tumor cells and the bone microenvironment.[38,54]

In summary, incorporating an acid-sensitive or cathepsin B cleavable linker in a doxorubicin bisphosphonate prodrug should be an effective way of releasing doxorubicin specifically in areas of bone metastases after uptake of the prodrug in skeletal metastases. As suitable maleimide-bearing prodrugs of doxorubicin we selected (a) the acid-sensitive albumin-binding prodrug, the (6-maleimidohexanoyl)hydrazone derivative of doxorubicin (DOXO-EMCH), in which doxorubicin is derivatized at its C-13 keto position with a thiol-binding linker and (b) the cathepsin B cleavable doxorubicin prodrugs EMC-Phe-Lys-PABC-DOXO or EMC-Val-Ala-PABC-DOXO (EMC = 6-maleimidocaproic acid), which incorporate a 1,6-self-immolative *p*-aminobenzyloxycarbonyl (PABC) linker between the drug and the enzymatically cleavable dipeptide.[50]

*Synthesis of Bisphosphonate Doxorubicin Prodrugs That Are Cleaved in a pH-Dependent Manner (**1**) and by Cathepsin B (**2** and **3**).* According to our synthetic approach, the construction of the prodrugs is based on the condensation of a thiol-bearing bisphosphonate moiety with a thiol-binding group conjugated with the anticancer drug.

The initial step in the development of our bisphosphonate prodrugs was the synthesis of a thiol-bearing aliphatic bisphosphonate compound, which allows the attachment of the thiol binding group of the doxorubicin prodrugs. The synthesis of the bisphosphonate **8** (Scheme 1) was performed according to published procedures,[51] with modifications in steps two and three (see Scheme 1 and Supporting Information). **5** was obtained by reacting commercially available tetraethyl methylene-bisphosphonate **4** with diethylamine and paraformaldehyde in methanol. Subsequent acid-catalyzed elimination furnished compound **6**. In this step, calcium hydride was substituted by powdered molecular sieve (4 Å), and the product was lyophilized from the aqueous layer. The bisphosphonate ester **6** was converted into the free acid **7** by using the dealkylating reagent bromotrimethyl silane ($BrSi(CH_3)_3$). The modification in this step was the use of methanol instead of 40% aqueous tetrabutylammonium hydroxide. Isolation of compound **8** was achieved by addition of 1,3-propanedithiol in a high yield (88% over the last two steps).

Scheme 1. Chemical Synthesis of the Thiol-Bearing Bisphosphonate 8[a]



[a]Reagents and conditions: (a) $HNEt_2$, $(HCHO)_n$, MeOH, reflux, 24 h; (b) toluene, *p*-TsOH, molecular sieve (4 Å), reflux, 24 h; (c) $BrSi(CH_3)_3$, DCM, 0 °C → rt, MeOH; (d) 1,3-propanedithiol, 1-butanol, reflux → rt, H_2O.

*Synthesis of the Acid-Sensitive Bisphosphonate Prodrug **1**.* The acid-sensitive bisphosphonate prodrug **1** was obtained in a rapid Michael addition from the thiol-bearing moiety **8** when coupled to the thiol-binding compound **9** (Scheme 2). Prodrug **1** was obtained as a water-soluble red solid after purification by preparative reverse phase chromatography and subsequent lyophilization.

*Synthesis of Bisphosphonate Doxorubicin Prodrugs with Cathepsin B Substrates (**2** and **3**).* A suitable thiol-binding doxorubicin derivative for synthesizing a cathepsin B cleavable bisphosphonate prodrug from thiol-bearing aliphatic bisphosphonate **8** is EMC-Phe-Lys-PABC-DOXO **10**, first published by Dubowchik et al.,[55] that we have previously synthesized as an albumin-binding prodrug.[50] **10** incorporates a self-immolative Phe-Lys-PABC-spacer that allows doxorubicin to be released efficiently after cleavage of Phe-Lys as the cathepsin B substrate. This compound bound to the cysteine-34 position of albumin was cleaved efficiently by cathepsin B releasing the free drug and exhibited superior antitumor efficacy in vivo compared to doxorubicin.[50]

Consequently, we coupled **10** to the bisphosphonate **8** as depicted in Scheme 3 and obtained the doxorubicin bisphosphonate **2**.

Although **2** was cleaved efficiently by cathepsin B (see Supporting Information), its low aqueous solubility (approximately 1 mM) does not make it an optimal candidate and limits its investigation for in vivo studies.

As an alternative to **2**, we decided to use another cathepsin B substrate, i.e., Val-Ala, described by Peterson et al.[56] Following our previous synthetic route for obtaining bisphosphonate prodrugs, we synthesized a new thiol-binding prodrug **16** (EMC-Val-Ala-PABC-DOXO) as depicted in Scheme 4. The self-immolative spacer of **12** was subsequently reacted with bis(*p*-nitrophenyl) carbonate (bis-PNP carbonate) to yield the activated carbonate **13**. Afterward, doxorubicin was introduced in the presence of *N*,*N*-diisopropylethylamine (DIEA) and subsequently piperidine was added to remove the Fmoc-protecting group. In a final step, the 6-maleimidocaproic-*N*-hydroxysuccinimide ester (EMC-OSu) was reacted with the amino-group of compound **15** to yield the cathepsin B cleavable compound **16**.

As shown in Scheme 5, the bisphosphonate prodrug **3** was obtained by reacting the thiol-bearing bisphosphonate **8** with

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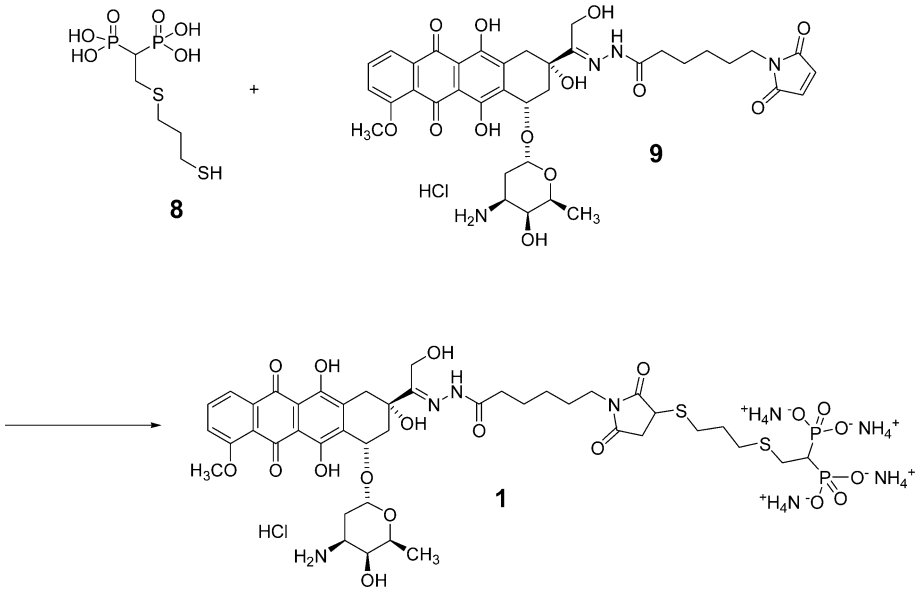
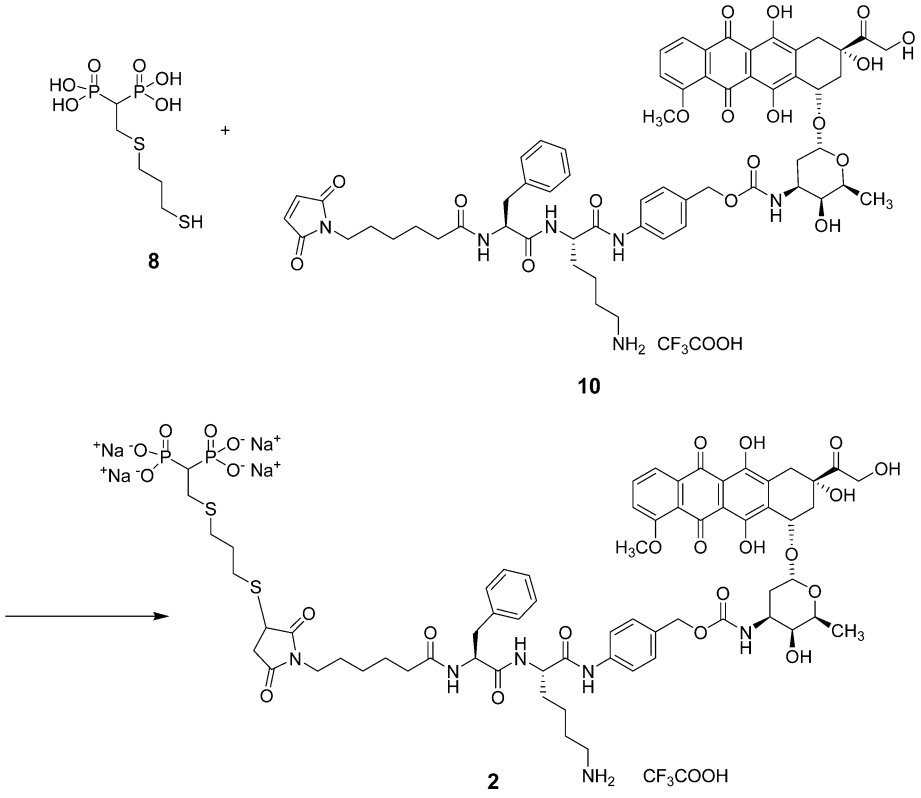
Scheme 2. Synthesis of the Acid-Sensitive Doxorubicin Bisphosphonate Prodrug 1[a]



[a]Reagents and conditions: 50 mM NH_4HCO_3 buffer (pH 7.4) including 25% *tert*-butanol, rt, 10 min; purification by C-18 RP chromatography.

Scheme 3. Synthesis of the Cathepsin B Cleavable Bisphosphonate Prodrug 2[a]



[a]Reagents and conditions: 50 mM sodium phosphate buffer (pH 7.4)/ethanol 1:1, 37 °C, 30 min.

16. **3** is formed within 15 min in a Michael addition as confirmed by HPLC.

Purification of **3** was achieved with preparative C-18 reverse phase chromatography and subsequent lyophilization yielded a red solid. Water solubility of **3** was considerably better than for **2** exceeding 5 mM.

Cleavage and Binding Properties of Novel Bisphosphonate Doxorubicin Prodrugs for Targeting Bone Metastases.

Cleavage Studies of the Bisphosphonate Prodrugs 1, 2, and 3. Following the synthesis of the prodrugs, we studied their cleavage properties in analogy to our previous studies.[42,50] On the basis of our rationale that the new bisphosphonate doxorubicin prodrugs should release doxorubicin under conditions prevailing in the tumor environment of bone metastases, we investigated the cleavage properties of

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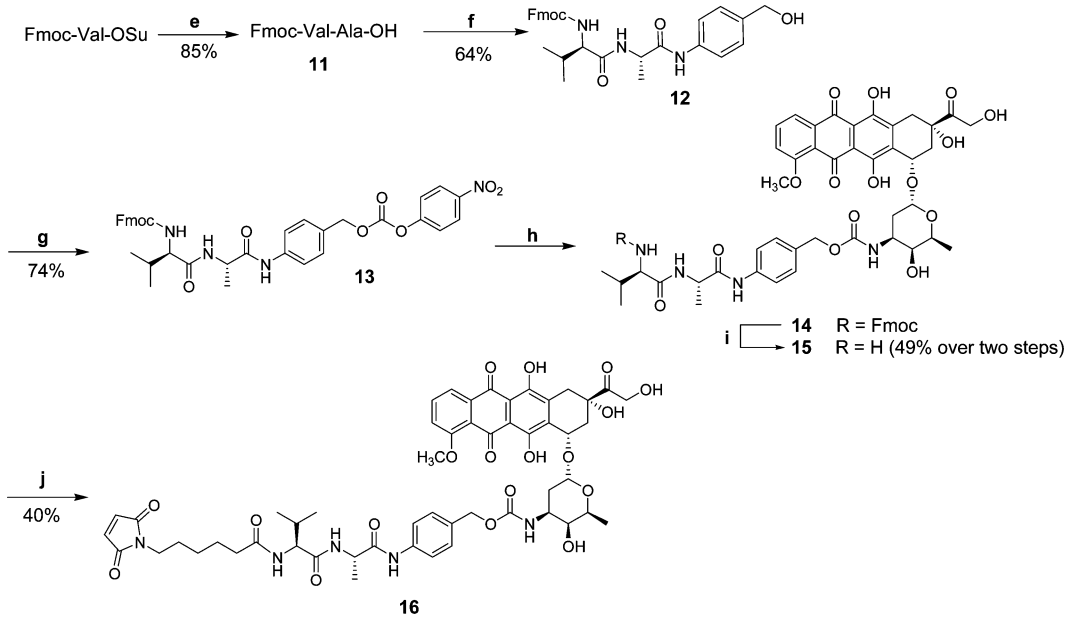
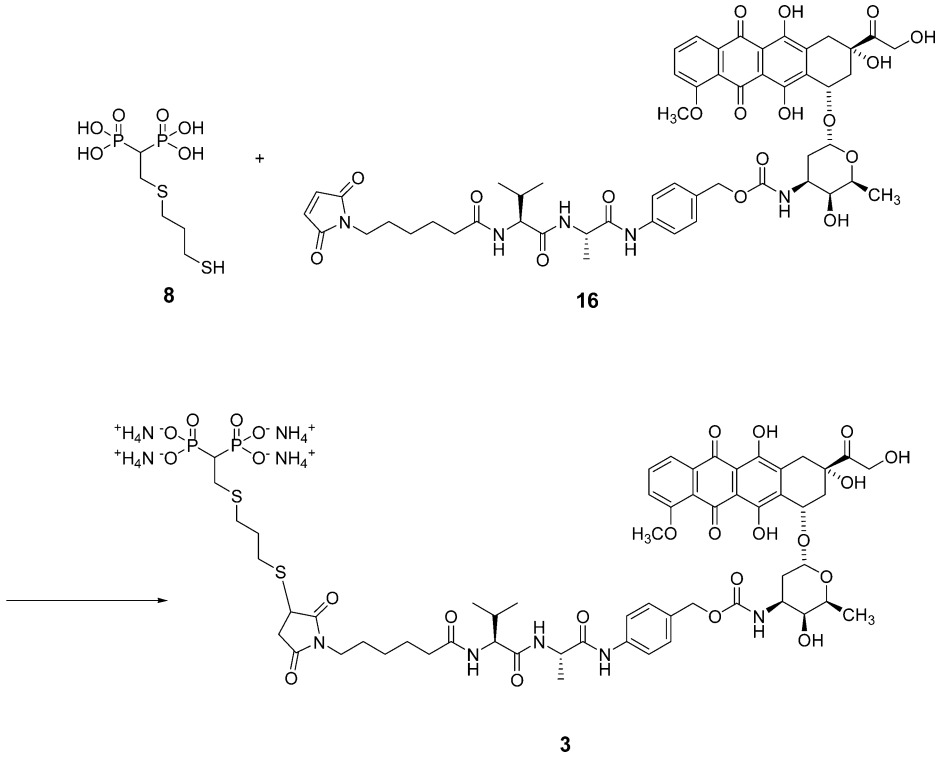
Scheme 4. Synthesis of EMC-Val-Ala-PABC-DOXO 16[a]



[a]Reagents and conditions: (e) H-Ala-OH, $NaHCO_3$, H_2O, THF, rt; (f) PABOH, EEDQ, DCM, rt; (g) DIEA, bis(*p*-nitrophenyl) carbonate, DCM, DMF, rt; (h) doxorubicin·HCl, DIEA, DMF, RT; (i) 20% piperidine in DMF, RT; (j) DCM, DMF, EMC-OSu, rt.

Scheme 5. Synthesis of Bisphosphonate Prodrug 3[a]



[a]Reagents and conditions: 50 mM NH_4HCO_3 buffer (pH 7.4)/ MeOH (1:1), rt; purification by C-18 RP chromatography.

the prodrugs according to the nature of the incorporated cleavage point.

The acid-sensitive hydrazone bond in **1** should allow doxorubicin to be released at the acidic pH values present in the resorption lacuna. Hence, we incubated compound **1** at pH 5.5 and analyzed the release of doxorubicin over a period of 5 h by HPLC (see Figure 5). Already after 5 min, an amount of ~4% doxorubicin was observed with a time-dependent decrease of the initial peak eluting at a retention time of ~3 min of the bisphosphonate prodrug **1.** The peak intensity of doxorubicin simultaneously increased. The half-life of **1** at pH 5.5 at room temperature was approximately 2.5 h.

To confirm that the release of doxorubicin from **1** was acid-promoted, we monitored the stability of **1** at pH 7.4 over 18 h.

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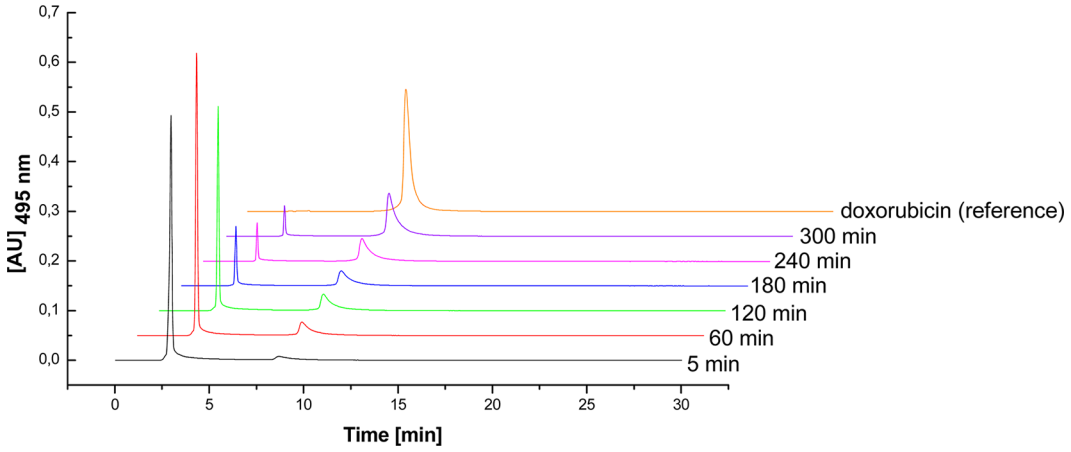

Figure 5. Chromatograms of an incubation study of compound **1** at pH 5.5 and room temperature over time. **1** was dissolved in 4 mM sodium phosphate buffer containing 150 mM sodium chloride (pH 7.4), and the pH value was adjusted to pH 5.5 with 50 mM sodium acetate buffer to obtain a final concentration of 2.4 mM of **1**. For HPLC injection, the solution was diluted to 600 μM. A chromatogram of doxorubicin is included as reference.

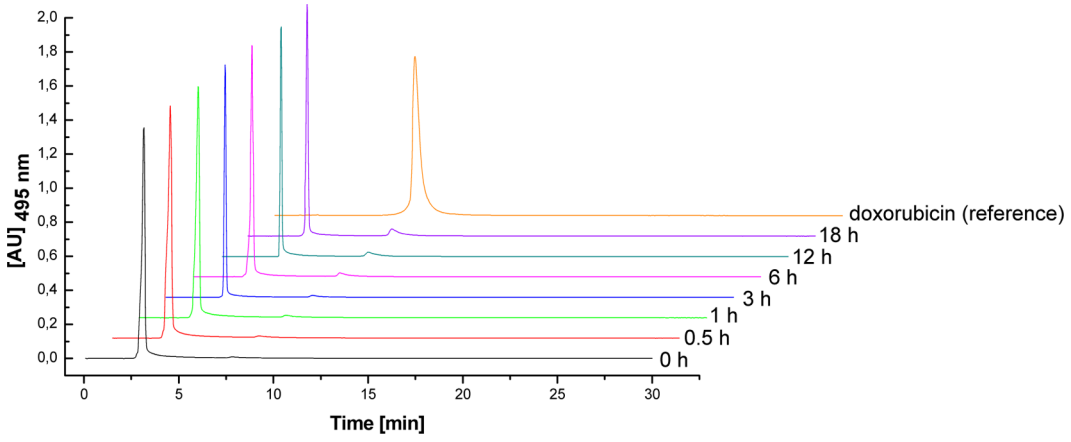

Figure 6. Chromatograms of a stability study of prodrug **1** (800 μM) at pH 7.4 (4 mM sodium phosphate buffer containing 150 mM sodium chloride) at room temperature.

As noted in Figure 6, **1** exhibited a considerably higher stability at this pH value with approximately 12% doxorubicin being released after an 18 h incubation period.

To assess the cleavage properties of prodrugs **2** and **3**, the respective doxorubicin bisphosphonate derivative was incubated with cathepsin B at pH 5.0 and pH 6.0 at 37 °C. Both prodrugs were cleaved to doxorubicin (see Supporting Information for **2** and Figure 7 for **3**). Under the conditions chosen (cathepsin B 0.4 mg/mL, 23.8 U/mg, buffer: 50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA, pH 5.0, containing 8 mM L-cysteine), the half-life for **3** was ~45 min, with the sole cleavage product being doxorubicin and completely released after 120 min (see Figure 7A). At pH 6.0 under the same conditions, cleavage was faster, with doxorubicin already being completely liberated after 40 min (see Figure 7C). Incubation of compound **3** without cathepsin B at pH 5.0 at 37 °C served as a control experiment (see Figure 7B) and showed no cleavage over 3 h. The pH optimum of cathepsin B is documented to vary in the range of pH 5.0–6.5 depending on the nature of the substrate.[57,58]

*Stability Studies of **1** and **3** in Human Plasma.* The plasma stability is an important criterion for the biological activity of a synthesized prodrug. It should be stable long enough to enable the transport of the active molecule to the tumor and tumor cells. To determine the stability of prodrugs **1** and **3** in plasma, these compounds were incubated in human plasma at 37 °C, and subsequently samples were taken over an incubation period of 6 h and analyzed by HPLC, as can be seen in Figure 8A,B.

While the cathepsin B cleavable prodrug **3** was stable under these conditions with no doxorubicin or other anthracycline derivatives being observed (Figure 8B), prodrug **1** showed a slight degradation in human plasma with small amounts of doxorubicin (retention time ~7 min) and an unidentified anthracycline derivative (~ 2.5 min) appearing over the 6 h incubation period (Figure 8A). Considering that the half-life in human beings of circulating bisphosphonates is short, between 0.5 and 2 h due to rapid bone uptake as well as renal clearance,[5] it can be surmised that the stability of both prodrugs is sufficient to allow them to accumulate in bone and bone metastases. In mice and rats, the half-life of bisphosphonates is considerably shorter than in humans, in the order of only a few minutes.[5,6,59]

Hydroxyapatite Binding Assay for Both Bisphosphonate Prodrugs at pH 6.0 and pH 7.4. A further aim of our work was to assess the affinity between our new bisphosphonate prodrugs and the bone mineral hydroxyapatite (HA). First, we incubated prodrug **3** with different equivalents of hydroxyapatite (30, 40, and 50 equiv) and monitored HA binding photometrically

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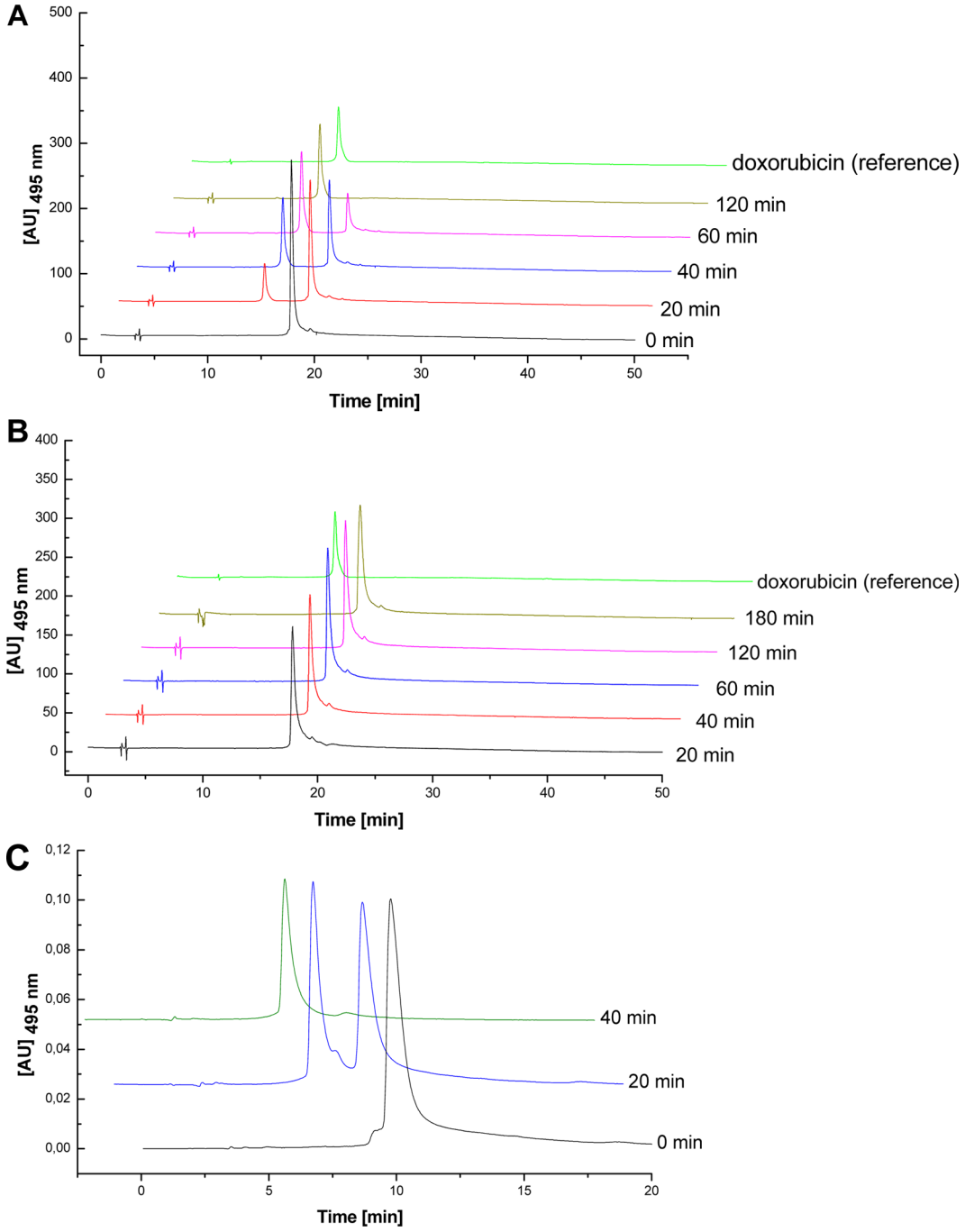

Figure 7. Chromatograms of an incubation study of compound **3** at pH 5.0 as well as at pH 6.0 (37 °C) with cathepsin B (A,C) and without cathepsin B (B). (A) A 176 μL stock solution of compound **3** (1.5 mM) was diluted with 9 μL of cathepsin B (0.4 mg/mL, 23.8 U/mg) and 264 μL of a buffer at pH 5.0 (50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA, pH 5.0) or (C) of a buffer at pH 6.0 (50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA, pH 6.0) containing L-cysteine (8 mM). The cleavage product doxorubicin is included as reference.

determining the decrease of **3** in the supernatant over time. Not unexpectedly, the kinetics of binding was dependent on the excess of hydroxyapatite that was used (see Figure S16 in Supporting Information). For further validation of the binding assay, we opted to use 30 equiv of HA. Binding to hydroxyapatite was carried out at pH 7.4 as well as pH 6.0 to simulate the pH values prevalent in plasma as well as the acidic bone lacunae. When doxorubicin was incubated with hydroxyapatite powder at 37 °C at both pH 6.0 and pH 7.4, no or only very weak binding of doxorubicin to hydroxyapatite could be observed (see Figures 9 and 10). In contrast to doxorubicin, the bisphosphonate prodrugs **1** and **3** were rapidly bound to hydroxyapatite under these conditions. At pH 7.4, approximately 50% of both prodrugs were bound to hydroxyapatite after 1 h (see Figure 9) and a concomitant red staining of the original white hydroxyapatite powder was observed. The binding of prodrug **3** increases with time, and it reached over 90% after 5 h and essentially 100% after 24 h. The acid sensitive prodrug **1** demonstrated a similar curve shape but saturation of HA binding occurred after 5 h with ~80% of **1** being bound to HA. This phenomenon could be explained by catalytic cleavage of the carboxylic hydrazone bond once **1** is

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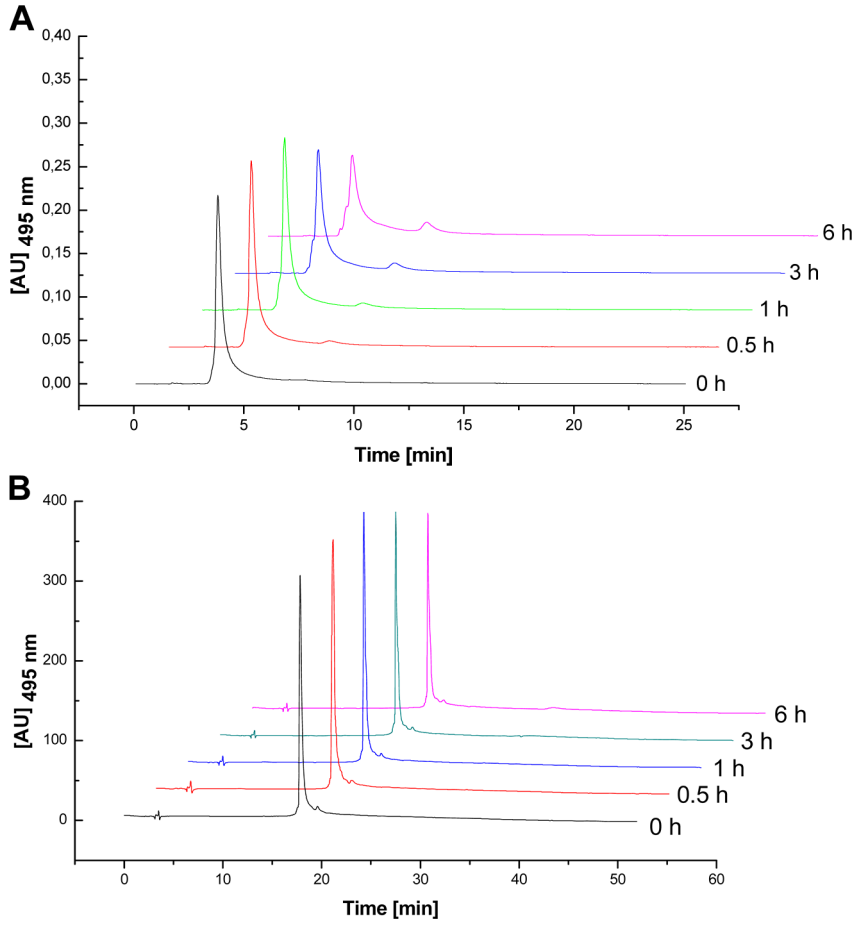

Figure 8. Chromatograms of an incubation study of prodrugs **1** (A) and **3** (B) in human plasma at 37 °C over 6 h, recorded at 495 nm.

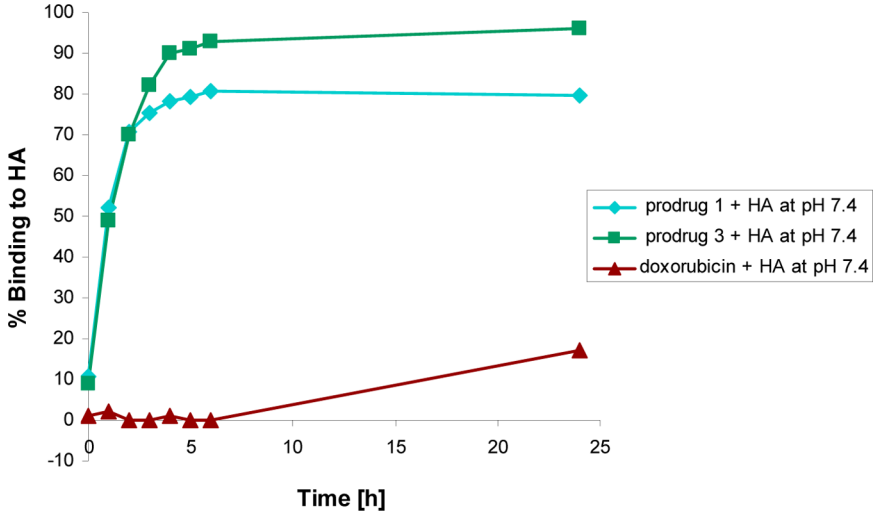

Figure 9. Binding of doxorubicin, of prodrugs **1** and **3** to hydroxyapatite (HA) at pH 7.4 and 37 °C.

bound to HA, releasing doxorubicin from the hydroxyapatite matrix.

Because of acidic pH value in the Howship's lacunae in bones, we also investigated the binding properties of the prodrugs and hydroxyapatite at pH 6.0 and at 37 °C (Figure 10). For both prodrugs, we observed a higher affinity for hydroxyapatite after 1 h with 65−70% of the prodrug being bound compared to ∼50% after incubation at pH 7.4. Not unexpectedly, after this initial fast binding phase, binding of the acid-sensitive prodrug **1** to HA did not increase, which can be explained by a competing acid-promoted cleavage to doxorubicin. In contrast, prodrug **3** showed a very high affinity to hydroxyapatite at pH 6.0 with over 90% being bound to HA.

Bone Binding Assay for Both Bisphosphonate Prodrugs. To confirm whether the new bisphosphonate prodrugs interact with the bone matrix, we determined their binding property to native bone. For this assay, bovine bone pieces were cut into very small fragments, washed several times with distilled water

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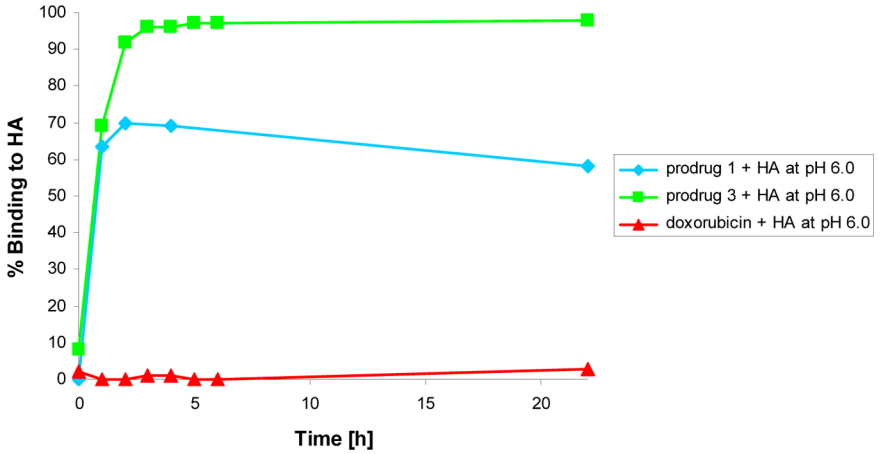

Figure 10. Binding of doxorubicin, of prodrugs **1** and **3** to hydroxyapatite (HA) at pH 6.0 and 37 °C.

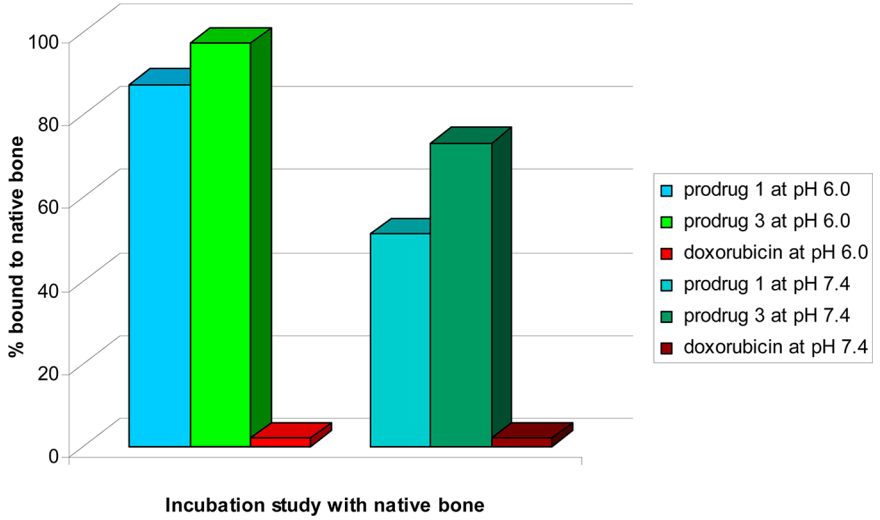

Figure 11. Incubation study of prodrugs **1** and **3** with native bone after 4 h at 37 °C and at pH 6.0 or pH 7.4.

and anhydrous ethanol, and dried at room temperature overnight. Both prodrugs were incubated with the bone matrices suspended in phosphate buffer at pH 7.4 and pH 6.0, respectively (at 37 °C). Figure 11 summarizes the data of native bone incubated with both prodrugs at pH 7.4 and at pH 6.0. Because of the results obtained in the hydroxyapatite binding assay, we incubated the prodrugs over a period of 4 h and measured the absorbance of the supernatant at 495 nm. Conventional doxorubicin was also incubated with the bone fragments serving as the negative control. As expected, we observed no binding of doxorubicin to the native bone. In contrast, 80−90% of both prodrugs were bound to the bone matrix after 4 h at pH 6.0 (Figure 11). At pH 7.4, binding to the bone was lower, with 50−70% of **1** or **3** being bound after 4 h incubation.

Orientating Toxicity Study of Prodrugs 1 and 3. For initiating an in vivo trial in a bone metastases animal model, we determined the systemic toxicity of **1** and **3** in female balb/C nude mice. Initially, two groups of three mice (groups 1 and 4) were treated intravenously once weekly with 8 mg/kg doxorubicin equivalents of compounds **1** or **3** (see Supporting Information) for three weeks. Within the first week after therapy, no weight losses or other drug-related side effects were observed (see Figure S17 of Supporting Information). Hence, the administration of prodrug **1** and **3** at higher doses (16 and 24 mg/kg doxorubicin equivalents) was carried out. Shortly after the initial treatment with both doses (16 and 24 mg/kg doxorubicin equivalents) of prodrug **3**, all animals died (group 2 and 3). Animals treated with 16 and 24 mg/kg doxorubicin equivalents of prodrug **1**, however, showed no acute side effects (groups 5 and 6). All animals were observed for four weeks, and behavior and body weight changes were documented. After 13 days after the last injection, all three animals of group 6 showed signs of anemia and one animal had died. Body weights remained constant or demonstrated the typical slight increase characteristic for adolescent animals during the term of the study. On the basis of this orientating toxicity study, the maximum tolerated dose, dosed on a weekly schedule, was estimated as 3 × 24 mg/kg for the acid-sensitive prodrug **1** and 3 × 8 mg/kg for **3**, which is slightly above the MTD of 2 × 8 mg/kg for doxorubicin in nude mice.

■ CONCLUSION

In this work, we report on the development of tailor-made prodrugs with bisphosphonates for treating bone metastases, a synthetic approach that has not been described to date. Two new water-soluble prodrugs which incorporated a bisphosphonate as the bone targeting moiety and doxorubicin as the anticancer agent were prepared that release the parent

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anthracycline either at an acidic pH value or enzymatically by cathepsin B. Sufficient stability in human plasma as well as high affinity for hydroxyapatite and native bone was demonstrated, making the novel prodrugs **1** and **3** suitable candidates for in vivo evaluation in a bone metastases model.

EXPERIMENTAL SECTION

General. Doxorubicin ·HCl was purchased from Yick-Vic (Hong Kong, China). The (6-maleimidohexanoyl)hydrazone derivative of doxorubicin (DOXO-EMCH) was prepared as described previously.[42] EMC-Phe-Lys-PABC-DOXO was prepared as described previously.[60] All other organic solvents and chemicals used were at least reagent grade and obtained from Merck (Damstadt, Germany), Sigma-Aldrich, and Fluka (Munich, Germany) or Roth (Karlsruhe, Germany) and used without further purification. The buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius, Göttingen, Germany) and thoroughly degassed prior to use. Enzymatically active cathepsin B (human liver) was obtained from Calbiochem (Schwalbach am Taunus, Germany). Hydroxyapatite (HA) was purchased from Sigma-Aldrich (Munich, Germany).

^{1}H NMR, ^{13}C NMR, and ^{31}P NMR were recorded on a Bruker Avance DRX spectrometer 400 MHz (400, 100, and 161 MHz for ^{1}H, ^{13}C , and ^{31}P, respectively). Analytical HPLC were performed either with a Gilson 321 pump, a Kontron 535 detector (at 495 nm), and a Bischoff Lambda 1010 detector (at 254 nm) or with a Waters system (pump, Waters 600E+; detector, Waters 996 photodiode array detector; controller, Waters 600E; auto sampler, Waters 717 plus). For peak integration, Geminyx software (version 1.91 by Goebel Instrumentelle Analytik GmbH, Germany) was used or Empower 2002 (version 1). UV/vis-spectrophotometry was carried out with a double-beam UV/vis-spectrophotometer U-2000 from Hitachi. Mass spectrometry (ESI-TOF-MS) was performed by A&M Labor für Analytik, Bergheim, Germany, using a LTQ-Orbitrap-XL (Thermo Fisher Scientific, San José, CA, USA), spray voltage 4.5 kV. Elementary analyses were performed by vario EL (Elementar Analysensysteme GmbH, Haunau, Germany). Melting points were determined using a Büchi 530 (Büchi Labortechnik GmbH, Essen, Germany). Purity of all compounds (≥95%) was determined by HPLC using one of the methods specified below.

Chromatography. Preparative separation of crude products was carried out by column chromatography using silica gel 60 (230−400 mesh, Roth, Karlsruhe, Germany) or Lichroprep RP-18 (Merck, Darmstadt, Germany). Analytical TLC was performed either on aluminum-coated plates using TLC silica gel 60 UV_{254} (Alugram Sil G, Roth, Karlsruhe, Germany) or on Merck glass plates using silica gel 60 RP-18 F_{254S}. Visualization of TLC plates was realized by suitable detection methods; spots were identified by UV light absorption λ = 254 nm and/or by treatment with a solution containing phosphormolybdic acid (25 mL), Cer(IV)-sulfate (10 g), water (940 mL), and concentrated sulfuric acid (60 mL) and subsequent heating at 150 °C.

Methods. *HPLC Studies.* Four different types of HPLC methods were used:

(a) Kontron System: Column, Waters, 300 Å, Symmetry C18 5 μm [4.6 mm × 250 mm] with precolumn [3.9 mm × 20 mm]. Chromatographic conditions: flow, 1.2 mL/min; mobile phase A, 20% CH_3CN, 80% 20 mM sodium phosphate buffer (pH 7.0); mobile phase B, 70% CH_3CN, 30% 20 mM sodium phosphate buffer (pH 7.0); injection volume, 20 μL; gradient, 0−5 min 100% mobile phase A; 5−40 min increase to 70% CH_3CN, 30% 20 mM sodium phosphate buffer; 40−50 min 100% mobile phase B; 50−60 min decrease to initial mobile phase; 60−65 min 100% mobile phase A.

(b) Waters System: Column, Waters, 300 Å, Symmetry C18 5 μm [4.6 mm × 250 mm] with precolumn [3.9 mm × 20 mm]. Chromatographic conditions: flow, 1.0 mL/min; mobile phase A, 15% CH_3CN, 85% 20 mM sodium phosphate buffer (pH 7.0); mobile phase B, 30% CH_3CN, 70% 20 mM sodium phosphate buffer (pH 7.0); injection volume, 50 μL; gradient, 0−1.5 min 100% mobile phase A; 1.5−20 min increase to 100% mobile phase B; 20−45 min 30% CH_3CN, 70% 20 mM sodium phosphate buffer; 45−55 min decrease to initial mobile phase; 55−58 min 100% mobile phase A.

(c) Waters System: Column, Waters, 100 Å, Symmetry C18 5 μm [4.6 mm × 250 mm] with precolumn [3.9 mm × 20 mm]. Chromatographic conditions: isocratic flow, 1.0 mL/min; mobile phase, 30% CH_3CN, 70% 10 mM sodium phosphate buffer (pH 7.8).

(d) Waters System: Column, Waters, 300 Å, Symmetry C18 5 μm [4.6 mm × 250 mm] with precolumn [3.9 mm × 20 mm]. Chromatographic conditions: isocratic flow, 1.0 mL/min; mobile phase, 30% CH_3CN, 70% 20 mM sodium phosphate buffer (pH 7.8).

*Incubation Studies with Human Plasma and Prodrugs **1** and **3** at 37 °C.* Human blood plasma (EDTA stabilized) was taken from healthy volunteers. **1** or **3** was dissolved in 10 mM sodium phosphate buffer (7.4) containing 0.15 M sodium chloride [3 mM]. The respective sample was added to human plasma (EDTA as anticoagulant) to obtain a final concentration of 600 μM, and the samples incubated for 0, 0.5, 1, 3, and 6 h at 37 °C. Then 100 μL of each sample was centrifuged in a 1.5 mL Eppendorf reaction tube at a maximum of 13000 rpm (duration 60 s), and a 20 μL sample was analyzed by HPLC using method (d) for compound **1** and method (a) for compound **3**.

*pH-Dependent Studies of Compound **1** at pH 5.5.* The stability of **1** was monitored by HPLC at pH 5.5 and 7.4. For studies at pH 7.4, **1** was dissolved in 4 mM sodium phosphate buffer containing 150 mM sodium chloride (pH 7.4) to obtain a final concentration of 800 μM. The samples were incubated at room temperature and measured by HPLC at t = 0, 0.5, 1, 3, 6, and 18 h (method c). For studies at pH 5.5, the pH value of the acid-sensitive bisphosphonate prodrug **1**, dissolved in 4 mM sodium phosphate, 150 mM NaCl, pH 7.4, at a final concentration of 2.4 mM, was adjusted to pH 5.5 with 50 mM sodium acetate (pH 4.5) and incubated at room temperature; samples were diluted to 600 μM and analyzed by HPLC (method c, injection volume: 50 μL) at t = 5, 60, 120, 180, 240, and 300 min.

*Cleavage Studies of Compounds **2** and **3** with Cathepsin B.* First, 100 μL of prodrug **2** (300 μM in 50 mM sodium phosphate buffer, pH 7.0) was added to a solution of a 50 mM sodium acetate buffer containing 100 mM NaCl, 4 mM EDTA containing L-cysteine (8 mM) (pH 5.0). Afterward, 6.7 μL of cathepsin B (0.4 mg/mL, 23.8 U/mg) were added and the solution was incubated for 1 h at 37 °C, and samples were analyzed by HPLC (method b) after 0 and 60 min. For **3**, a 176 μL stock solution of compound **3** (1.5 mM) was diluted with 9 μL of cathepsin B (0.4 mg/mL, 23.8 U/mg) and 264 μL of buffer (50 mM sodium acetate, 100 mM NaCl, 4 mM EDTA, pH 5.0) containing L-cysteine (8 mM). The mixture was incubated at 37 °C, and aliquots (65 μL) were taken after 5, 20, 40, 60, and 120 min and analyzed by HPLC (method a). As control, the incubation study was performed with **3** in the absence of cathepsin B under the same conditions as described above.

*Hydroxyapatite Binding Assay of Compounds **1** and **3** at Different pH Values.* General procedure: In a falcon tube, the test compound was diluted in phosphate buffer (4 mM sodium phosphate containing 150 mM NaCl, pH 6.0 and pH 7.4) to obtain a solution with a concentration of 300 μM, confirmed photometrically (λ = 495 nm, ε = 10650 M^{-1} cm^{-1}). Hydroxyapatite (30 equiv) was added, and the suspension was stirred well and incubated at 37 °C. Another solution of the samples without hydroxyapatite was used as control. In each case, after 0, 1, 2, 3, 4, 5, 6, and 20 h the samples were centrifuged (4000 rpm, duration 60 s, Heraeus Megafuge 1.0) and the absorbance of the supernatant was measured with a spectrophotometer at a wavelength of 495 nm (ε_{495}(doxorubicin) = 10650 M^{-1} cm^{-1}). Each result represents the arithmetic average of three measurements. Percent HA binding was calculated as following: [(sample concentration without HA − sample concentration with HA)]/(sample concentration without HA) × 100%.

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*Bone Assay of Prodrugs **1** and **3** at Different pH Values.* A bone fragment was isolated from the backbone of a cow, and the cortical bone was cut into small pieces (6 mm ×4 mm ×1 mm). The bone pieces were left untreated, washed with water (bidest) and absolute ethanol, and dried. For evaluating binding, the prodrugs were dissolved in a falcon tube in phosphate buffer (4 mM Na_2HPO_4 containing 150 mM NaCl, pH 6.0 and pH 7.4) at a concentration of 300 μM, which was monitored with a spectrophotometer (Hitachi U2000). Approximately 50 mg of an untreated bone piece was added to the clear-red solution, and the samples were incubated under stirring at 37 °C. As control, a solution without a bone piece was prepared. Every hour, over a period of 6 h and after 20 h, the samples were centrifuged (4000 rpm, duration 60 s, Heraeus Megafuge 1.0), and the absorbance of the supernatant was measured with a spectrophotometer at a wavelength of 495 nm (ε_{495}(doxorubicin) = 10650 M^{-1} cm^{-1}). Percent bone binding was calculated as following: [(sample concentration without bone − sample concentration with bone)]/(sample concentration without bone) × 100%.

*Orientating Toxicity Study of Prodrugs **1** and **3**.* All experiments were performed using five-week-old female Balb/C mice (Charles River GmbH, Sulzfeld, Germany) with an approximate weight of 20 g. Mice were maintained in individual ventilated cages (three mice/cage) at constant temperature and humidity. Experimental protocols had been approved by the Ethics Committee for Animal Experimentation, University Freiburg. **1** and **3** were dissolved in a sterile buffer containing 10 mM sodium phosphate and 0.15 mM sodium chloride (pH 7.4) at a concentration of 3.4 mM. Bisphosphonate prodrugs **1** and **3** were intravenously administered to six groups of three mice each. Doses of 8, 16, and 24 mg/kg doxorubicin equivalents were administered once weekly over a period of three weeks. During this time, animal behavior and welfare was monitored daily and changes in body weight were checked every two days.

*Synthesis of Compound **8**.* Synthesis of compound **8** including analytical data is described in the Supporting Information.

*Synthesis of Compound **16**.* Synthesis of compound **16** including analytical data is described in the Supporting Information.

*Synthesis of Bisphosphonate Prodrug **1**.* First, 50.7 mg of compound **8** (171 μmol) was dissolved in 43 mL of 50 mM ammonium bicarbonate buffer (pH 7.4) to obtain a final concentration of 4 mM. This solution contained approximately 80% free thiol groups as assessed with the Ellmann's test.[61] Subsequently, a solution of 82 mg of DOXO-EMCH (1 equiv) dissolved in a mixture of 40 mL of 50 mM ammonium bicarbonate buffer (pH 7.4) and *tert*-butanol (1:1) was added dropwise to the bisphosphonate solution over 3 min. The mixture was stirred at room temperature for 10 min, frozen with liquid nitrogen, and lyophilized, yielding 236 mg as a red solid. The solid was dissolved in mobile phase which was acetonitrile/10 mM sodium phosphate buffer (pH 7.8) 20:80 and purified by C-18 RP chromatography, and the pure fractions were collected and lyophilized. The purity of **1** as assessed by HPLC (method c) was $A_{495\ nm} \geq 99\%$ of peak area. Due to the fact that the lyophilized product contained salts, the content of doxorubicin in the sample was determined using the ε-value for doxorubicin (ε_{495} (pH 7.4) = 10650 M^{-1} cm^{-1}). $C_{42}H_{56}N_4O_{19}P_2S_2$ HRMS ESI-TOF: calculated $[M + H]^+$ 1047.2528, found 1047.2514. The HPLC chromatogram and mass spectrum is included as Supporting Information.

*Synthesis of the Bisphosphonate Prodrug **2**.* First, 60 mg (3.4 mmol, 1 equiv) of EMC-Phe-Lys-PABC-Doxo **10** dissolved in 14 mL of ethanol was added dropwise to a solution of 16.7 mg of 7 (3.4 mmol, 1 equiv) dissolved in 14 mL of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C, and the sample was stirred for 30 min. Then the reaction mixture was centrifuged, and the solvent removed in high vacuo, yielding 140 mg of **2** as a red powder. The purity of **2** was monitored by HPLC (method b), $A_{495\ nm} \geq 95\%$ of peak area. Due to the fact that the lyophilized product contained salts, the content of doxorubicin in the sample was determined using the ε-value for doxorubicin (ε_{495} (pH 7.4) = 10650 M^{-1} cm^{-1}). $C_{65}H_{81}N_6O_{24}P_2S_2$ HRMS ESI-TOF: calculated $[M + H]^+$ 1457.4370, found 1457.4377. The HPLC chromatogram and mass spectrum is included as Supporting Information.

*Synthesis of the Bisphosphonate Prodrug **3**.* First, 35 mg of compound **8** was dissolved in 29 mL of 50 mM ammonium bicarbonate buffer (pH 7.4) to obtain a thiol concentration of 3.2 mM. Then a solution of 99 mg of EMC-Val-Ala-PABC-DOXO **16** dissolved in 40 mL of 10 mM ammonium bicarbonate buffer (pH 7.4, 3.2 mM) was added dropwise to the bisphosphonate solution over 5 min. The mixture was stirred at room temperature for 15 min and was frozen with liquid nitrogen. After lyophilization, the red crude product was purified by flash chromatography (RP 18) eluting with CH_3CN/H_2O (HPLC grade) 1:1 to give **3** as a red solid. HPLC analysis (method a): $A_{495\ nm} \geq 98\%$ of peak area. Due to the fact that the lyophilized product contained salts, the content of doxorubicin in the sample was determined using the ε-value for doxorubicin (ε_{495} (pH 7.4) = 10650 M^{-1} cm^{-1}). The HPLC chromatogram and mass spectrum is included as Supporting Information. $C_{58}H_{75}N_5O_{24}P_2S_2$ HRMS ESI-TOF: calculated $[M + H]^+$ 1352.3791, found 1352.3778.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses of compounds **8** and **16** including analytical data, chromatographic methods, HPLC purities, and mass spectra for compounds **1**, **2**, **3**, **12**, **13**, **15**, and **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMDP, *cis*-diammine[nitrilotris(methylphosphonato)(2)-O^1,N^1]-platinum(II); BAD, 4-[4-[bis(2-chloroethyl)amino]-phenyl]-1-hydroxybutane-1,1-bisphosphonic acid; BCMP, 3-[bis-(2-chloroethyl)-amino]-4-methylphenyl-hydroxymethane-1,1-bisphosphonic acid; BP, bisphosphonate; DDU, diglycidyl-[3-(3,3-diphosphonate-3-hydroxy-propylamino)-2-hydroxy-propyl]-urazol; DIEA, *N*,*N*-diisopropylethylamine; DOXO, doxorubicin; DOXO-EMCH, 6-maleimidohexanoyl)hydrazone derivative of doxorubicin; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; EMC, 6-maleimidocaproic acid; EMC-OSu, 6-maleimidocaproic-*N*-hydroxysuccinimide ester; Gem/BP, bisphosphonate conjugated to gemcitabine; HA, hydroxyapatite; MTX-BP, a bisphosphonate derivative of methotrexate; PABC, *p*-aminobenzyloxycarbonyl; PNP, *p*-nitrophenyl; *p*-TsOH, *p*-toluenesulfonic acid; uPA, urokinase-type plasminogen activator

■ REFERENCES

(1) Hong, W. K.; Bast, R. C.; Hait, W.; Kufe, D. W.; Holland, J. F. et al. *Holland-Frei Cancer Medicine*; 8th ed.; BC Decker: Shelton, CT, 2010.

(2) Rubens, R. D. Bone metastases—the clinical problem. *Eur. J. Cancer* **1998**, *34*, 210−213.
(3) Coleman, R. E. Skeletal complications of malignancy. *Cancer* **1997**, *80*, 1588−1594.
(4) Rubens, R. D. Metastatic breast cancer. *Curr. Opin. Oncol.* **1995**, *7*, 523−526.
(5) Fleisch, H. Bisphosphonates in bone disease: from the laboratory to the patient; 3rd ed.; The Parthenon Publishing Group: London, 1997.
(6) Lin, J. H. Bisphosphonates: a review of their pharmacokinetic properties. *Bone* **1996**, *18*, 75−85.
(7) Bartl, R.; von Treschkow, E.; Bartl, C. Bisphosphonat-Manual: Wirkungen-Indikationen-Strategien; Springer-Verlag: Berlin−Heidelberg, 2006.
(8) Goeckeler, W. F.; Edwards, B.; Volkert, W. A.; Holmes, R. A.; Simon, J.; et al. Skeletal localization of samarium-153 chelates: potential therapeutic bone agents. *J. Nucl. Med.* **1987**, *28*, 495−504.
(9) Bayouth, J. E.; Macey, D. J.; Kasi, L. P.; Fossella, F. V. Dosimetry and toxicity of samarium-153-EDTMP administered for bone pain due to skeletal metastases. *J. Nucl. Med.* **1994**, *35*, 63−69.
(10) Hirabayashi, H.; Sawamoto, T.; Fujisaki, J.; Tokunaga, Y.; Kimura, S.; et al. Relationship between physicochemical and osteotropic properties of bisphosphonic derivatives: rational design for osteotropic drug delivery system (ODDS). *Pharm. Res.* **2001**, *18*, 646−651.
(11) Wingen, F.; Eichmann, T.; Manegold, C.; Krempien, B. Effects of new bisphosphonic acids on tumor-induced bone destruction in the rat. *J. Cancer Res. Clin. Oncol.* **1986**, *111*, 35−41.
(12) Wingen, F.; Sterz, H.; Blum, H.; Moller, H.; Pittermann, W.; et al. Synthesis, antitumor activity, distribution and toxicity of 4-[4-[bis(2-chloroethyl)amino]phenyl]-1-hydroxybutane-1 1-bisphosphonic acid (BAD), a new lost derivative with increased accumulation in rat osteosarcoma. *J. Cancer Res. Clin. Oncol.* **1986**, *111*, 209−219.
(13) Wingen, F.; Pool, B. L.; Klein, P.; Klenner, T.; Schmahl, D. Anticancer activity of bisphosphonic acids in methylnitrosourea-induced mammary carcinoma of the rat—benefit of combining bisphosphonates with cytostatic agents. *Invest. New Drugs* **1988**, *6*, 155−167.
(14) Pool, B. L.; Berger, M.; Schlehofer, J. R.; Wingen, F. In vivo and in vitro investigations on biological effects of aromatic bis-(2-chloroethyl)amino-bisphosphonic acids, new agents proposed for chemotherapy of bone tumors: cytostatic activity in rat osteosarcoma; toxicity and genotoxicity in liver and bone marrow; mutagenicity in *S. typhimurium*. *Invest. New Drugs* **1988**, *6*, 67−78.
(15) Klenner, T.; Valenzuela-Paz, P.; Keppler, B. K.; Angres, G.; Scherf, H. R.; et al. Cisplatin-linked phosphonates in the treatment of the transplantable osteosarcoma in vitro and in vivo. *Cancer Treat. Rev.* **1990**, *17*, 253−259.
(16) Klenner, T.; Wingen, F.; Keppler, B. K.; Krempien, B.; Schmahl, D. Anticancer-agent-linked phosphonates with antiosteolytic and antineoplastic properties: a promising perspective in the treatment of bone-related malignancies? *J. Cancer Res. Clin. Oncol.* **1990**, *116*, 341−350.
(17) Sturtz, G. L.; Appere, G.; Breistol, K.; Fodstad, O.; Schwartsmann, G.; et al. *Eur. J. Med. Chem.* **1992**, *27*, 825−833.
(18) Sturtz, G. L.; Couthon, H. M.; Fabulet, O.; Mian, M.; Rosini, S. Synthesis of *gem*-bisphosphonic methotrexate conjugates and their biological response towards Walker's osteosarcoma. *Eur. J. Med. Chem.* **1993**, *28*, 899−903.
(19) Hosain, F.; Spencer, R. P.; Couthon, H. M.; Sturtz, G. L. Targeted delivery of antineoplastic agent to bone: biodistribution studies of technetium-99m-labeled *gem*-bisphosphonate conjugate of methotrexate. *J. Nucl. Med.* **1996**, *37*, 105−107.
(20) Fabulet, O.; Sturtz, G. L. Synthesis of *gem*-bisphosphonic doxorubicin conjugates. *Phosphorus, Sulfur Silicon Relat. Elem.* **1995**, *101*, 225−234.
(21) El-Mabhouh, A. A.; Angelov, C. A.; Cavell, R.; Mercer, J. R. A 99mTc-labeled gemcitabine bisphosphonate drug conjugate as a probe to assess the potential for targeted chemotherapy of metastatic bone cancer. *Nucl. Med. Biol.* **2006**, *33*, 715−722.
(22) El-Mabhouh, A. A.; Mercer, J. R. 188Re-labelled gemcitabine/bisphosphonate (Gem/BP): a multifunctional, bone-specific agent as a potential treatment for bone metastases. *Eur. J. Nucl. Med. Mol. Imaging* **2008**, *35*, 1240−1248.
(23) El-Mabhouh, A. A.; Nation, P. N.; Abele, J. T.; Riauka, T.; Postema, E.; et al. A conjugate of gemcitabine with bisphosphonate (Gem/BP) shows potential as a targeted bone-specific therapeutic agent in an animal model of human breast cancer bone metastases. *Oncol. Res.* **2011**, *19*, 287−295.
(24) Erez, R.; Ebner, S.; Attali, B.; Shabat, D. Chemotherapeutic bone-targeted bisphosphonate prodrugs with hydrolytic mode of activation. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 816−820.
(25) Miller, K.; Eldar-Boock, A.; Polyak, D.; Segal, E.; Benayoun, L.; et al. Antiangiogenic antitumor activity of HPMA copolymer−paclitaxel−alendronate conjugate on breast cancer bone metastasis mouse model. *Mol. Pharmaceutics* **2011**, *8*, 1052−1062.
(26) Segal, E.; Pan, H.; Benayoun, L.; Kopeckova, P.; Shaked, Y.; et al. Enhanced antitumor activity and safety profile of targeted nanoscaled HPMA copolymer−alendronate−TNP-470 conjugate in the treatment of bone malignances. *Biomaterials* **2011**, *32*, 4450−4463.
(27) Miller, K.; Erez, R.; Segal, E.; Shabat, D.; Satchi-Fainaro, R. Targeting bone metastases with a bispecific anticancer and antiangiogenic polymer−alendronate−taxane conjugate. *Angew. Chem., Int. Ed. Engl.* **2009**, *48*, 2949−2954.
(28) Pan, H.; Sima, M.; Kopeckova, P.; Wu, K.; Gao, S.; et al. Biodistribution and pharmacokinetic studies of bone-targeting *N*-(2-hydroxypropyl)methacrylamide copolymer−alendronate conjugates. *Mol. Pharmaceutics* **2008**, *5*, 548−558.
(29) Blair, H. C.; Teitelbaum, S. L.; Ghiselli, R.; Gluck, S. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **1989**, *245*, 855−857.
(30) Palokangas, H.; Mulari, M.; Vaananen, H. K. Endocytic pathway from the basal plasma membrane to the ruffled border membrane in bone-resorbing osteoclasts. *J. Cell Sci.* **1997**, *110* (Pt 15), 1767−1780.
(31) Baron, R.; Neff, L.; Louvard, D.; Courtoy, P. J. Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100 kD lysosomal membrane protein at the osteoclast ruffled border. *J. Cell Biol.* **1985**, *101*, 2210−2222.
(32) Kingsley, L. A.; Fournier, P. G.; Chirgwin, J. M.; Guise, T. A. Molecular biology of bone metastasis. *Mol. Cancer Ther.* **2007**, *6*, 2609−2617.
(33) Teitelbaum, S. L. Bone resorption by osteoclasts. *Science* **2000**, *289*, 1504−1508.
(34) Arnett, T. Regulation of bone cell function by acid−base balance. *Proc. Nutr. Soc.* **2003**, *62*, 511−520.
(35) Miyake, H.; Hara, I.; Yamanaka, K.; Arakawa, S.; Kamidono, S. Elevation of urokinase-type plasminogen activator and its receptor densities as new predictors of disease progression and prognosis in men with prostate cancer. *Int. J. Oncol.* **1999**, *14*, 535−541.
(36) Woodward, J. K. L.; Holen, I.; Coleman, R. E.; Buttle, D. J. The roles of proteolytic enzymes in the development of tumour-induced bone disease in breast and prostate cancer. *Bone* **2007**, *41*, 912−927.
(37) Cher, M. L.; Biliran, H. R., Jr.; Bhagat, S.; Meng, Y.; Che, M.; et al. Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7847−7852.
(38) Podgorski, I.; Linebaugh, B. E.; Sameni, M.; Jedeszko, C.; Bhagat, S.; et al. Bone microenvironment modulates expression and activity of cathepsin B in prostate cancer. *Neoplasia* **2005**, *7*, 207−223.
(39) Littlewood-Evans, A. J.; Bilbe, G.; Bowler, W. B.; Farley, D.; Wlodarski, B.; et al. The osteoclast-associated protease cathepsin K is expressed in human breast carcinoma. *Cancer Res.* **1997**, *57*, 5386−5390.
(40) Brubaker, K. D.; Vessella, R. L.; True, L. D.; Thomas, R.; Corey, E. Cathepsin K mRNA and protein expression in prostate cancer progression. *J. Bone Miner. Res.* **2003**, *18*, 222−230.

(41) Kratz, F.; Muller-Driver, R.; Hofmann, I.; Drevs, J.; Unger, C. A novel macromolecular prodrug concept exploiting endogenous serum albumin as a drug carrier for cancer chemotherapy. *J. Med. Chem.* **2000**, *43*, 1253−1256.
(42) Kratz, F.; Warnecke, A.; Scheuermann, K.; Stockmar, C.; Schwab, J.; et al. Probing the cysteine-34 position of endogenous serum albumin with thiol-binding doxorubicin derivatives. Improved efficacy of an acid-sensitive doxorubicin derivative with specific albumin-binding properties compared to that of the parent compound. *J. Med. Chem.* **2002**, *45*, 5523−5533.
(43) Kratz, F.; Mansour, A.; Soltau, J.; Warnecke, A.; Fichtner, I.; et al. Development of albumin-binding doxorubicin prodrugs that are cleaved by prostate-specific antigen. *Arch. Pharm. (Weinheim)* **2005**, *338*, 462−472.
(44) Chung, D.-E.; Kratz, F. Development of a novel albumin-binding prodrug that is cleaved by urokinase-type plasminogen activator (uPA). *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5157−5163.
(45) Schmid, B.; Chung, D.-E.; Warnecke, A.; Fichtner, I.; Kratz, F. Albumin-binding prodrugs of camptothecin and doxorubicin with an Ala-Leu-Ala-Leu-linker that are cleaved by cathepsin B: synthesis and antitumor efficacy. *Bioconjugate Chem.* **2007**, *18*, 702−716.
(46) Kratz, F. DOXO-EMCH (INNO-206): the first albumin-binding prodrug of doxorubicin to enter clinical trials. *Expert Opin. Invest. Drugs* **2007**, *16*, 855−866.
(47) Kratz, F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J. Controlled Release* **2008**, *132*, 171−183.
(48) Gianni, L.; Grasselli, G.; Cresta, S.; Locatelli, A.; Vigano, L.; et al. Anthracyclines. *Cancer Chemother. Biol. Response Modif.* **2002**, *20*, 59−69.
(49) Kratz, F.; Warnecke, A.; Schmid, B.; Chung, D. E.; Gitzel, M. Prodrugs of anthracyclines in cancer chemotherapy. *Curr. Med. Chem.* **2006**, *13*, 477−523.
(50) Abu Ajaj, K.; Graeser, R.; Fichtner, I.; Kratz, F. In vitro and in vivo study of an albumin-binding prodrug of doxorubicin that is cleaved by cathepsin B. *Cancer Chemother. Pharmacol.* **2009**, *64*, 413−418.
(51) Bansal, G.; Wright, J. E. I.; Zhang, S.; Zernicke, R. F.; Uludag, H. Imparting mineral affinity to proteins with thiol-labile disulfide linkages. *J. Biomed. Mater. Res., Part A* **2005**, *74*, 618−628.
(52) Woodward, J. K.; Holen, I.; Coleman, R. E.; Buttle, D. J. The roles of proteolytic enzymes in the development of tumour-induced bone disease in breast and prostate cancer. *Bone* **2007**, *41*, 912−927.
(53) Webb, S. D.; Sherratt, J. A.; Fish, R. G. Alterations in proteolytic activity at low pH and its association with invasion: a theoretical model. *Clin. Exp. Metastasis* **1999**, *17*, 397−407.
(54) Arkona, C.; Wiederanders, B. Expression, subcellular distribution and plasma membrane binding of cathepsin B and gelatinases in bone metastatic tissue. *Biol. Chem.* **1996**, *377*, 695−702.
(55) Dubowchik, G. M.; Firestone, R. A.; Padilla, L.; Willner, D.; Hofstead, S. J.; et al. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjugate Chem.* **2002**, *13*, 855−869.
(56) Peterson, J. J.; Meares, C. F. Cathepsin substrates as cleavable peptide linkers in bioconjugates, selected from a fluorescence quench combinatorial library. *Bioconjugate Chem.* **1998**, *9*, 618−626.
(57) Barrett, A. J.; Kirschke, H. Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* **1981**, *80* (Pt C), 535−561.
(58) Swanson, A. A.; Martin, B. J.; Spicer, S. S. Human placental cathepsin B1. Isolation and some physical properties. *Biochem. J.* **1974**, *137*, 223−228.
(59) Fleisch, H. Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. *Drugs* **1991**, *42*, 919−944.
(60) Abu Ajaj, K.; Biniossek, M. L.; Kratz, F. Development of protein-binding bifunctional linkers for a new generation of dual-acting prodrugs. *Bioconjugate Chem.* **2009**, *20*, 390−396.
(61) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.