Synthesis and Biological Evaluation of Novel Prodrugs of Anthracyclines for Selective Activation by the Tumor-Associated Protease Plasmin

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New prodrugs of daunorubicin and doxorubicin designed for selective activation by the serine protease plasmin are described. The low toxic prodrugs **3**, **4**, and **5** are converted to the corresponding cytotoxic drugs upon proteolysis by the tumor-associated protease plasmin. Application of a self-eliminating spacer was essential for enzyme activation. A prodrug containing a chloro-substituted spacer was synthesized with the aim of enhancing the rate of conversion by plasmin. All prodrugs were highly stable in buffer solution and in serum and on the average 15-fold less cytotoxic than the parent drugs in seven human tumor cell lines. A marked in vitro selectivity was demonstrated by incubation of the doxorubicin prodrugs with a plasmin generating MCF-7 breast cancer cell line transfected with urokinase-type plasminogen activator (u-PA) in comparison with the nontransfected nonplasmin generating cell line. Prodrugs **4** and **5** showed the same cytotoxic effect as the free parent drug doxorubicin in the u-PA transfected cells, indicating complete conversion of the prodrug by plasmin. Addition of the plasmin inhibitor Trasylol drastically increased the ID_{50} values in the u-PA transfected MCF-7 cells for both prodrugs **4** and **5**.

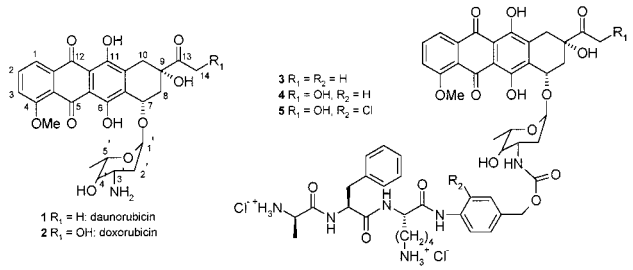
Introduction

Chemotherapeutic anticancer agents show significant toxicity toward proliferating normal cells. The lack of selectivity leads to severe side effects, which is still a serious drawback in conventional cancer chemotherapy. A promising approach to increase selectivity is to exploit the existence of tumor-associated enzymes that are able to activate a pharmacologically inactive prodrug to the corresponding active parent drug in the vicinity of the tumor. A high concentration of a toxic anticancer agent at the tumor site can be generated via this concept of "monotherapy". An attractive feature of prodrug monotherapy, unlike "antibody-directed enzyme prodrug therapy",[1] is that no antibody-enzyme conjugates are needed, which renders it relatively straightforward and commercially more interesting.[2]

In preceding investigations by us and by others it was shown that "prodrug monotherapy" works well with anthracycline prodrugs that are activated by β-glucuronidase.[3,4] β-Glucuronidase can be found in elevated concentrations in necrotic areas of tumor tissue.[5] The promising in vivo results that we obtained with the involved anthracycline prodrugs have stimulated us to develop new anthracycline prodrugs that are to be activated by the tumor-associated protease plasmin. Anthracyclines such as daunorubicin (**1**) and doxorubicin (**2**) (Chart 1) are still widely used cytotoxic agents in cancer chemotherapy due to their very broad activity spectrum and their high degree of efficacy against many human tumors.[7]

The plasmin system is recognized to play a key role in tumor invasion and metastasis by its matrix degrading activity and its involvement in tumor growth, most likely by its participation in growth factor activation and angiogenesis.[8−11] Active plasmin catalyzes the breakdown of extracellular matrix proteins and, thus, contributes to migration, invasion, and metastasis of tumor cells.[12,13] In the body plasmin is predominantly present in its inactive pro-enzyme form plasminogen. Active plasmin is formed locally at or near the surface of tumor cells by urokinase-type plasminogen activator (u-PA), produced by the cancer and/or stroma cells.[14,15] Plasmin activity is kept localized because cell-bound urokinase can activate cell-bound plasminogen into active plasmin, which stays cell-bound. Active urokinase and active plasmin do not occur in the blood circulation since they are very rapidly inhibited by inhibitors such as PAI-1 and α_2-antiplasmin, respectively. Many tumor cell lines and tumors have a significantly higher u-PA level than that of their normal counterparts,[16] and u-PA was shown to be correlated with invasive behavior and to be a strong prognostic factor for reduced survival and increased relapse in many types of tumors.[17−20] Plasmin is a very promising enzyme for exploitation in a tumor-specific prodrug approach because the proteolytically active form is localized at tumor level.

Chart 1



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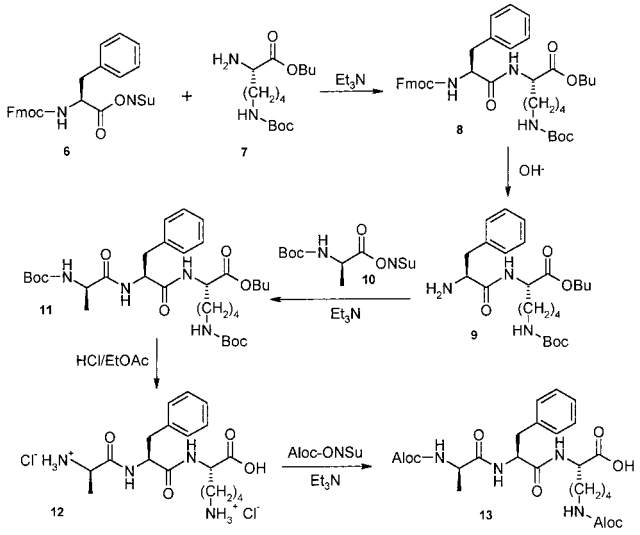
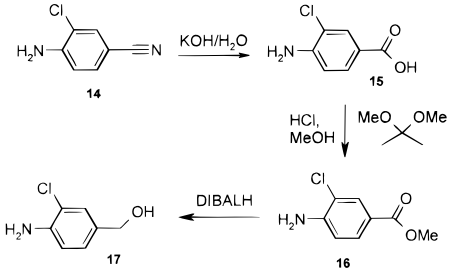
Scheme 1



Scheme 2



The first anthracycline prodrugs that were designed for specific activation by plasmin were reported by Katzenellenbogen et al.[21,22] These bipartate anthracycline prodrugs consisted of a tripeptide coupled to the anthracycline amino function. A major limitation of these prodrugs was that they were very inefficiently activated by plasmin. There was most likely too much steric hindrance for the protease to cleave the tripeptide. In a recent report, plasmin served as the target enzyme for tripartate prodrugs of alkylating agents that consisted of tripeptide specifiers coupled to the drug via a cyclization spacer.[23,24]

Recently, peptide conjugates of anthracyclines were reported which contained a monoclonal antibody that was needed for tumor specificity.[25] In these conjugates, dipeptides were applied for activation by lysosomal enzymes (in particular cathepsin B, C, or D are mentioned) after internalization by the cell. For optimization of cathepsin B cleavage after internalization, some model prodrugs containing several tripeptides were tested for their half-lives.[26] These prodrugs were claimed to be stable extracellularly.[27]

The prodrugs described in this paper are designed for extracellular activation by plasmin. The synthesized tripartate prodrugs contain a self-immolative 1,6-elimination spacer[28] placed between the tripeptide and the drug to make the proteolytic cleavage possible. Prodrug cytotoxicity was determined in seven human tumor cell lines. To obtain ultimate proof of in vitro selectivity, the prodrugs were tested in a MCF-7 breast cancer cell line that was transfected with u-PA and in MCF-7 control cells.

Chemistry

The prodrugs of the present paper were prepared by attaching the drug moiety to the spacer end of the tripeptide-spacer moieties. Doubly protected tripeptides **13** were synthesized via well-established peptide chemistry (Scheme 1).

Allyloxycarbonyl (Aloc) was chosen as protecting group. In the first step H-Lys(Boc)-OBu (**7**) was coupled with Fmoc-Phe-ONSu (**6**) (Scheme 1). After Fmoc deprotection under basic conditions, the dipeptide **9** was coupled with the third amino acid Boc-D-Ala-ONSu (**10**). Deprotection of the Boc groups and the *tert*-butyl ester under acidic conditions and following protection of both amines using Aloc-ONSu under basic conditions yielded the doubly protected tripeptide **13**.

The chloro-substituted aminobenzyl alcohol was not commercially available, and therefore, it was synthesized starting from 4-amino-2-chloro-benzonitrile **14** (Scheme 2).

The cyano functionality was hydrolyzed to the corresponding carboxylic acid **15** by heating under reflux in 50% potassium hydroxide. Treatment of amino acid **15** with 2,2-dimethoxypropane in the presence of hydrochloric acid gave the methylester **16**, which on reduction with DIBALH gave the desired amino alcohol **17**.

To synthesize anthracycline prodrugs **3**−**5**, 4-aminobenzyl alcohol **18** and the chloro-substituted spacer **17** were linked to the protected tripeptide **13** using isobutyl chloroformate as a coupling agent (Scheme 3).

Several coupling agents, i.e., 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) were also tried, but the highest yields were obtained with the chloroformate. The benzylic alcohols **19** and **20** were converted subsequently into the activated analogues **21** and **22** by reacting them with 4-nitrophenyl chloroformate. Several coupling methods were tried, but the best results were obtained via this route. For the construction of the tripartate anthracycline prodrugs, the activated 4-nitrophenyl carbonates were reacted with daunorubicin and doxorubicin to yield protected prodrugs **23**, **24**, and **25**. In the final step the Aloc proctecting groups were removed smoothly with a palladium catalyst in the presence of morpholine. Treatment with hydrochloric acid provided the corresponding prodrugs **3**, **4**, and **5** as their double ammonium salt.

Biological Data

Prodrugs **3**, **4**, and **5** were highly stable in a Tris buffer solution (pH 7.3). No degradation products were detected after 3 days of incubation, and all prodrugs remained completely intact upon incubation in bovine serum for 3 days.

In contrast to plasmin prodrugs of anthracyclines without a self-immolative spacer,[21] prodrugs **3**, **4**, and **5** were indeed converted to yield the corresponding anthracyclines upon incubation with human plasmin. To determine the kinetics of enzymatic hydrolysis, prodrugs **3**, **4**, and **5** were incubated at a concentration of 100 μM with 50 μg/mL human plasmin in Tris buffer solution. The half-lives of enzymatic hydrolysis for prodrugs **3**, **4**, and **5** were 14, 19, and 7 min, respectively, and they were determined using an analytical RP-HPLC column. As was expected, the chloro-substituted prodrug **5** showed a clearly enhanced rate of

Scheme 3

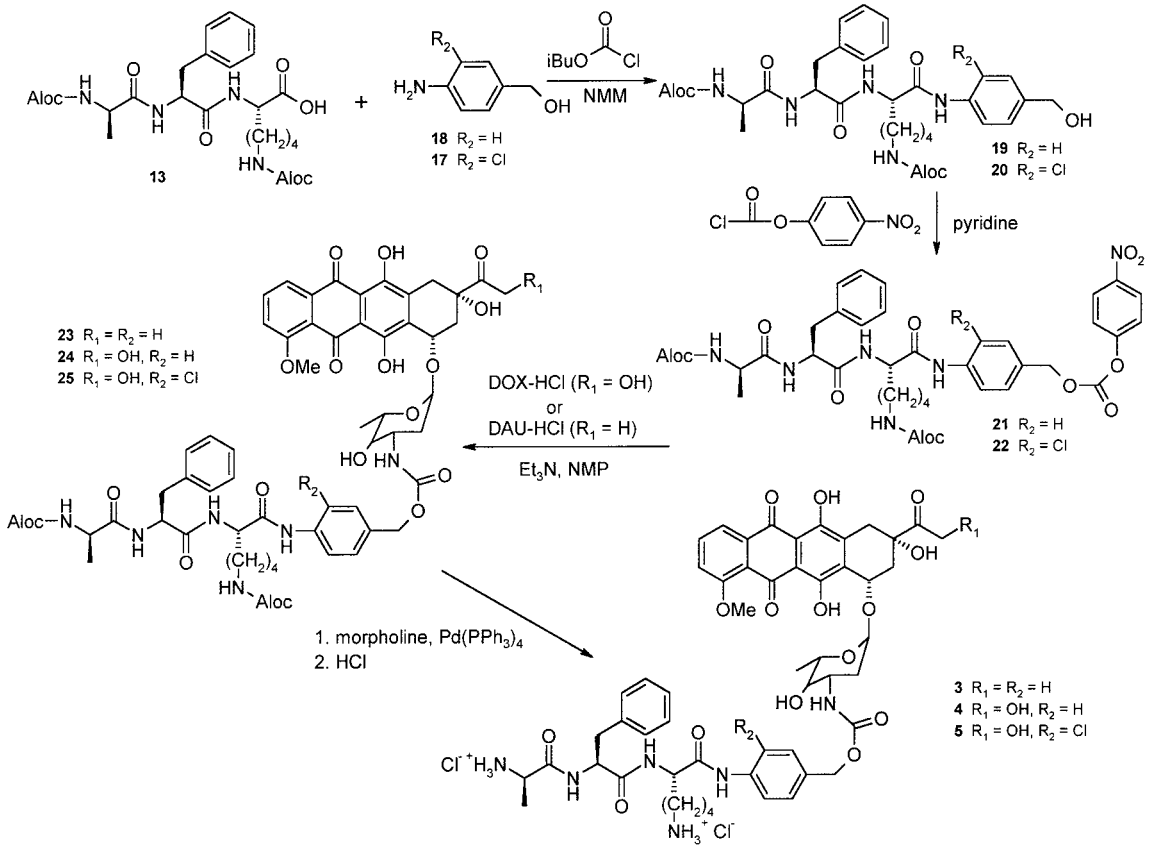


Table 1. Cytotoxicity (ID_{50} Values[a,b] (ng/mL)) of Daunorubicin, Doxorubicin, and Prodrugs **3**−**5** in Various Tumor Cell Lines

	MCF-7	EVSA-T	WIDR	IGROV	M19	A498	H226
daunorubicin (**1**)	329	48	38	113	309	487	243
doxorubicin (**2**)	10	8	11	60	16	90	199
prodrug **3**	1214	1195	2150	225	1578	1158	1698
prodrug **4**	176	141	413	590	437	473	896
prodrug **5**	72	248	140	224	350	763	1429

[a] Drug dose that inhibited cell growth by 50% compared to untreated control cultures. [b] SRB cell viability test.

conversion by plasmin, indicating that the electron withdrawing substituent facilitates the enzymatic cleavage by plasmin.

All prodrugs showed a strongly reduced cytotoxicity in seven well-characterized human tumor cell lines in comparison with the corresponding parent drugs (Table 1).[29]

An average 15-fold reduction of cytotoxicity was observed, the reduction ranging from 2 to 56 times. These data may suggest that the seven cell cultures express different concentrations of u-PA or plasmin, although these levels are expected to be low. Unfortunately, the plasmin generating activity of these cell lines is unknown so that no clear conclusions can be drawn with respect to plasmin-dependent hydrolysis of the prodrugs.

To test the in vitro selectivity in cell lines with well-defined plasmin generating activity, the prodrugs **4** and **5** and doxorubicin were incubated for 24 h in a MCF-7 breast cancer cell culture that was transfected with wildtype u-PA and in MCF-7 control cells lacking u-PA. The parent compound doxorubicin was equally cytotoxic for both control nontransfected MCF-7 cells and u-PA producing MCF-7 cells. Addition of the plasmin inhibitor Trasylol had no effect (Figure 1).

However, in cells cultured with prodrugs **4** or **5** a much higher cytotoxicity was observed in u-PA producing MCF-7 cells as compared with control cells (ID_{50} = 7.8 μM versus 49 μM for prodrug **4** and 4.1 μM versus 57 μM for prodrug **5**). Prodrugs **4** and **5** show the same cytotoxic effect as the free parent drug doxorubicin in the u-PA transfected cells, indicating complete conversion of the prodrug by plasmin. Furthermore, the addition of the plasmin inhibitor Trasylol, which had no effect on doxorubicin treated cells, drastically increased the ID_{50} values in the u-PA transfected cells for both prodrugs **4** and **5** (ID_{50} = 7.8 μM versus 84 μM for prodrug **4** and 4.1 μM versus 107 μM for prodrug **5**).

Discussion

The prodrugs of the present paper show an average 15-fold reduced cytotoxicity in seven human tumor cell cultures. It may be concluded that the synthesized prodrugs are being selectively activated upon incubation with plasmin generating tumor cells. The findings that prodrugs **4** and **5** show much higher growth inhibition potency in the u-PA transfected cell line in comparison with the nontransfected cell line strongly suggest that it is plasmin activity that is responsible for the observed cytotoxicity in this assay. The evidence for plasmin-

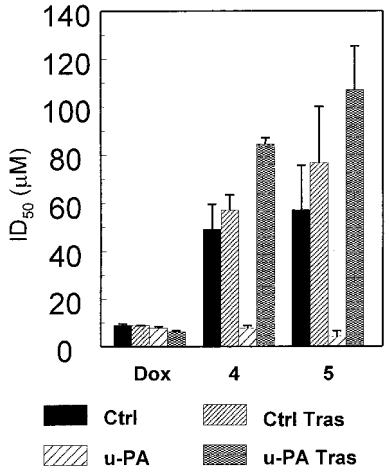


Figure 1. ID_{50} values (μM) for doxorubicin and prodrugs **4** and **5** in u-PA producing MCF-7 cells and MCF-7 control cells, with and without the addition of plasmin inhibitor Trasylol. Control MCF-7 cells (first and second bar) and u-PA transfected cells (third and fourth bar) were cultured with and without 400 KIE/mL Trasylol in the presence of doxorubicin (left) or prodrugs **4** (middle) and **5** (right). Surviving cells were determined after 24 h as described in the Experimental Section.

mediated prodrug activation is further supported by the results obtained by incubation with the specific plasmin inhibitor Trasyslol. As doxorubicin prodrugs **4** and **5** show high in vitro selectivity and potency, further in vivo evaluation will be performed with these prodrugs.

Experimental Section

Chemistry. ^{1}H NMR spectra were measured on a Bruker AM-300 (300 MHz, FT) spectrometer in the given solvent. Chemical shift values are reported as δ-values in parts per million in comparison with TMS as an internal standard. The assignment of protons is partial for the prodrugs. Mass spectra were obtained with a double-focusing VG 7070E spectrometer. Elemental analyses were carried out in triplicate on a Carlo Erba Instruments CHNSO EA 1108 element analyzer and were within 0.4% of the theoretical values calculated for C, H, and N. Melting points were measured on a Reichert Thermopan microscope and are uncorrected. TLC was performed using precoated silica gel ($60F_{254}$) plates, and spots were examined with UV light, an ammonium molybdic solution, or with a Chloro-TDM test. Chromatography was carried out on Baker silica gel.

Fmoc-Phe-Lys(Boc)-OBu (8). To a solution of 2.50 g of Fmoc-Phe-ONSu (**6**) (5.16 mmol) in dry dichloromethane under an argon atmosphere were added at 0 °C 0.791 mL of triethylamine (1.1 equiv) and 1.92 g of H-Lys(Boc)-OBu·HCl (**7**, 1.1 equiv). The mixture was stirred at room temperature for 5 h, then dichloromethane was added, and the organic layer was washed with 10% citric acid, saturated sodium bicarbonate and water, dried (Na_2SO_4), and evaporated. The white solid (3.08 g, 89%) was used without further purification: mp 93 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.10−1.90 (m, 24H, 6 CH_2-Lys and 18 *t*-Bu), 3.06 (m, 4H, N-CH_2-Lys and Bn), 4.19 (t, 1H, J = 6.8 Hz, Fmoc), 4.25−4.55 (m, 4H, 2 Fmoc and 2 Hα), 7.19−7.78 (m, 13H, aromatic) ppm; MS (FAB) *m/e* 672 (M + H)$^+$, 694 (M + Na)$^+$; Anal. ($C_{39}H_{49}N_3O_7$) C, H, N.

Boc-D-Ala-Phe-Lys(Boc)-OBu (11). Fmoc-Phe-Lys(Boc)-OBu (**8**) (3.08 g, 4.58 mmol) was dissolved in 100 mL of dioxane/methanol/2 N sodium hydroxide (70/25/5) and stirred at room temperature for 1 h. The mixture was neutralized with acetic acid (0.571 mL), and organic solvents were evaporated. Water and dioxane were added, and the solution was freeze-dried. Diisopropyl ether was added, and after filtration the filtrate was evaporated. The product was dissolved in dry dichloromethane and added at 0 °C to a solution of 1.19 g (4.16 mmol) of Boc-D-Ala-ONSu (**10**) and 0.634 mL (1.1 equiv) of triethylamine in dry dichloromethane. The mixture was stirred for 16 h after which dichloromethane was added. The organic layer was washed with 10% citric acid, saturated sodium bicarbonate, and water, dried (Na_2SO_4), and evaporated. The product was purified by means of column chromatography (chloroform−methanol; 20/1) to afford 2.56 g (4.13 mmol, 99%) of tripeptide **11** as a white foam: mp 59 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (d, 3H, J = 3.2 Hz, CH_3-Ala), 1.43 (br s, 27H, *t*-Bu), 1.00−1.90 (m, 6H, CH_2-Lys), 2.80−3.30 (m, 4H, N-CH_2-Lys and Bn), 4.15 (m, 1H, Hα), 4.35 (m, 1H, Hα), 4.64 (br d, 1H, J = 7.1 Hz, Hα), 7.15−7.35 (m, 5H, aromatic) ppm; MS (FAB) *m/e* 621 (M + H)$^+$, 643 (M + Na)$^+$. Anal. ($C_{32}H_{52}N_4O_8 \cdot {}^1/_2H_2O$) C, H, N.

D-Ala-Phe-Lys-OH(·2HCl) (12). Boc-D-Ala-Phe-Lys(Boc)-OBu (**11**) (2.56 g, 4.13 mmol) was stirred in a solution of hydrochloric acid in ethyl acetate (3 M). After 5 h the solvent was evaporated, and *tert*-butyl alcohol was added and evaporated twice to remove remaining hydrochloric acid. The product was freeze-dried in dioxane/water to yield a cream colored powder, which was used without further purification: ^{1}H NMR (300 MHz, D_2O) δ 0.94 (d, 3H, J = 7.0 Hz, CH_3-Ala), 1.10−1.85 (m, 6H, CH_2-Lys), 2.75−2.84 (m, 3H, N-CH_2-Lys and Bn), 3.09 (dd, 1H, J = 5.4 Hz, J = 14.1 Hz, Bn), 3.54 (q, 1H, J = 7.0 Hz, Hα), 3.98 (dd, 1H, J = 5.4 Hz, J = 7.8 Hz, Hα), 4.54 (dd, 1H, J = 5.4 Hz, J = 9.8 Hz, Hα), 7.10−7.22 (m, 5H, aromatic) ppm; MS (FAB) *m/e* 365 (M + H)$^+$.

Aloc-D-Ala-Phe-Lys(Aloc)-OH (13). To a solution of 706 mg (1.61 mmol) of D-Ala-Phe-Lys-OH (**12**) in water/acetonitrile was added triethylamine until a pH of 9−9.5 was reached. Then a solution of 704 mg (2.2 equiv) of Aloc-ONSu in acetonitrile was added, and the mixture was kept basic by adding triethylamine. After the pH of the mixture did not alter anymore, a 0.5 M solution of hydrochloric acid in ethyl acetate was added until a pH of 3 was reached. The mixture was thoroughly extracted with dichloromethane. The organic layer was washed with water, dried (Na_2SO_4), and evaporated to result in the desired product (**13**) as a cream colored foam (742 mg, 86%): mp 141 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.10−1.95 (m, 6H, CH_2-Lys), 1.21 (d, 3H, J = 6.5 Hz, CH_3-Ala), 2.90−3.30 (m, 4H, N-CH_2-Lys and Bn), 4.20 (m, 1H, Hα), 4.55 (m, 5H, Hα and 4 Aloc), 4.76 (br d, 1H, J = 5.2 Hz, Hα), 5.17−5.31 (m, 4H, Aloc), 5.83−5.92 (m, 2H, Aloc), 7.20−7.28 (m, 5H, aromatic) ppm; MS (FAB) *m/e* 533 (M + H)$^+$, 555 (M + Na)$^+$; Anal. ($C_{26}H_{36}N_4O_8$) C, H, N.

Methyl 4-Amino-3-chlorobenzoate (16). Starting compound **14** (2.32 g, 15.2 mmol) was dissolved in 100 mL of a 50% potassium hydroxide solution and refluxed for 19 h. The mixture was diluted with 200 mL water and filtrated. The filtrate was acidified with concentrated hydrochloric acid, and the mixture was freeze-dried. To the product was added methanol, and the inorganic salt was filtered. The filtrate was evaporated to dryness, and 50 mL of dimethoxypropane, 3 mL of concentrated hydrochloric acid, and 6 mL methanol were added. The mixture was stirred under refluxing conditions for 6 h and additionally stirred for 18 h. Then, ethyl acetate was added, and the organic layer was washed with a saturated sodium bicarbonate solution and brine, dried (Na_2SO_4), and evaporated resulting in a brown oil. Upon the addition of hexane, a solid precipitated. After filtration, product **16** (1.49 g, 53% for two steps) was obtained as a green powder: mp 86 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.86 (s, 3H, Me), 4.07 (br s, 2H, NH_2), 6.52 (dd, 1H, J = 2.3 Hz, J = 8.6 Hz, aromatic), 6.70 (d, 1H, J = 2.3 Hz, aromatic), 7.78 (d, 1H, J = 8.5 Hz, aromatic) ppm; MS (EI) *m/e* 185 (M$^+$). Anal. ($C_8H_8NO_2Cl$) C, H, N.

(4-Amino-3-chlorophenyl)methanol (17). To a solution of 840 mg (4.53 mmol) of **16** in dry THF was added 7.55 mL of DIBALH (2.5 equiv, 1.5 M in toluene) under an argon atmosphere, and the mixture was stirred for 16 h. The solvent was evaporated, and dichloromethane was added together with 10% citric acid. Then to the water layer was added potassium hydroxide, and the water layer was extracted with dichloromethane. The organic layer was washed with saturated

sodium bicarbonate and brine, dried (Na_2SO_4), and evaporated. The residual oil was purified by means of column chromatography (chloroform−methanol; 9/1) to afford 343 mg (48%) of the amino alcohol (**22**): mp 99 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.61 (s, 2H, Bn), 6.59 (dd, 1H, J = 2.3 Hz, J = 8.2 Hz, aromatic), 6.72 (d, 1H, J = 2.3 Hz, aromatic), 7.20 (d, 1H, J = 8.2 Hz, aromatic) ppm; MS (EI) *m/e* 157 (M^+). Anal. (C_7H_8NOCl) C, H, N.

General Procedure for the Coupling of Spacers 17 and 18 to the Protected Tripeptide 13. Aloc-D-Ala-Phe-Lys(Aloc)-PAB-OH (19). A solution of 730 mg of **13** (1.37 mmol) was dissolved in dry THF under an argon atmosphere and cooled to −40 °C. *N*-Methylmorpholine (166 μL, 1.1 equiv) and isobutylchloroformate (196 μL, 1.1 equiv) were added. The reaction mixture was stirred for 3 h at a temperature below −30 °C. A solution of 4-aminobenzyl alcohol (203 mg, 1.2 equiv) and *N*-methylmorpholine (181 μL, 1.2 equiv) in dry THF was added dropwise to the reaction mixture. After 2 h, THF was evaporated and dichloromethane was added. The organic layer was washed with saturated sodium bicarbonate, a 0.5 N potassium bisulfate solution, and brine, dried (Na_2SO_4), and evaporated. The residual pale yellow solid was purified by means of column chromatography (chloroform−methanol; 9:1) to afford 812 mg (93%) of the desired product **19** as a cream colored powder: mp 156 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.96 (d, 3H, J = 7.1 Hz, CH_3-Ala), 1.10−1.85 (m, 6H, CH_2-Lys), 2.77 (dd, 1H, J = 10.2 Hz, J = 13.6 Hz, Bn Phe), 2.97 (br d, 2H, J = 5.9 Hz, N-CH_2-Lys), 3.09 (br d, 1H, J = 10.4 Hz, Bn Phe), 4.00 (t, 1H, J = 7.1 Hz, Hα), 4.20−4.60 (m, 8H, 2 Hα and 4 Aloc and CH_2-OH), 5.00−5.35 (m, 4H, Aloc), 5.76−5.95 (m, 2H, Aloc), 7.05−7.30 (m, 7H, aromatic), 7.41 (d, 1H, J = 7.0 Hz, NH), 7.56 (d, 2H, J = 8.4 Hz, aromatic), 8.12 (d, 1H, J = 7.7 Hz, NH), 8.18 (d, 1H, J = 8.1 Hz, NH), 9.80 (s, 1H, NH anilide) ppm; MS (FAB) *m/e* 638 $(M + H)^+$, 660 $(M + Na)^+$. Anal. ($C_{33}H_{43}N_5O_8 \cdot {}^1/_2H_2O$) C, H, N.

General Procedure for the Activation of Benzylic Alcohols 19 and 20 with 4-Nitrophenyl Chloroformate. Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PNP (21). To a solution of 384 mg (0.602 mmol) of **19** in dry THF/dichloromethane under an argon atmosphere were added 4-nitrophenylchloroformate (182 mg, 1.5 equiv) and dry pyridine (73 μL, 1.5 equiv). The mixture was stirred at room temperature for 48 h, and then ethyl acetate was added. The organic layer was washed with 10% citric acid, brine, and water, dried (Na_2SO_4), and evaporated yielding a yellow solid. The product was purified by means of column chromatography (dichloromethane−methanol; 30:1) to afford 324 mg (67%) of carbonate **21**: ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.21 (d, 3H, J = 7.1 Hz, CH_3-Ala), 1.25−2.05 (m, 6H, CH_2-Lys), 2.95 (br d, 1H, J = 9.7 Hz, Bn Phe), 3.13 (br t, 2H, J = 5.6 Hz, N-CH_2-Lys), 3.27 (dd, 1H, J = 4.2 Hz, J = 16.2 Hz, Bn Phe), 4.08 (q, 1H, J = 7.1 Hz, Hα), 4.25 (dd, 1H, Hα), 4.30−4.65 (m, 5H, Hα and 4 Aloc), 5.04−5.35 (m, 4H, Aloc), 5.26 (s, 2H, CH_2-OH), 5.65−6.00 (m, 2H, Aloc), 7.10−7.35 (m, 5H, aromatic), 7.39−7.43 (2 * d, 4H, aromatic), 7.71 (d, 2H, J = 8.3 Hz, aromatic), 8.28 (d, 2H, J = 9.1 Hz, aromatic) ppm; MS (FAB) *m/e* 803 $(M + H)^+$, 825 $(M + Na)^+$.

General Procedure for the Coupling of Doxorubicin and Daunorubicin to Carbonates 21 and 22. Aloc-D-Ala-Phe-Lys(Aloc)-PABC-DAU (23). 4-Nitrophenyl carbonate **21** (51 mg, 0.0635 mmol) and daunorubicin-HCl (37.6 mg, 1.05 equiv) in *N*-methylpyrrolidinone were treated at room temperature with triethylamine (10.0 μL, 1.05 equiv). The reaction mixture was stirred in the dark for 16 h and was then diluted with 10% 2-propanol/ethyl acetate. The organic layer was washed with brine and water and was dried (Na_2SO_4). After evaporation of the solvents, the crude product was purified by means of circular chromatography using a chromatotron supplied with a 2 mm silica plate (chloroform−methanol; 60:1 and 20:1 respectively) to yield 73 mg (97%) of **23**: mp 119−125 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.23 (d, 3H, J = 6.6 Hz, sugar CH_3), 1.26 (d, 3H, J = 6.6 Hz, CH_3-Ala), 1.25−2.00 (m, 7H, CH_2-Lys and 2′), 2.10 (dd, 1H, J = 4.1 Hz, J = 14.5 Hz, 2′), 2.32 (br d, 1H, J = 14.9 Hz, 8), 2.42 (s, 3H, 14), 2.44 (br d, 1H, 8), 2.90−3.35 (m, 6H, Bn Phe and N-CH_2-Lys and 10), 3.63 (s, 1H, 4′), 3.88 (br d, 1H, J = 8.2 Hz, 3′), 4.06 (br s, 4H, OMe and 5′), 4.20 (m, 2H, Hα), 4.35−4.70 (m, 5H, Hα and 4 Aloc), 4.96 (2 * d, 2H, J = 11.7 Hz, Bn spacer), 5.05−5.35 (m, 4H, Aloc), 5.46 (m, 2H, 1′ and 7), 5.60−6.00 (m, 2H, Aloc), 7.10−7.35 (m, 7H, aromatic), 7.39 (d, 1H, J = 8.5 Hz, 3), 7.56 (d, 2H, J = 7.5 Hz, aromatic), 7.78 (t, 1H, J = 8.2 Hz, 2), 8.02 (d, 1H, J = 7.6 Hz, 1) ppm; MS (FAB) *m/e* 1213 $(M + Na)^+$. Anal. ($C_{61}H_{70}N_6O_{19} \cdot 2{}^1/_2H_2O$) C, H, N.

General Procedure for the Deprotection of Protected Prodrugs 3−5. D-Ala-Phe-Lys-PABC-DAU(·2HCl) (3). To a solution of 19 mg (0.016 mmol) of protected prodrug **23** in dry THF/dichloromethane under an argon atmosphere was added morpholine (14 μL, 10 equiv) together with a catalytic amount of $Pd(PPh_3)_4$. The reaction mixture was stirred for 1 h in the dark. The red precipitate was collected by means of centrifugation. Ethyl acetate was added, and the mixture was acidified using 0.5 mL of 1 M hydrochloric acid/ethyl acetate. The precipitate was collected by means of centrifugation and washed several times with ethyl acetate. *tert*-Butyl alcohol was added and evaporated, and the resulting red film was freeze-dried in water yielding 18 mg (100%) of daunorubicin prodrug **3**: mp 128 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.20 (d, 3H, J = 6.9 Hz, sugar CH_3), 1.28 (d, 3H, J = 6.6 Hz, CH_3-Ala), 1.25−2.00 (m, 8H, CH_2-Lys and 2′), 2.09 (dd, 1H, J = 6.1 Hz, 8), 2.33 (br d, 1H, J = 15.2 Hz, 8), 2.42 (s, 3H, 14), 2.89−3.02 (m, 3H, N-CH_2-Lys and 10), 3.19−3.38 (m, 3H, Bn Phe and 10), 3.62 (s, 1H, 4′), 3.87 (m, 1H, 3′), 4.05 (s, 3H, OMe), 4.18 (m, 2H, 5′ and Hα), 4.52 (br d, 1H, J = 6.6 Hz, Hα), 4.65 (m, 1H, Hα), 4.95 (2 * d, 2H, J = 12.2 Hz, Bn spacer), 5.24 (br s, 1H, 1′), 5.48 (br s, 1H, 7), 7.05−7.35 (m, 7H, aromatic), 7.39 (d, 1H, J = 8.4 Hz, 3), 7.52 (d, 2H, J = 8.2 Hz, aromatic), 7.77 (t, 1H, J = 8.1 Hz, 2), 8.01 (d, 1H, J = 7.7 Hz, 1) ppm; MS (FAB) *m/e* 1023 $(M + H)^+$, 1045 $(M + Na)^+$. Anal. ($C_{53}H_{62}N_6O_{15} \cdot 6.4HCl$) C, H, N.

Aloc-D-Ala-Phe-Lys(Aloc)-PABC-DOX (24): yield (76%); mp 124 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.22 (d, 3H, J = 7.2 Hz, sugar CH_3), 1.28 (d, 3H, J = 6.6 Hz, CH_3-Ala), 1.25−2.00 (m, 8H, CH_2-Lys and 2′), 2.15 (dd, 1H, J = 3.3 Hz, J = 13.9 Hz, 8), 2.35 (br d, 1H, 8), 3.05 (br d, 1H, J = 18.9 Hz, 10), 2.90−3.35 (m, 5H, Bn Phe and N-CH_2-Lys and 10), 3.61 (m, 1H, 4′), 3.73 (m, 1H, 3′), 3.85 (m, 1H, Hα), 4.08 (s, 3H, OMe), 4.07−4.32 (m, 2H, Hα and 5′), 4.35−4.70 (m, 5H, Hα and 4 Aloc), 4.76 (s, 2H, 14), 4.97 (m, 2H, Bn spacer), 5.03−5.38 (m, 4H, Aloc), 5.49 (br s, 1H, 1′), 5.55 (br d, 1H, J = 8.3 Hz, 7), 5.60−6.00 (m, 2H, Aloc), 7.05−7.35 (m, 7H, aromatic), 7.41 (d, 1H, J = 8.2 Hz, 3), 7.56 (d, 2H, J = 7.3 Hz, aromatic), 7.79 (t, 1H, J = 8.2 Hz, 2), 8.04 (d, 1H, J = 7.6 Hz, 1) ppm; MS (FAB) *m/e* 1230 $(M + Na)^+$. Anal. ($C_{61}H_{70}N_6O_{20} \cdot 3{}^1/_2H_2O$) calculated C 57.68%, H 6.11%, N 6.62%; measured C 57.77%, H 5.74%, N 6.19%.

D-Ala-Phe-Lys-PABC-DOX(·2HCl) (4): yield (96%); mp >320 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.21 (d, 3H, J = 7.0 Hz, sugar CH_3), 1.29 (d, 3H, J = 6.4 Hz, CH_3-Ala), 1.30−2.10 (m, 8H, CH_2-Lys and 2′), 2.18 (m, 1H, 8), 2.36 (d, 1H, J = 14.1 Hz, 8), 2.80−3.40 (m, 6H, Bn Phe and N-CH_2-Lys and 10), 3.62 (br s, 1H, 4′), 3.91 (m, 1H, 3′), 4.00−4.15 (m, 4H, OMe and 5′), 4.21 (br d, 1H, J = 7.3 Hz, Hα), 4.51 (m, 1H, Hα) 4.64 (m, 1H, Hα), 4.77 (s, 2H, 14), 4.97 (2 * d, 2H, J = 11.9 Hz, Bn spacer), 5.29 (br s, 1H, 1′), 5.49 (br s, 1H, 7), 7.10−7.35 (m, 7H, aromatic), 7.44 (d, 1H, J = 8.3 Hz, 3), 7.54 (d, 2H, J = 8.4 Hz, aromatic), 7.82 (t, 1H, J = 8.1 Hz, 2), 8.04 (d, 1H, J = 7.8 Hz, 1) ppm; MS (FAB) *m/e* 1039 $(M + H)^+$. Anal. ($C_{53}H_{62}N_6O_{16} \cdot 4.7HCl$) C, H, N.

Aloc-D-Ala-Phe-Lys(Aloc)-PAB(Cl)-OH (20): yield (55%); mp 173 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.23 (d, 3H, J = 7.1 Hz, CH_3-Ala), 1.25−2.00 (m, 6H, CH_2-Lys), 2.99 (dd, 1H, J = 9.2 Hz, J = 13.8 Hz, Bn Phe), 3.14 (br d, 2H, J = 5.7 Hz, N-CH_2-Lys), 3.25 (dd, 1H, J = 4.3 Hz, Bn Phe), 4.07 (br d, 1H, J = 7.0 Hz, Hα), 4.30 (dd, 1H, J = 5.0 Hz, Hα), 4.35−4.65 (m, 5H, Hα and 4 Aloc), 4.69 (s, 2H, CH_2-OH), 5.10−5.31 (m, 4H, Aloc), 5.65−6.00 (m, 2H, Aloc), 7.10−7.32 (m, 5H, aromatic), 7.37−7.49 (m, 2H, aromatic), 7.79 (br s, 1H, aromatic)

ppm; MS (FAB) *m/e* 672 $(M + H)^+$, 694 $(M + Na)^+$. Anal. ($C_{33}H_{41}N_5O_8Cl \cdot H_2O$) C, H.

Aloc-D-Ala-Phe-Lys(Aloc)-PABC(Cl)-PNP (22): yield (96%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (d, 3H, J = 6.8 Hz, CH_3-Ala), 1.15−2.10 (m, 6H, CH_2-Lys), 2.90−3.40 (m, 4H, N-CH_2-Lys and Bn Phe), 4.02 (m, 1H, Hα), 4.21 (m, 1H, Hα), 4.36 (dd, 1H, J = 5.5 Hz, J = 13.1 Hz, Hα), 4.45−4.75 (m, 4H, Aloc), 4.95−5.35 (m, 4H, Aloc), 5.36 (s, 2H, CH_2-OH), 5.66 (m, 1H, Aloc), 5.91 (m, 1H, Aloc), 7.10−7.45 (m, 9H, aromatic), 7.61 (br d, 1H, aromatic), 7.97 (br s, 1H, aromatic), 8.27 (d, 2H, J = 9.1 Hz, aromatic) ppm; MS (FAB) *m/e* 837 $(M + H)^+$, 859 $(M + Na)^+$. Anal. ($C_{40}H_{44}N_6O_{12}Cl \cdot 1^1/_4H_2O$) calculated C 55.94%, H 5.46%, N 9.79%; measured C 56.32%, H 5.42%, N 9.31%.

Aloc-D-Ala-Phe-Lys(Aloc)-PABC(Cl)-DOX (25): yield (75%); mp 111 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.23 (d, 3H, J = 6.9 Hz, sugar CH_3), 1.28 (d, 3H, J = 6.5 Hz, CH_3-Ala), 1.20−2.05 (m, 8H, CH_2-Lys and 2′), 2.14 (dd, 1H, J = 4.1 Hz, J = 14.9 Hz, 8), 2.35 (br d, 1H, 8), 2.90−3.35 (m, 6H, Bn Phe and N-CH_2-Lys and 10), 3.63 (br s, 1H, 4′), 3.86 (m, 1H, 3′), 4.06 (s, 3H, OMe), 4.00−4.35 (m, 3H, 2 Hα and 5′), 4.35−4.75 (m, 5H, Hα and 4 Aloc), 4.76 (s, 2H, 14), 5.00−5.38 (m, 6H, Bn spacer and 4 Aloc), 5.48 (br s, 1H, 1′), 5.59 (br d, 1H, J = 8.5 Hz, 7), 5.55−6.05 (m, 2H, Aloc), 7.05−7.35 (m, 6H, aromatic), 7.28−7.47 (m, 2H, aromatic), 7.70−7.85 (m, 2H, aromatic), 8.01 (d, 1H, J = 7.7 Hz, 1) ppm; MS (FAB) *m/e* 1263 $(M + Na)^+$. Anal. ($C_{61}H_{69}N_6O_{20}Cl \cdot 4H_2O$) calculated C, H, N 6.40%; measured C, H, N 5.93%.

D-Ala-Phe-Lys-PABC(Cl)-DOX(·2HCl) (5): yield (92%); mp 201 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.20 (d, 3H, J = 6.9 Hz, sugar CH_3), 1.29 (d, 3H, J = 6.4 Hz, CH_3-Ala), 1.30−2.05 (m, 8H, CH_2-Lys and 2′), 2.17 (dd, 1H, J = 3.9 Hz, J = 14.9 Hz, 8), 2.37 (br d, 1H, J = 14.8 Hz, 8), 2.85−3.45 (m, 6H, Bn Phe and N-CH_2-Lys and 10), 3.63 (br s, 1H, 4′), 3.89 (m, 1H, 3′), 4.08 (s, 3H, OMe), 4.21 (m, 2H, Hα and 5′), 4.51 (m, 1H, Hα), 4.66 (m, 1H, Hα), 4.78 (s, 2H, 14), 5.08 (2 * d, 2H, J = 13.1 Hz, Bn spacer), 5.27 (br s, 1H, 1′), 5.49 (br s, 1H, 7), 7.10−7.35 (m, 6H, aromatic), 7.35−7.50 (m, 2H, aromatic), 7.70−7.88 (m, 2H, aromatic), 8.03 (d, 1H, J = 7.5 Hz, 1) ppm; MS (FAB) *m/e* 1073 $(M + H)^+$. Anal. ($C_{53}H_{61}N_6O_{16}Cl \cdot 4.7HCl$) C, H, N.

Biological Characterization. Stability of the Prodrug in Serum and Buffer. Prodrugs **3**, **4**, and **5** were incubated at concentrations of 100−270 μM in 0.1 M Tris/hydrochloric acid buffer (pH 7.3) for 3 days and showed no formation of parent drug (TLC, RP_{18}; acetonitrile−water−acetic acid: 19:19:2). Prodrugs **3**, **4**, and **5** were incubated at 100 μM for 72 h in bovine serum and showed again no degradation products (TLC, RP_{18}; acetonitrile−water−acetic acid: 19:19:2).

Kinetics of Enzymatic Hydrolysis. Hydrolysis of prodrugs **3**, **4**, and **5** was investigated by incubation at a prodrug concentration of 100 μM in 0.1 M Tris/hydrochloric acid buffer (pH 7.3) in the presence of 50 μg/mL human plasmin (Fluka). Analysis was carried out with the following HPLC system using a Chrompack Microsphere-C18 column (3 μm, 2 × 100 × 4.6 mm). Elution of the analytical column was performed using 7:3 methanol/50 mM Et_3N−formate buffer (pH 3.0). Detection was performed using a UV detector (λ = 500 nm).

In Vitro Cytotoxicity in Seven Human Tumor Cell Lines. The antiproliferative effect of prodrugs **3**, **4**, and **5**, daunorubicin, and doxorubicin was determined in vitro applying seven well-characterized human tumor cell lines and the microculture sulforhodamine B (SRB) test.[30] The antiproliferative effects were determined and expressed as ID_{50} values, which are the (pro)drug concentrations that gave 50% inhibition when compared to control cell growth after 5 days of incubation. Results were averaged from experiments that were performed in quadruplicate.

In Vitro Selectivity. MCF-7 breast carcinoma cells, producing no or small amounts (<10 ng/10^5 cells in 24 h) of t-PA and u-PA, and MCF-7 cells transfected with u-PA cDNA (MCF-7.WT.u-PA) expressing u-PA protein at levels of 260 ng/10^5 cells in 24 h were cultured in HEPES buffered Dulbecco's Modified Eagle's Medium (DMEM) containing 1 g/L glucose and Glutamax, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. All cells were grown at 37 °C in a humid atmosphere with 5% (v/v) CO_2. At the beginning of the experiment 5×10^4 MCF-7 and MCF-7.WT.u-PA cells were seeded into wells of a 24-well plate. The medium was aspirated the next day and replaced with 500 μL of fresh medium containing 10% FCS, 0.15 μM human plasminogen, and (pro)drugs **4**, **5**, and doxorubicin in a concentration of 0, 0.1, 0.3, 1, 3, 10, 20, 40, 60, 80, 100, and 150 μM. After 24 h, medium was aspirated and cells were fixed with 0.5 mL of 2.5% (v/v) glutaraldehyde and stained with 0.2% (v/v) crystalviolet. The area covered by cells was determined by video microscope analysis (Optimas software). Cultures without doxorubicin or analogue were used as 100% control. From dose−response curves for doxorubicin and both prodrugs ID_{50} values were determined, and the results were averaged from three experiments. The bars in Figure 1 indicate the standard error of the mean (SEM).

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