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Correlation of the acid-sensitivity of polyethylene glycol daunorubicin conjugates with their in vitro antiproliferative activity

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Abstract—Polyethylene glycol conjugates with linkers of varying acid-sensitivity were prepared by reacting five maleimide derivatives of daunorubicin containing an amide bond (**1**) or acid-sensitive carboxylic hydrazone bonds (**2**–**5**) with α-methoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 20000) or α,ω-bis-thiopropionic acid amide poly(ethylene glycol) (MW 20000). The polymer drug derivatives were designed to release daunorubicin inside the tumor cell by acid-cleavage of the hydrazone bond after uptake of the conjugate by endocytosis. In subsequent cell culture experiments, the order of antitumor activity of the PEG daunorubicin conjugates correlated with their acid-sensitivity as determined by HPLC (cell lines: BXF T24 bladder carcinoma and LXFL 529L lung cancer cell line; assay: propidium iodide fluorescence assay). The acid-sensitivity of the link between PEG and daunorubicin is therefore an important parameter for in vitro efficacy.

1. Introduction

In the past 20 years, numerous drug polymer conjugates with anticancer agents have been developed with the aim of improving cancer chemotherapy. Passive targeting of macromolecules to solid tumors is mediated by the pathophysiology of tumor tissue, characterized by a high metabolic turnover, angiogenesis, a defective vascular architecture, and an impaired lymphatic drainage.[1]

One of the key issues which has been addressed for establishing structure–activity relationships of drug polymer conjugates is the significance of the chemical bond between the drug and the polymer. Primarily, two types of bonds have been investigated in some detail: (a) acid-cleavable bonds[2] and (b) peptide bonds which can be cleaved by lysosomal enzymes.[3] Essentially, both types of bonds exploit the cellular uptake mechanism for macromolecules, that is, endocytosis, which allows the macromolecular bound drug to be released in intracellular compartments.

In our initial work on acid-sensitive drug conjugates, we developed transferrin and albumin conjugates with the anticancer drugs doxorubicin, daunorubicin, and chlorambucil which have shown promising in vitro and in vivo antitumor activity.[4–10] In these conjugates, the drug is linked to the protein through a maleimide spacer molecule, which incorporates a carboxylic hydrazone bond as a predetermined breaking point allowing the bound drug to be released in the acidic environment of endosomes and/or lysosomes after cellular uptake of the conjugate.

Recently, we extended our therapeutic approach to the synthetic polymer polyethylene glycol (PEG) and developed PEG conjugates with doxorubicin, paclitaxel, and methotrexate.[11–13] High-molecular weight PEGs are non-ionic, water-soluble synthetic polymers which are potential drug carriers due to their synthetic diversity, their recognized biocompatability, and tumor accumulation.[14]

Keywords: Daunorubicin; Polyethylene glycol; Drug polymer conjugates; Acid-sensitivity; In vitro activity.
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In this paper, we report on the synthesis and antiproliferative activity of daunorubicin conjugates with PEGs of MW 20 kDa which contain an amide bond or hydrazone bonds of varying acid-sensitivity. The main objective of the present work was to investigate whether the acid-lability of these conjugates correlated with their inhibitory effects in tumor cell lines, an issue which has not been addressed to date.

2. Results and discussion

2.1. Synthesis of maleimide derivatives of daunorubicin

The five maleimide derivatives of daunorubicin (**1**–**5**) which were coupled to sulfhydryl containing PEGs are shown in Figure 1. Compounds **1**–**5** differ in the site (3′-amino or 13-keto position) and stability (benzamide or substituted as well as non-substituted benzoyl and phenylacetyl hydrazone bonds) of the chemical link between daunorubicin and the spacer molecule. Compounds **1**, **2**, and **3** were synthesized as described previously.[10] The route of preparing **4** and **5** is depicted in Scheme 1. In a first step, 2-aminobenzoic acid or 2-amino-4,5-difluorobenzoic acid was reacted with *tert*-butyl carbazate in the presence of the coupling reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC) to yield $\mathbf{a}_1$ and $\mathbf{a}_2$, respectively. The maleimide derivatives $\mathbf{b}_1$ and $\mathbf{b}_2$ were then obtained by reaction of 3-maleimidobenzoic acid chloride with $\mathbf{a}_1$ or $\mathbf{a}_2$ with 1 equiv of triethylamine and isolating the compounds through chromatography on a silica gel column. In a next step, the *tert*-butoxycarbonyl group was cleaved with CF_3COOH to yield the hydrazides $\mathbf{c}_1$ and $\mathbf{c}_2$, respectively. Finally, the carboxylic hydrazone derivatives **4** and **5** were obtained by reacting daunorubicin HCl with an excess of $\mathbf{c}_1$ or $\mathbf{c}_2$ in anhydrous methanol and isolating the products through crystallization. All newly synthesized compounds were characterized by NMR spectroscopy and mass spectrometry (see Section 3). NMR spectra in d_6-DMSO indicated the presence of *E*(*Z*)-isomers in this solvent.

2.2. Preparation of PEG daunorubicin conjugates PEG20000-1 to PEG20000-5 and PEG20000-$(1)_2$ to PEG20000-$(5)_2$

Polyethylene glycol conjugates of daunorubicin were prepared by reacting a twofold excess of **1**–**5** with

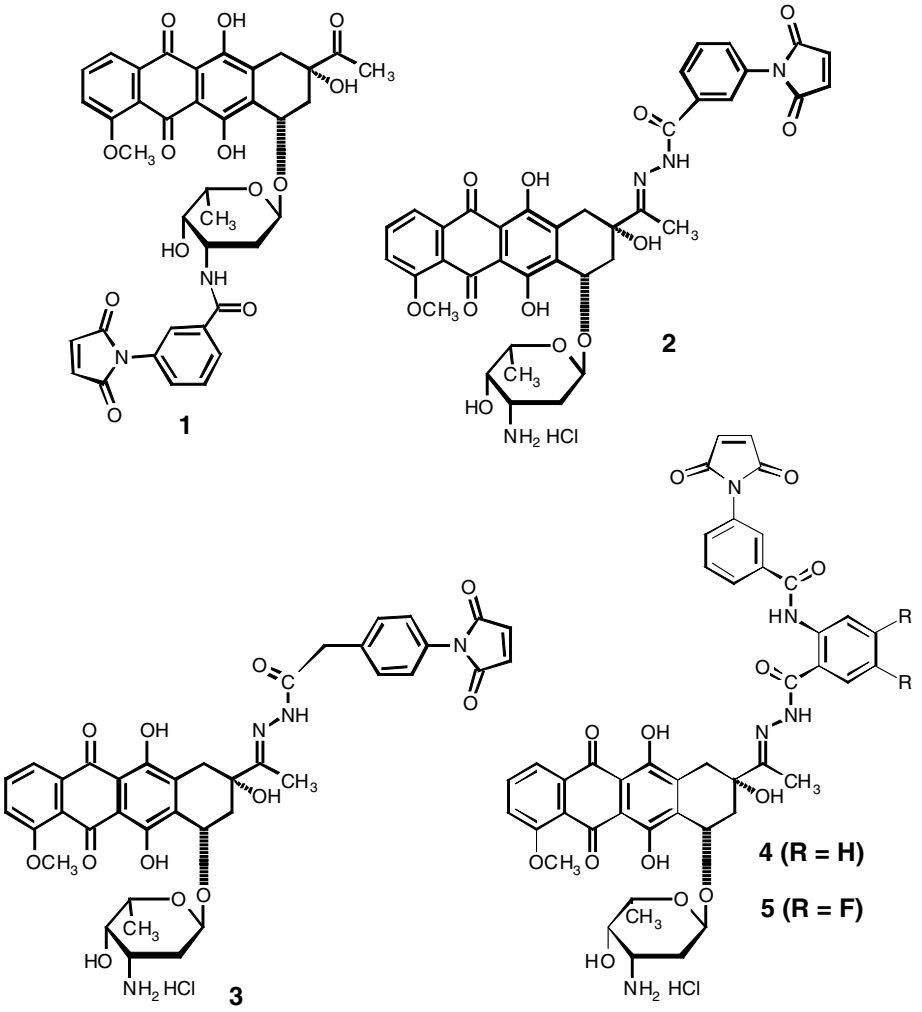
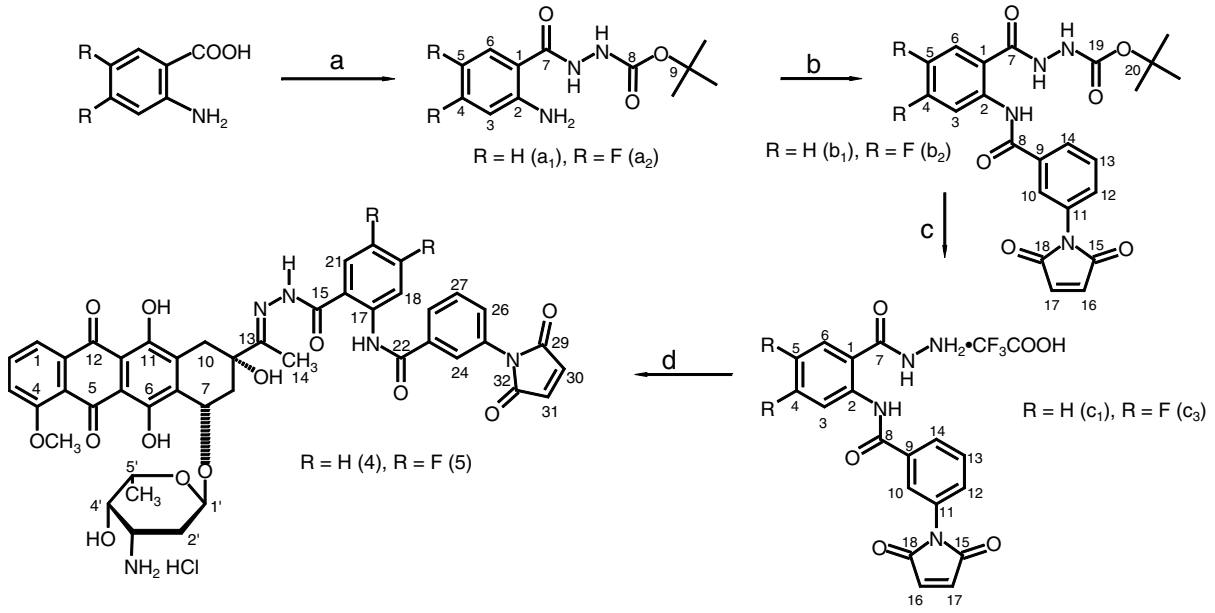


Figure 1. Structures of the maleimide derivatives of daunorubicin **1**–**5**.



Scheme 1. Reagents: (a) $H_2NHN\text{-}COO\text{-}C(CH_3)_3$, DMAP, DCC, THF; (b) 3-maleimidobenzoic acid chloride, Et_3N, THF; (c) 1—CF_3COOH, 2—Et_2O (d) daunorubicin HCl, MeOH.

α-methoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 20,000) or α,ω-bis-thiopropionic acid amide poly(ethylene glycol) (MW 20,000) in aqueous media. The HS-group in the polymer adds to the double bond of the maleimide group in a fast and selective reaction forming a stable thioether bond. Subsequently, the resulting PEG daunorubicin conjugates were isolated through size-exclusion chromatography over Sephadex® G-25 in phosphate buffer or over LH20® in methanol for NMR studies (structures of the synthesized conjugates are shown in Fig. 2).

The purity of the samples after gel filtration was determined with an analytical HPLC-size-exclusion column (Nucleogel® aqua-OH 40-8) and showed no free daunorubicin or unreacted maleimide daunorubicin derivatives. Retention times of the daunorubicin PEG conjugates are between 7 and 10 min on this column, free daunorubicin elutes as a peak at ~70 min (data not shown). UV/vis-spectra of the daunorubicin PEG conjugates in phosphate buffer showed the typical absorption maxima at $\lambda = 496$, 475, 290, and 234 nm (data not shown). Selected vacuum-dried samples [PEG20000-5 and PEG20000-$(1)_2$] which were obtained over Sephadex® LH20 in methanol were investigated with ^1H NMR spectroscopy in $CDCl_3$ (400 MHz). Ethylene signals of the polyethylene glycol backbone were decoupled at 3.5 ppm. Analysis of the spectra revealed that distinct signals of the anthraquinone ring could be assigned, that is, the HO-6 (~14 ppm, s) and HO-14 (~13.3 ppm, s) protons as well as the aromatic protons of ring A together with two proton signals of the spacer (7.5–8.0 ppm). In addition, the NH-proton of the carboxylic hydrazone bond showed a characteristic peak at ~10.5 ppm. The characteristic strong singlet signal of the maleimide double bond (~7.2 ppm) was no longer present in the spectra, indicating that the HS-group had reacted with the maleimide group. Assignment of the proton signals of the sugar ring of daunorubicin was not possible, however, due to a very broad signal of $-CH_2$-groups of the polymer despite our decoupling attempts.

2.3. pH-dependent stability studies

Previous stability studies have demonstrated an acid-sensitive character of the carboxylic hydrazone bond.[4–10] In order to assess distinct differences between the stability of individual PEG daunorubicin conjugates, we performed pH-dependent stability studies with the conjugates at pH 5.0 and 7.4 on our Nucleogel® column with the aid of HPLC.The decrease in the peak area of the conjugate recorded at $\lambda = 495$ nm was used as a measure of daunorubicin release.

Whereas the amide derivatives PEG20000-1 did not release daunorubicin at pH 5.0 or 7.4 after 48 h, the conjugates containing a carboxylic hydrazone bond showed good stability at pH 7.4 (less than 10% release of daunorubicin after 48 h) but released daunorubicin at pH 5.0 in the order PEG-2 > PEG-4 > PEG-3 ≫ PEG-5 with half-lives ranging from 7 to >72 h (see Table 1). No noteworthy difference in acid-sensitivity was observed between the PEG daunorubicin conjugates which had one daunorubicin molecule or two daunorubicin molecules bound to the polymer. Introduction of the fluor substituents in the benzoyl hydrazone ring had a pronounced effect on the acid-sensitivity of the PEG conjugates (see Table 1). PEG20000-5 and PEG20000-$(5)_2$ showed only marginal acid-sensitivity at pH 5.0. When the pH was lowered to pH 3.5, half-lives were of the order of ~20 h.

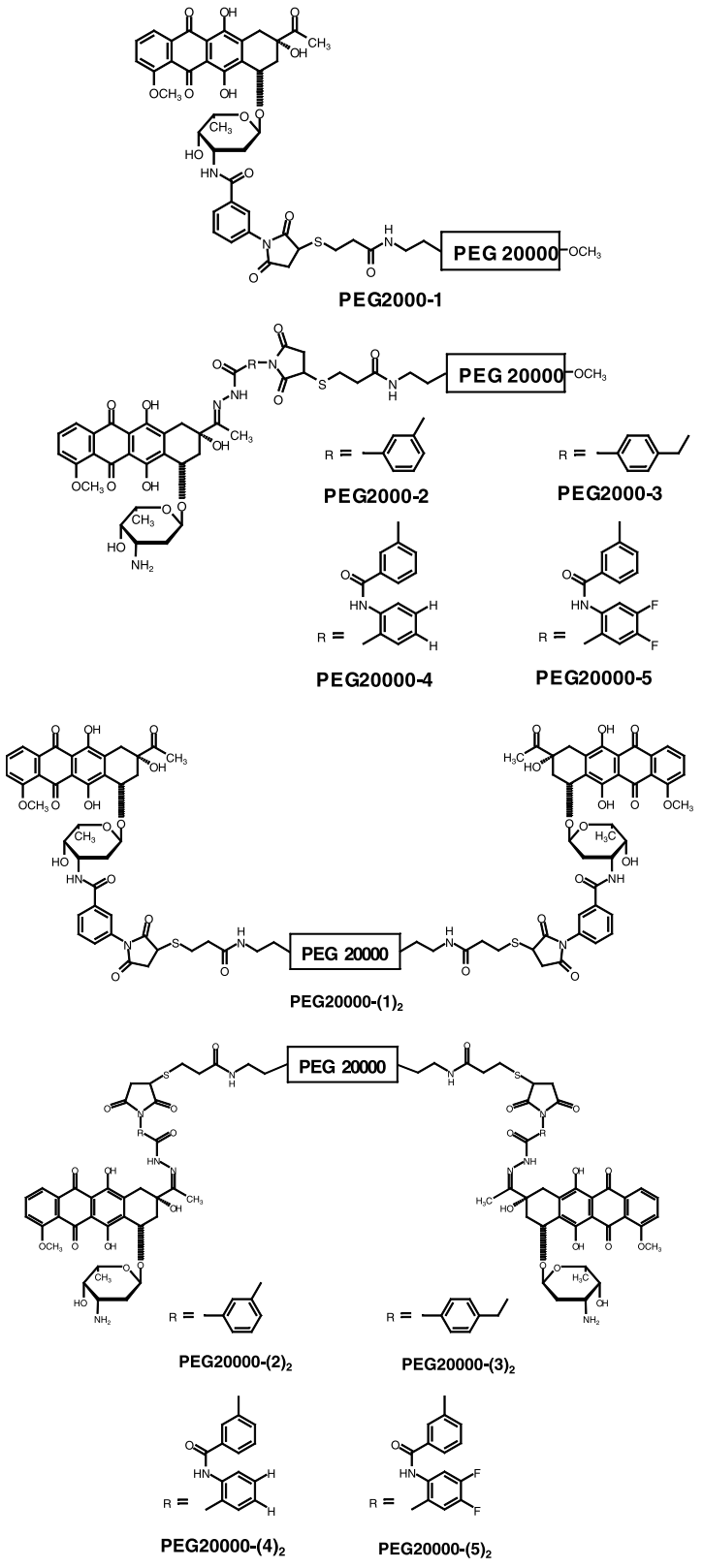


Figure 2. Structures of PEG daunorubicin conjugates PEG20000-1 to PEG20000-5 and PEG20000-$(1)_2$ to PEG20000-$(5)_2$.

2.4. Cell culture experiments

The newly synthesized daunorubicin PEG conjugates and unbound daunorubicin were subsequently evaluated for inhibitory effects in two human tumor cell lines (BXF T24 bladder carcinoma and LXFL 529 lung cancer cells) using the propidium iodide fluorescence assay. Respective IC_{50} values are summarized in Table 1. Unbound polyethylene glycols had only marginal influence on cell growth in both cell lines (data not shown). The

Table 1. In vitro antitumor activity and half-lives at pH 5.0 of daunorubicin PEG conjugates in two human tumor cell lines (bladder carcinoma T24, lung carcinoma LXFL 529)[a]

Compound	IC_{50} (LXFL 529L) (μM)	IC_{50} (BXF T24) (μM)	$t_{1/2}$ (pH 5.0) (h)
Daunorubicin	0.008	0.08	—
PEG20000-1	>10	>10	>72
PEG20000-2	0.2	0.04	~7
PEG20000-3	0.3	0.5	~10
PEG20000-4	0.5	1.2	~22
PEG20000-5	4.0	8.8	>72[b]
PEG20000-$(1)_2$	>10	>10	>72
PEG20000-$(2)_2$	0.06	0.2	~7
PEG20000-$(3)_2$	0.08	0.2	~12
PEG20000-$(4)_2$	0.4	0.6	~24
PEG20000-$(5)_2$	2.8	4.9	>72[b]

[a] Similar results were obtained in a second experiment.
[b] Half-lives at pH 3.5 were ~20 h.

conjugates PEG-2 and PEG-3 which contain a benzoyl hydrazone bond in *meta*-position to the maleimide group and a phenylacetyl hydrazone bond in *para*-position to the maleimide group are the most active conjugates followed by PEG-4 which contains a benzoyl hydrazone bond in *ortho*-position to the maleimide spacer. The difference in the IC_{50} values between PEG-2 and PEG-4 which both contain a benzoyl hydrazone bond can be best explained by the different substitution position of the maleimide moiety in the aromatic ring and/or the different nature of the attached chemical group (maleimide group versus maleimide spacer bound through an amide bond to the phenyl ring). The decrease in the IC_{50} values between PEG-2, PEG-3, and PEG-4 correlates with the increase in the half-lives of the conjugates at pH 5.0. PEG-5, which is analogous to PEG-4 but contains 2 fluorine atoms in the benzoyl hydrazone ring, is significantly less active than the other acid-sensitive conjugates; this observation is in line with a considerable decrease in the acid-sensitivity of PEG20000-5 and PEG20000-$(5)_2$ which is attributed to the electron-withdrawing effect on the benzoyl hydrazone bond (see Table 1). The amide conjugate PEG-1 showed no activity at the concentrations tested (0.001–10 μM). The PEG daunorubicin conjugates which had two daunorubicin molecules bound to the polymer were generally more active than the congeners that contain one daunorubicin molecule. Free daunorubicin is the most active compound in both cell lines.

In summary, we have addressed the relationship between the pH-dependent stability of the bond between daunorubicin and PEG in five PEG daunorubicin conjugates and their in vitro antitumor activity against two tumor cell lines. We observed a consistent correlation between the acid-sensitivity of the conjugates and their antiproliferative effects, increasing acid-sensitivity being paralleled by enhanced cytotoxicity. Macromolecular prodrugs with PEG, like other drug polymer conjugates, are taken up by the tumor cell through endocytosis.[3,7,11] During internalization of the conjugate, the pH is reduced from 7.4 to 5.0 in endosomes and pH 4.0 in lysosomes, and this pH change can be exploited through acid-cleavage of a predetermined breaking point allowing the drug to be released inside the tumor cell. Our fluorescence microscopy studies with acid-sensitive doxorubicin PEG conjugates have shown that doxorubicin is primarily accumulated in the cytoplasm of tumor cells in contrast to the nucleus which is the predominant organelle that shows fluorescence after cell exposure to free doxorubicin.[11]

The potential clinical significance of high-molecular weight acid-sensitive anthracycline prodrugs is currently being addressed: the (6-maleimidocaproyl)hydrazone derivatives of doxorubicin, used as an albumin-binding prodrug or coupled to the BR96-antibody, are acid-sensitive prodrugs of doxorubicin that are undergoing phase I/II studies.[15–18]

3. Experimental

3.1. Chemicals, materials, and spectroscopy

^{1}H NMR and ^{13}C NMR: Bruker 400 MHz AM 400 or Varian Unity 300 (internal standard: TMS); ^{13}C NMR spectra were obtained with broad-band decoupling; mass spectra were obtained on a Finnigan MAT 312 with associated MAT SS 200 data system using electron impact or electro spray ionization; analytical HPLC: HPLC studies were performed on an analytical HPLC column (*Nucleogel® aqua-OH 40-8*, 300 mm × 7.7 mm, from Macherey & Nagel, FRG); mobile phase: 0.15 M NaCl, 0.01 M sodium phosphate, 10% v/v CH_3CN, and 30% v/v MeOH, pH 7.0. A Lambda 1000 UV/vis monitor from Bischoff (at λ = 495 or 280 nm), an autosampler Merck Hitachi AS400, and an Integrator Merck Hitachi D2500 were used, silica gel chromatography on silica gel 60 (0.063–0.100 mm) from Merck AG; TLC: silica-coated plates 60 F_{254} from Merck AG; daunorubicin HCl was a gift from Aventis, FRG; organic solvents: HPLC grade (Merck) or analytical grade (gift from BASF AG)—other organic or inorganic compounds: Merck AG, FRG. Compounds **1**, **2**, and **3** were prepared previously.[10] PEGs were purchased from Rapp Polymer, FRG; the buffers used were vacuum-filtered through a 0.2 μm membrane (Sartorius, FRG). Cell culture media, supplements (L-glutamine, antibiotics, and trypsin versene/EDTA), and fetal calf serum (FCS) were purchased from Bio Whittaker (Serva, Heidelberg, FRG). Propidium iodide was purchased from Aldrich–Sigma-Chemie, FRG. All culture flasks were obtained from Greiner Labortechnik (Frickenhausen, FRG).

3.2. Methods for the preparation of conjugates

FPLC for preparation of conjugates: P-500 pump, LCC 501 Controller (Pharmacia), and LKB 2151 UV-monitor (at λ = 280 nm); buffer: 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4.

3.3. Synthesis of 4 and 5

Compounds $\mathbf{a}_1$ and $\mathbf{a}_2$: 17.3 mmol 2-amino-4,5-difluorobenzoic acid or 2-aminobenzoic acid, 9.15 g (69.2 mmol,

4 equiv) *tert*-butylcarbazate and 21.2 mg (0.17 mmol) DMAP were dissolved in 50 ml THF to which a solution of 3.93 g (19.0 mmol, 1.1 equiv) *N*,*N*′-dicylohexylcarbodiimide in 30 ml THF was added during a period of 90 min at 4 °C. The mixture was stirred for 12 h at 4 °C, filtered, and the solvent evaporated in vacuo. The residue was dissolved in 100 ml ethyl acetate and extracted six times with 50 ml H_2O. After drying over $MgSO_4$, the organic layer was concentrated to a volume of 50 ml and *n*-hexane added until a slight turbidity appeared. The resulting suspension was allowed to stand at −20 °C for 12 h to afford **a**$_1$ and **a**$_2$ as white crystals which were washed with diethyl ether and dried under high vacuum.

Analytical data. Compound **a**$_1$: yield 3.73 g (20.2 mmol, 40%)—^{1}H NMR (300 MHz, MSO-d_6/TMS): δ = 9.8/8.8 (2s, 2H, NH–NH), 7.6 (d, 1H, 6-H), 7.2 (t, 1H, 4-H), 6.8 (d, 1H, 5-H), 6.6 (t, 1H, 3-H), 6.4 (br s, 2H, NH_2), 1.4 (s, 9H, 3 CH_3)—^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 168.4 (C-7), 156.4 (C-8), 149.8 (C-6), 132.1 (C-1), 127.9 (C-4), 116.3 (C-2), 114.4 (C-5), 112.2 (C-3), 79.9 (C-9), 28.0 (3 *C*H$_3$) MS (EI, 220 °C, 70 eV, 1 mA, 2 kV): *m*/*z* = 251(18) [$M^{+\bullet}$], 196 (33) [$(M+1)^{+\bullet}-C_4H_8^+$], 147 (32) [$(M+1)^{+\bullet}-(CO_2, -C_4H_8^+)$]—($C_{12}H_{17}N_3O_3$) M = 251.28 g/mol.

Compound **a**$_2$: yield 3.85 g (13.4 mmol, 78%)—^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 9.9/8.8 (2s, 2H, N*H*-N*H*), 7.6 (dd, 1H,. 6-*H*, $^3J_{6\text{-H},5\text{-F}}$ = 11.9 Hz, $^4J_{6\text{-H},4\text{-F}}$ = 9.2 Hz), 6.7 (dd, 1H, 3-*H*, $^3J_{3\text{-H},4\text{-F}}$ = 13.2 Hz, $^4J_{3\text{-H},5\text{-F}}$ = 7.2 Hz), 6.6 (br s, 2H, N*H*$_2$), 1.4 (s, 9H, 3 C*H*$_3$)—^{13}C NMR (DMSO-d_6/TMS): δ = 166.7 (C-7), 155.5 (C-8), 152.1 (C-4, $^1J_{(4\text{-C}/4\text{-F})}$ = 249.5 Hz), 148.3 (C-5, $^1J_{(5\text{-C}/5\text{-F})}$ = 233.4 Hz), 139.7 (C-2), 116.1 (C-1), 107.1 (C-6, $^2J_{(6\text{-C}/5\text{-F})}$ = 14.1 Hz), 103.48 (C-3, $^2J_{(3\text{-C}/4\text{-F})}$ = 14.1 Hz), 79.2 (C-9), 28.0 (3 *C*H$_3$)—MS (EI, 220 °C, 70 eV, 1 mA, 2 kV): *m*/*z* = 287 (18) [$M^{+\bullet}$], 231 (67) [$(M + 1)^{+\bullet}-C_4H_8^+$], 187 (32) [$(M + 1)^{+\bullet}-(CO_2, -C_4H_8^+)$], 156 (100) [$M^{+\bullet}-(NH–NH–C_4H_9)^+$], ($C_{12}H_{15}F_2N_3O_3$) M = 287.27 g/mol.

Compounds **b**$_1$ and **b**$_2$: 3.63 g (15.4 mol, 1.1 equiv) 3-maleimidobenzoic acid chloride dissolved in 90 ml THF was added during a period of 90 min at room temperature to a stirring solution of 14 mmol **a**$_1$ or **a**$_2$ and 2.15 ml (15.4 mmol, 1.1 equiv) triethylamine in 140 ml THF. The reaction mixture was stirred for 72 h, the triethylammonium chloride salt removed by filtration, and the mixture concentrated to an oily residue. Purification was performed by chromatography (silica gel in ethyl acetate/hexane, 1.5:1) to yield **b**$_1$ and **b**$_2$ as yellow crystals.

Analytical data. Compound **b**$_1$: yield 4.41 g (9.2 mmol, 70%)—^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 11.9/10.6 (s, 1H, C7-NH), 9.1/8.7 (s,1H, C19-NH), 8.6 (s, 1H, C2-NH), 7.95–7.6 (m, 7H, arom. H), 7.2 (s, 2H, 16-H/17-H), 1.4 (s, 9H, 3 CH_3)—^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 169.7 (C-18/C-21), 166.5 (C-7), 163.5 (C-11), 155.2 (C-8), 134.7 (C-19/C-20), 139.0/132.8/132.4/131.8/131.5/130.8/129.3/128.0/127.3/125.7/123.2/ 120.7 (arom. C), 79.5 (C-9), 27.9 (3 CH_3)—MS (ESI: Spray 3.8 kV, 200 °C, Sheatgas 30 psi): *m*/*z* = 473 (75) [$M^{+\bullet}$ + Na^+], 451 (10) [M^+ + 1]−($C_{23}H_{22}N_4O_6$) M = 450.45 g/mol.

Compound **b**$_2$: yield 3.5 g (7.2 mmol, 51.4%)—^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 10.9 (s, 1H, C7-N*H*), 7.6 (dd, 1H, 6-H, $^3J_{6\text{-H},5\text{-F}}$ = 11.9 Hz, $^4J_{6\text{-H},4\text{-F}}$ = 9.2 Hz), 7.64- 7.55 (m, 4H, arom. *H*), 7.2 (s, 2H, 16-H/17-H), 6.8 (dd, 1H, 3-H, $^3J_{3\text{-H},4\text{-F}}$ = 13.7 Hz, $^4J_{3\text{-H},5\text{-F}}$ = 7.8 Hz), 6.7 (br s, 2H, C2-N*H*, C19-N*H*), 1.2 (s, 9H, 3 C*H*$_3$)—^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 169.8 (C-15/C-18), 166.3 (C-8), 151.1 (C-4, $^1J_{(4\text{-C}/4\text{-F})}$ = 249.5 Hz), 149.2 (C-7), 148.9 (C-19), 140.1 (C-5, $^1J_{(5\text{-C}/5\text{-F})}$ = 233.4 Hz), 136.8 (C-2), 136.7/131.9/130.0/129.2/126.8/125.8 (arom. *C*), 134.9 (C-16/C-17), 116.5 (C-1), 105.9 (C-6, $^2J_{(6\text{-C}/5\text{-F})}$ = 14.1 Hz), 104.0 (C-3, $^2J_{(3\text{-C}/4\text{-F})}$ = 14.1 Hz), 84.1 (C-9), 27.2 (3 *C*H$_3$)—MS (ESI: Spray 4-5 kV, 200–250 °C, Sheatgas 20 psi): *m*/*z* = 471 (100) [$M^{+\bullet}-CH_3^+$], 439 (56) [$M^{+\bullet}-(CO, F^+)$], 423 (66) [$M^+-(CO_2, F^+)$], 389 (24) [$M^{+\bullet}-C_4H_2NO_2^+$]—($C_{23}H_{20}F_2N_4O_6$) (486.4 g/mol).

Compounds **c**$_1$ and **c**$_2$: 2.7 mmol **b**$_1$ or **b**$_2$ was suspended in 3 ml trifluoroacetic acid and stirred at room temperature until all of the compound was dissolved. Excess of trifluoroacetic acid was evaporated in vacuo and the oily residue triturated with diethyl ether to afford **c**$_1$ and **c**$_2$ as a yellow powder.

Analytical data. Compound **c**$_1$: yield 1.17 g (2.5 mmol, 96%)—^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 11.6 (br s, 1H, $NH_2{\cdot}CF_3COOH$), 8.4 (s, 1H, C7-NH), 8.0–7.3 (m, 5H, 4 arom. H, C8-NH), 7.21 (s, 2H, 16-H/17-H)—^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 169.8 (C-15/C-18), 167.5 (C-7), 164.0 (C-8), 138.2/135.5/133.0/132.3/130.3/129.4/128.5/126.0/125.3/123.9/122.2/120.4 (arom. C), 135.2 (C-16/C-17), 123.8/118.0/ 114.1/111.5 (CF_3)—MS (ESI: Spray 3.8 kV, 200 °C, Sheatgas 30 psi): *m*/*z* = 351 (100) [$M^{+\bullet}-CF_3COO^-$], 319 (58) [M^+−NH-$NH_3CF_3COO^-$]—($C_{20}H_{13}F_5N_4O_6$) M = 464.35 g/mol.

Compound **c**$_2$: yield 0.98 g (2 mmol, 75%)—mp 192 °C; ^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 10.47 (s, 1H, C7-NH), 10.2 (br s, 1H, $NH_2{\cdot}CF_3$-COOH), 8.05–7.56 (m, 5H, arom. H, C8-NH), 7.58 (dd, 1H, 6-H, $^3J_{6\text{-H},5\text{-F}}$ = 11.9 Hz, $^4J_{6\text{-H},4\text{-F}}$ = 9.2 Hz), 7.21 (s, 2H, 16-H/17-H), 6.7 (dd, 1H, 3-H, $^3J_{3\text{-H},4\text{-F}}$ = 13.7 Hz, $^4J_{3\text{-H},5\text{-F}}$ = 7.8 Hz)—^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 169.6 (C-15/C-18), 166.7 (C-8), 165.1 (C-7), 152.2 (C-4, $^1J_{(4\text{-C}/4\text{-F})}$ = 249.5 Hz), 148.3 (C-2), 139.8 (C-5, $^1J_{(5\text{-C}/5\text{-F})}$ = 233.4 Hz), 136.7/131.9/130.0/129.2/126.8/125.8 (arom. C), 134.9 (C-16/C-17), 116.34 (CF_3), 116.24 (C-6, $^2J_{(6\text{-C}/5\text{-F})}$ = 14.1 Hz), 105.2 (C-1), 104.5 (C-3, $^2J_{(3\text{-C}/4\text{-F})}$ = 14.1 Hz)—($C_{20}H_{13}F_5N_4O_6$) M = 500.33 g/mol.

Compounds **4** and **5**: 50 mg (0.089 mmol) daunorubicin hydrochloride and 0.44 mmol (5 equiv) **c**$_1$ or **c**$_2$ were dissolved in 45 ml anhydrous methanol to which 50 μl trifluoroacetic acid was added. The solution was stirred in the dark for 96 h at room temperature and then concentrated to a volume of approximately 15–20 ml in vacuo.

Acetonitrile was added to the red dark solution until a slight turbidity appeared. The resulting suspension was allowed to stand at −20 °C for crystallization of the product. The red hydrazone was collected by centrifugation. The supernatant was evaporated to a small volume and treated with acetonitrile as above. The hydrazone fractions were combined and re-crystallized from methanol/acetonitrile to yield a red microcrystalline powder.

Analytical data. Compound **4**: yield 60 mg (0.065 mmol, 72%)—^{1}H NMR (400 MHz, DMSO-d_6/TMS): δ = 14.08 (s, 1H, C-6-O*H*), 13.30 (s, 1H, C-11-O*H*), 11.74 (s, 1H, N-N*H*), 10.75 (s, 1H, N-N*H*), 10.21 (s, 1H, C17-NH), 8.21-7.45 (m, 10H, arom. H: 1-H/3-H/2-H/H-18/H-19/H20/21-H/24-H/26-H/27-H/28-H), 7.86 (m, 1H, NH_2·HCl), 7.21 (s, 2H, 30-H/31-H), 7.2 (s, 2H, 30-H/31-H), 5.49 (m, 1H, 1′-H), 5.28 (br s, 1H, C-9-O*H*), 4.50 (br s, 1H, 7-H), 4.32 (m, 1H, C4′-O*H*), 4.27 (d, 1H, 5′-H), 3.96 (s, 3H, –OCH_3), 3.61 (m, 1H, 4′-H), 2.87 (m, 2H, 10-H), 2.28 (m, 1H, 3′-H), 2.17 (m, 2H, 8-H), 2.07 (m, 2H, 2′-H), 1.86 (s, 3H, 14-H), 1.7 (s, 3H, 14-H), 1.1 (m, 3H, 5′-CH_3); ^{13}C NMR (Varian 300, 75.4 MHz, DMSO-d_6/TMS): δ = 186.3 (C-5), 186.1 (C-12), 169.7 (C-29/C-32), 167.5 (C*-29/C*-32), 163.9 (C-4), 160.6 (C-15), 156.7 (C-11), 156.3 (C-6), 138.3 (C-13), 138.1 (C*-13), 135.1 (C-2), 135.0 (C-6a), 134.7 (C-12a), 134.6 (C-30/C-31), 133.0 (C-10a), 132.2 (C-17), 135.8/131.1/130.2/129.3/128.7/127.4/126.1/125.9/125.7/123.8/120.3/118.4 (arom. C), 122.0 (C-1), 119.8 (C-4a), 118.9 (C-3), 110.3 (C-5a), 110.0 (C-11a), 109.2 (C-1′), 79.5 (C-9), 78.2 (C-9), 76.8 (C-7), 66.2 (C-5′), 65.9 (C-4′), 56.5 (-OCH_3), 46.5 (C-3′), 40.2 (C-8), 39.9 (C-8), 38.8 (C-14), 23.4 (C*-14), 28.4 (C-10), 27.2 (C-2′), 16.7 (C-5-CH_3) (* = splitting of the proton or carbon signals that are indicative of the presence of *E*(*Z*)-isomers in DMSO). MS (ESI: Spray 3.8 kV, 200 °C, Sheatgas 30 psi, CH_3OH): *m*/*z* = 892 (100) [M + 1 (CH_3OH)]$^+$ (methanol adduct), 860 (2) [M+1]$^+$—($C_{45}H_{42}N_5O_{13}$) M = 859.8 g/mol.

Compound **5**: yield 60 mg (0.065 mmol, 72%)—^{1}H NMR (300 MHz, DMSO-d_6/TMS): δ = 14.08 (s, 1H, C-6-OH), 13.30 (s, 1H, C-11-OH), 10.92 (s, 1H, N-NH), 10.30 (s, 1H, N-NH*), 10.21 (s, 1H, C17-H), 8.21/7.94 (m, 2H, 1-H/3-H), 7.86 (m, 1H, NH_2·HCl), 7.74 (m, 1H, 2-H), 7.68–7.45 (m, 5H, 21-H/24-H/26-H./27-H/28-H), 7.21 (s, 2H, 30-H/31-H), 7.2 (s, 2H, 30-H*/31-H*), 6.68 (dd, 1H, 18-H), 5.49 (m, 1H, 1′-H), 5.28 (br s, 1H, C-9-OH), 4.88 (br s, 1H, C-9-OH*), 4.80 (br s, 1H, 7-H), 4.72 (m, 1H, C4′-OH), 4.47 (d, 1H, 5′-H), 3.96 (s, 3H, –OCH_3), 3.61 (m, 1H, 4′-H), 2.87 (m, 2H, 10-H), 2.48 (m, 1H, 3′-H), 2.27 (m, 2H, 8-H), 1.90 (m, 2H, 2′-H), 1.76 (s, 3H, 14-H), 1.7 (s, 3H, 14-H*), 1.1 (m, 3H, 5′-CH_3)—^{13}C NMR (Varian 300, DMSO-d_6/TMS): δ = 186.5 (C-5), 186.4 (C-12), 169.7 (C-29/C-32), 168.3 (C*-29/C*-32), 166.0 (C-22), 160.4 (C-4), 159.3 (C-15), 156.3 (C-11), 154.9 (C-6), 151.9 (C-19), 144.2 (C-13), 144.1 (C*-13), 140.5 (C-20), 136.1 (C-2), 135.5 (C-6a), 135.4 (C-12a), 134.8 (C-30/C-31), 134.7 (C-10a), 133.2 (C-17), 132.4/131.9/130.2/129.4/127.4/126.1 (C-23/C-24/C-25/C-26/C-27/C-28), 120.0 (C-1), 119.7 (C-4a), 118.9 (C-3), 115.8 (C-16), 110.5 (C-5a), 110.4 (C-11a), 110.3 (C-1′), 108.8 (C-21), 101.7 (C-18), 81.1 (C-9), 80.7 (C*-9), 75.8 (C-7), 68.5 (C-5′), 66.2 (C-4′), 56.6 (-OCH_3), 46.2 (C-3′), 38.6 (C-8), 38.4 (C*-8), 33.9 (C-14), 20.5 (C*-14), 29.4 (C-10), 27.8 (C-2′), 16.6 (C-5-CH_3) (* = splitting of the proton or carbon signals that are indicative of the presence of *E*(*Z*)-isomers in DMSO)—MS (ESI: Spray 4–5 kV, 200–250 °C, Sheatgas 20 psi, CH_3OH): *m*/*z* = 928 (100) [M + 1 (CH_3OH)]$^+$ (methanol adduct), 896 (2) [M + 1]$^+$, 799 (27) [M−$C_4H_2NO_2$]$^+$— ($C_{45}H_{40}F_2N_5O_{13}$) M = 895.8 g/mol.

3.4. Synthesis of PEG daunorubicin conjugates

All reactions were performed at room temperature unless otherwise stated. Data for one representative experiment are given.

3.4.1. Preparation of PEG 20000-(2)$_2$. Eight milligrams (0.01 mmol) of **2** was dissolved in 250 μl dimethylformamide and added to 50 mg (0.0025 mmol) PEG-20000$(SH)_2$ dissolved in 5 ml buffer (0.004 M sodium phosphate, 0.15 mol NaCl, pH 6.8). The mixture was homogenized and kept at room temperature for 30 min. After centrifuging the slightly turbid mixture for 5 min with a Sigma 112 centrifuge, the supernatant was loaded on a *Sephadex*® G 25 column (100 mm × 20 mm, loop size: 5 ml). The conjugate eluted with a retention time of 5–10 min (flow: 1.0 ml/min, buffer: 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4). Concentration of the conjugate to a volume of approximately 2 ml was carried out with CENTRIPREP®-10-concentrators from Amicon, FRG (60 min at 4 °C and 4500 rpm). The concentration of daunorubicin in the conjugate was adjusted to c = 300 ± 20 μM using the ε-value for daunorubicin in physiological buffer (ε_{495} = 9280 M^{-1} cm^{-1})^{xy}. The conjugate was stored at −80 °C. Daunorubicin polyethylene glycol conjugates for NMR studies were chromatographed over *Sephadex*® LH20 Gel (100 mm × 10 mm, loop size: 2 ml, flow: 1.0 ml/min, retention time: 3–6 min, eluent: 100% methanol HPLC grade).

3.4.2. pH-dependent stability studies with the PEG daunorubicin conjugates at pH 5.0 and 7.4. Fifty microliters of the stock solutions of the conjugates (c 300 ± 20 μM) in phosphate buffer was added to 450 μL of buffer, pH 5.0 (0.15 M NaCl, 0.004 M sodium phosphate adjusted to pH 5.0 with hydrochloric acid) or pH 7.4 (0.15 M NaCl, 0.004 M sodium phosphate). The solutions were incubated at room temperature and 50 μL samples were analyzed at λ = 495 nm every 2–4 h over a period of 72 h on an analytical HPLC column (*Nucleogel® aqua-OH 40-8*, 300 mm × 7.7 mm, from Macherey & Nagel, FRG); mobile phase: 0.15 M NaCl, 0.01 M sodium phosphate, 10% v/v CH_3CN, 30% v/v MeOH, pH 7.0. A Lambda 1000 UV/vis monitor from Bischoff (at λ = 495 nm), an autosampler Merck Hitachi AS400, and an Integrator Merck Hitachi D2500 were used.

3.4.3. Biology. Human tumor cells were grown at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in monolayer RPMI 1640 culture medium with phenol red

supplemented with 10% heat-inactivated FCS, 300 mg/L glutamine, and 1% antibiotic solution (5.000 μg gentamycin/mL). Cells were trypsinized and maintained twice a week. The concentration of free daunorubicin and daunorubicin in the stock solution of the conjugates was 300 μM.

3.4.4. Propidium iodide fluorescence assay. The fluorescence assay was performed according to the method of Dengler et al.[19] Briefly, cells were harvested from exponential phase cultures growing in RPMI culture medium by trypsinization, counted, and plated in 96-well flat-bottomed microtiter plates (50 μL cell suspension/well, 1.0×10^5 cells/mL). After a 24 h recovery in order to allow cells to resume exponential growth, 100 μL culture medium (6 control wells per plate) or culture medium containing drug was added to the wells. Each drug concentration was plated in triplicate. After 6 days of continuous drug exposure, nonviable cells were stained by addition of 25 μL of a propidium iodide solution (50 μg/mL). Fluorescence (FU_1) was measured using a Millipore Cytofluor 2350 microplate reader (excitation 530 nm, emission 620 nm). Microplates were then kept at −18 °C for 24 h, which resulted in a total cell kill. After thawing of the plates and a second fluorescence measurement (FU_2), the amount of viable cells was calculated by $FU_2 - FU_1$. Growth inhibition was expressed as treated/control × 100 (%T/C).

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