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Synthesis and In Vitro Efficacy of Acid-Sensitive Poly(ethylene glycol) Paclitaxel Conjugates

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Abstract—Three maleimide derivatives of the anticancer drug paclitaxel that incorporate an acid-sensitive carboxylic hydrazone linker were prepared and coupled to bifunctional PEGs (MW 20,000 g/mol). The conjugates showed in vitro activity in three human cancer lines in the low micromolar range.

Paclitaxel is currently one of the most important anticancer agents with promising antitumor activity against ovarian, breast, and lung cancers.[1–4] Because of its extremely poor aqueous solubility, paclitaxel is formulated as a concentrated solution containing a 1:1 ratio of Cremophor EL and ethanol.[5] Unfortunately, Cremophor, a polyoxyethylated castor oil, has been associated with a number of side effects including hypersensitive reactions and neurotoxicity.[6] Furthermore, paclitaxel, like other anticancer agents shows severe side effects due to a lack of selectivity towards tumor cells.[4]

To date, numerous attempts have been undertaken in order to improve the chemotherapeutic potency and water solubility of paclitaxel.[7–11] A promising strategy of modifying the biodistribution, of reducing drug toxicity and thus improving the therapeutic efficacy of anticancer agents is the attachment of antitumor drugs to macromolecules. In recent years a number of investigations have shown that macromolecules with molecular weight (M_r) > 20,000 g/mol accumulate in solid tumors in experimental animal models.[12,13] Tumor uptake of synthetic polymers and serum proteins seems to be mediated by the vascular network of tumor tissue which is characterized by an enhanced permeability of tumor blood vessels for circulating macromolecules combined with subsequent retention due to a deficient lymphatic recovery system in tumor tissue. Consequently, macromolecular drug conjugates with anticancer agents have been developed with the aim of improving cancer chemotherapy.

Paclitaxel has been coupled to natural polymers such as albumin as well as to synthetic polymers, for example poly(ethylene glycol) (PEG) and *N*-(2-Hydroxypropyl)-methacrylamide copolymers.[14–17] These conjugates, in which paclitaxel was either directly bound to the polymer or through a suitable aliphatic linker, were designed to release paclitaxel by hydrolytic or enzymatic cleavage of the conjugate and showed promising in vitro and in vivo activity.

However, no examples of polymer paclitaxel conjugates have been reported that incorporate an acid-sensitive bond as a predetermined breaking point for releasing paclitaxel. In our recent work, we have developed acid-sensitive albumin, transferrin as well as PEG conjugates with the anticancer drugs doxorubicin, daunorubicin and chlorambucil which showed high in vitro and in vivo activity.[18–22] 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.

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In this work, we thus developed acid-sensitive paclitaxel conjugates with polyethylene glycols of M_r 20,000. Polyethylene glycols (PEGs) are non-ionic, water-soluble synthetic polymers which are potential drug carriers due to their synthetic diversity and recognized biocompatability.[23]

Based on our experience with maleimide derivatives and acid-sensitive linkers, we synthesized maleimide paclitaxel derivatives that incorporate carboxylic hydrazone bonds which were then coupled to PEG20000 bearing two thiol groups. For developing acid-sensitive maleimide derivatives of paclitaxel, we selected three maleimide hydrazides, **2a**, **2b** and **2c**, as thiol-binding spacer molecules (see Scheme 2 and ref 24). Because the sole C-9 keto-position of paclitaxel is sterically hindered and can only be derivatized under strong conditions,[7] an ester-derivative of paclitaxel at the C-2′-OH-position was first prepared with the commercially available compound 4-acetylbenzoic acid. The resulting paclitaxel-derivative (**1**) containing an aromatic ester linkage provided a suitable carbonyl group for preparing maleimide paclitaxel derivatives with a carboxylic hydrazone bond. The method of preparing **1** is depicted in Scheme 1.

Compound **1** was synthesized by reacting paclitaxel with 4-acetylbenzoic acid chloride and two equivalents of triethylamine in dichloromethane for 2 h at room temperature. Isolation of the compound was performed by chromatography on silica gel to obtain an analytical pure sample (see refs 25, 26). The keto-derivative **1** was then reacted with an excess of the maleimide hydrazide spacers **2a**, **2b** and **2c** in anhydrous methanol yielding the maleimide carboxylic hydrazone derivatives of paclitaxel **3a**, **3b** and **3c** as shown in Scheme 2 (see ref 27).

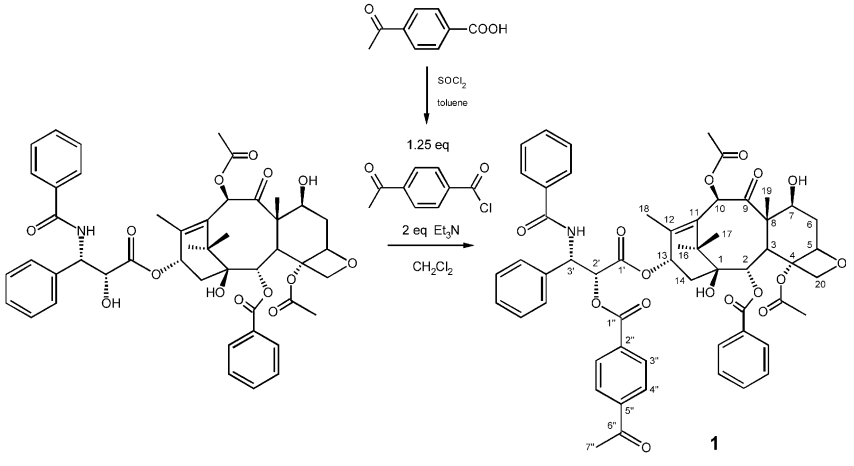
Compounds **3a**, **3b** and **3c** were isolated through chromatography on silica gel. The isolated compounds **1**, **3a**, **3b** and **3c** were characterized through ^{1}H NMR and ^{13}C NMR spectroscopy, mass spectrometry and HPLC. The ^{1}H NMR spectra of **3a**, **3b** and **3c** in DMSO-d_6 showed the characteristic peaks of the protons of the introduced maleimide group at 7.1–7.2 ppm. The shift of the C-2′-H proton from 4.7–4.8 ppm in the spectrum of paclitaxel to 5.4–5.7 ppm in the spectra of **3a**, **3b** and **3c** confirmed the esterification of paclitaxel at the C-2′-OH group. In the ^{13}C NMR spectra the characteristic peaks of the carbon atoms of the maleimide double bond and the respective carbonyl atoms of **1**, **3a**, **3b** and **3c** were observed at 134–135 ppm and at 169–170 ppm. The ^{1}H and ^{13}C NMR signals of paclitaxel could be assigned by comparison with the literature data (see refs 26, 27).

The presence of a set of two signals in the NMR spectra of the maleimide paclitaxel derivatives for the protons (7″-H at 2.38/2.35 ppm, 19″-H at 3.63/3.59 ppm) and the carbon atoms (C-6″ at 150.05/146.05 ppm, C-19″ at 75.21/74.65 ppm) adjacent to the C=N double bond indicate the presence of (*E*)/(*Z*)-isomers in DMSO-d_6 (ratio of the splitting 2/1). Based on previous investigations with hydrazone isomers of the anticancer drug chlorambucil and model compounds, the thermodynamically (*E*)-isomer is favored.[21,28]

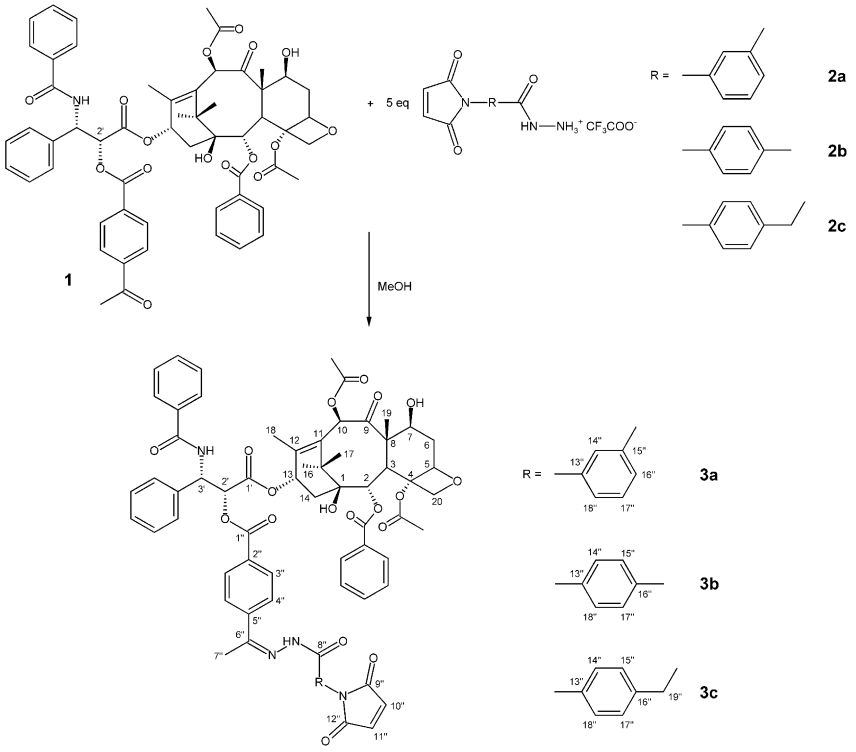
The presence of the two isomers of the maleimide derivatives of paclitaxel was confirmed by analysis of the derivatives through HPLC on a reversed phased C_{18}-column (see ref 29). Each chromatogram of **3a**, **3b** and **3c** showed a main peak at a retention time of 9.4–9.9 min (82–90% integrated area) as well as a small peak at 7.9–8.0 min (5–15% integrated area) which were attributed to the (*E*)- and (*Z*)-isomers of the maleimide paclitaxel derivatives, respectively.

UV spectra of **1**, **3a**, **3b** and **3c** were recorded in THF in order to determine the ε-value at the absorbance maximum of each compound in THF. The paclitaxel derivatives showed maxima at λ = 280–309 nm [λ (**1**) = 280 nm, λ (**3a**) = 309 nm, λ (**3b**) = 309 nm, λ (**3c**) = 303 nm]; the ε-value at the respective maximum are listed in ref 30.

The PEG conjugates of paclitaxel were prepared by reacting the maleimide derivatives of paclitaxel **3a**, **3b** and **3c** with α,ω-bis-thiopropionic acid amide poly(ethylene glycol) (M_r 20,000 g/mol) in a solution containing 50% buffer (0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4) and 50% dimethylformamide for 1 h. The HS-group in the polymer adds to the double bond of the maleimide group in a fast and selective reaction forming



Scheme 1.



Scheme 2.

a stable thioether bond. Subsequently, the resulting PEG paclitaxel conjugates were isolated through size-exclusion chromatography over Sephadex® LH20 in methanol (see ref 31). The structures of PEG20000-(**3a**)$_2$, PEG20000-(**3b**)$_2$, PEG20000-(**3c**)$_2$ are shown in Figure 1.

The purity of the samples was determined with a semi-analytical FPLC-size exclusion column (Sephadex® LH20) at $\lambda = 280$ nm. Retention times of the paclitaxel PEG conjugates were between 15 and 16 min (**3a** = 15.6 min, **3b** = 15.2 min and **3c** = 15.8 min). The chromatograms did not show peaks corresponding to the free maleimide derivatives of paclitaxel (retention time at 35–40 min eluting as broad peaks).

UV spectra of the paclitaxel PEG conjugates in THF showed the typical absorption maxima of the maleimide paclitaxel derivatives. Based on the assumption that the two thiol groups of the polymer (M_r 20,000) have reacted with the maleimide paclitaxel derivatives and employing the ε-values obtained for the individual maleimide

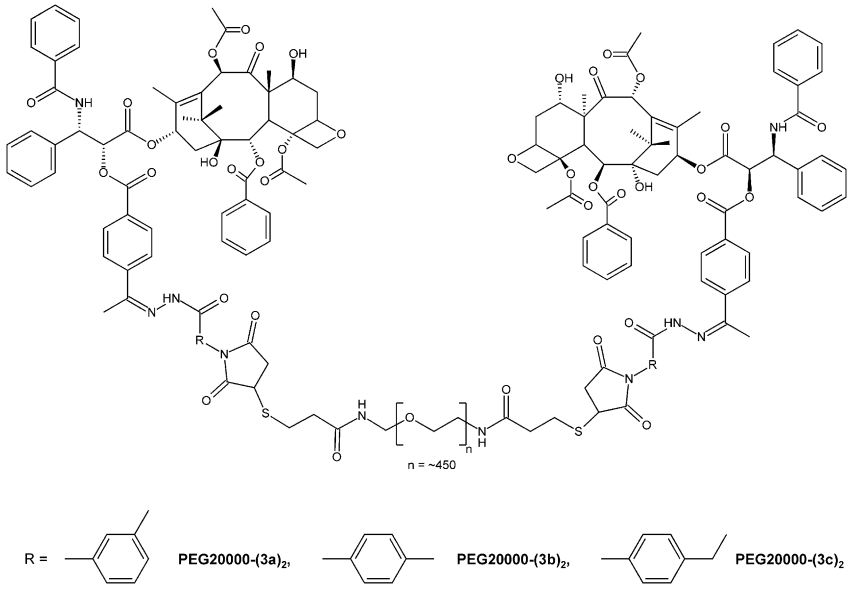


Figure 1.

Table 1. IC_{50} values[a] of the paclitaxel keto-derivative **1**, the paclitaxel PEG conjugates PEG20000-(**3a**)$_2$ PEG20000-(**3b**)$_2$, PEG20000-(**3c**)$_2$ and of paclitaxel in three human cancer cell lines (MACL MCF7, MEXF 514 and RXF 944) as well as the half-lives at pH 4.0 and the percent of release of the PEG paclitaxel conjugates at pH 7.4 after 48 h

Compd	Percent of release at pH 7.4 after 48 h	$t_{1/2}$ (h) 4.0	MACL MCF7 IC_{50} (μM)	MEXF 514 IC_{50} (μM)	RXF 944 IC_{50} (μM)
Paclitaxel		—	0.04±0.003	1.03±0.11	0.05±0.004
1		—	0.13±0.01	0.22±0.01	0.09±0.001
PEG20000-(**3a**)$_2$	<10%	~24	0.79±0.08	3.91±0.37	1.94±0.2
PEG20000-(**3b**)$_2$	<10%	~28	1.49±0.15	5.45±0.51	1.75±0.17
PEG20000-(**3c**)$_2$	<10%	~17	0.75±0.65	3.64±0.35	1.17±0.11

[a]IC_{50} values (50% inhibitory concentration) are listed as mean values±standard deviation of three independent experiments. Concentrations refer to the amount of paclitaxel present.

derivatives, the concentration of paclitaxel in the conjugate samples was determined showing an almost quantitative loading of the polymers with the maleimide paclitaxel derivatives. In addition, no free thiol groups could be determined in the isolated PEG conjugates using Ellman's reagent.[32]

The pH-dependent stability of the PEG conjugates PEG20000-(**3a**)$_2$, PEG20000-(**3b**)$_2$, PEG20000-(**3c**)$_2$ were studied at pH values of 4.0 and 7.4 with the aid of FPLC (Sephadex® G25) at room temperature and chromatograms recorded over a period of 48 h (see ref 33). The decrease in the peak area ($\lambda = 280$ nm) of the paclitaxel PEG conjugate derivative was used as a measure of hydrolysis. Half-lives at pH 4.0 are shown in Table 1 and fall in the range of 17–28 h, which is comparable to other drug polymer conjugate that contain hydrazone bonds.[34] In contrast, the conjugates showed less than 10% decomposition at pH 7.4 after 48 h.

In an orientating study, we also assessed the stability of PEG20000-(**3c**)$_2$ in cell-conditioned medium that had been exposed to MCF-7 cells for 48 h. For this purpose paclitaxel or PEG20000-(**3c**)$_2$ were incubated with cell-conditioned medium for 18 h at 37 °C and a sample extracted with CH_2Cl_2 at 0 and 18 h and subsequently analyzed by HPLC (see ref 35). Whereas almost identical amounts of paclitaxel could be detected after incubation with free paclitaxel at both time points, only traces of paclitaxel could be observed after incubation of PEG20000-(3c)$_2$ after 18 h. Furthermore, stability of **1** at pH 4.0 and pH 7.4 was monitored on a reversed phase C_{18}-column at $\lambda = 230$ nm for 24 h (see ref 36). No release (pH 4.0) or only traces (<5%, pH 7.4) of paclitaxel were observed indicating that the ester bond of **1** is sufficiently stable under these conditions.

Paclitaxel, **1** and the PEG conjugates PEG20000-(**3a**)$_2$, PEG20000-(**3b**)$_2$, PEG20000-(**3c**)$_2$ were subsequently tested for biological activity in three human tumor cell lines (MACL MCF 7 mamma carcinoma, MEXF 514 melanoma carcinoma and RXF 944 renal carcinoma) using the propidium iodide fluorescence assay (see ref 37). Respective IC_{50} values of cell culture experiments—performed three times in triplicate—are summarized in Table 1.

The PEG conjugates showed dose-dependent in vitro activity in the three cancer cell lines at the concentrations tested (0.003–30 μM) with IC_{50} values in the range of 0.75–5.45 μM. In comparison to free paclitaxel or **1**, the paclitaxel PEG conjugates showed a 20- to 40-fold decrease in activity.

In conclusion we have synthesized acid-sensitive paclitaxel PEG conjugates which show in vitro activity in the low micromolecular range that is relevant for further preclinical assessment. Due to our experience with related acid-sensitive conjugates of anthracyclines and chlorambucil, the incorporation of an acid-sensitive linker is an effective way of retaining or improving the in vitro and in vivo efficacy of the parent compound.[18–21] The fact that paclitaxel derivative **1** shows similar in vitro activity to paclitaxel indicates that the ester linkage is either hydrolyzed in the lysosomes/endosomes or that **1** might be as active as paclitaxel per se after cellular uptake of the paclitaxel derivatives. The precise mechanism of intracellular release as well as the in vivo activity of the paclitaxel conjugates will be the next issues that need to be addressed.

Acknowledgements

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24. The synthesis of maleimide spacer derivatives **2a** and **2c** were described previously. **2b** was prepared according to **2a** and **2c**. (see Beyer, U.; Krüger M.; Schumacher, P.; Unger, C.; Kratz, F., *Monatshefte Chemie* **1997**, *128*, 91).
25. Paclitaxel was purchased from Hande Tech USA, Inc. *Preparation of 1:* To 6 g (36.6 mmol) 4-acetylbenzoic acid dissolved in 250 mL toluene were added 80 mL (0.11 mol) thionyl chloride, and the mixture was refluxed for 1 h. Thionyl chloride as well as toluene were removed in vacuo, and the residue crystallized in 100 mL diethylether to yield the acid chloride as yellow crystals.
26. To 700 mg (0.85 mmol) paclitaxel and 240 μL (1.7 mmol) triethylamine dissolved in 50 mL CH_2Cl_2 were added dropwise 193 mg (1.06 mmol) 4-acetylbenzoic acid chloride dissolved in 10 mL CH_2Cl_2 over a period of 30 min. The mixture was stirred for 12 h in the dark. The solvent was removed in vacuo and the residue purified through chromatography on silica gel (0.4–0.6 Å//CH_2Cl_2/methanol = 97:3) to yield **1** as a colorless solid. *Analytical data*: **1**: yield 781 mg (0.78 mmol); R_f 0.7 (THF: iso/hexane = 2:1); ^{1}H NMR data: 300 MHz ($CDCl_3$) for **1**: δ = 8.12 (dd, 2H, arom. H: O–Bz), 8.02/8.03 (dd, 4H, arom. H: 3″-H/4″-H), 7.75 (dd, 2H, arom. H: N–Bz), 7.62–7.3 (m, 11H, arom. H), 7.05 (d, 1H, NH), 6.33 (s, 1H, 10-H), 6.26 (t, 1H, 13-H), 6.07 (dd, 1H, 3′-H), 5.71 (d, 1H, 2′-H), 5.67 (d, 1H, 2-H), 5.30 (s, 1H, 1-OH), 5.15 (s, 1H, 7-OH), 4.97 (dd, 1H, 5-H), 4.45 (dd, 1H, 7-H), 4.31/4.18 (dd, 2H, 20α-H/20β-H), 3.82 (d, 1H, 3-H), 2.64 (s, 3H, 7″-H), 2.54 (m, 1H, 6α-H), 2.45 (s, 3H, 4-OCOCH$_3$), 2.34/2.14 (m, 2H, 14-H), 2.22 (s, 3H, 10-OCOCH$_3$), 1.97 (s, 3H, 18-H), 1.85 (m, 1H, 6β-H), 1.67 (s, 3H, 19-H), 1.23 (s, 3H, 16-H), 1.13 (s, 3H, 17-H). ^{13}C NMR-data: (75.4 MHz, $CDCl_3$): δ = 203.80 (C-9), 197.30 (C-6″), 171.28 (C-1′), 169.80 (10-OCOCH$_3$), 167.99 (4-OCOCH$_3$), 167.21 (3′-NHCOPh), 167.03 (2′-OCOPh), 164.89 (C-1″), 142.66 (C-12), 140.96 (C-11), 136.71/133.67/132.90/132.23/132.12/130.22/130.13/129.22/128.81/128.75/128.46/127.07/126.67 (C-arom.) 84.46 (C-5), 81.09 (C-4), 79.19 (C-1), 77.21 (C-2′), 76.44 (C-20), 75.59 (C-10), 75.11 (C-2), 72.12 (C-13), 72.11 (C-7), 58.52 (C-8), 53.20 (C-3′), 45.59 (C-3), 43.19 (C-15), 35.55 (C-6), 35.54 (C-14), 26.90 (C-7″), 26.83 (C-17), 22.69 (4-OCOCH$_3$), 22.14 (C-16), 20.83 (10-OCOCH$_3$), 14.88 (C-18), 9.61 (C-19). MS (ESI: 4.5 kV, 250 °C) on a Finnigan MAT 312 mass spectrometer: m/z = 1022 (100) [$M^+ \cdot + Na^+$].
27. Data for one representative experiment is given: *Preparation of* **3a**: 100 mg (0.1 mmol) **1**, 173 mg (0.5 mmol) 3-maleimidobenzoic acid hydrazide trifluoroacetate and 77 μL (1 mmol) trifluoroacetic acid were dissolved in 10 mL methanol and stirred for a period of 14 h at room temperature in the dark. The solvent was removed in vacuo and the residue dissolved in 1.5 mL THF. Purification was performed by chromatography (silica gel, 0.6–1.0 Å, in THF/iso-hexane = 2:1) to yield **3a** as a colorless solid. *Analytical data*: **3a**: yield 54 mg (0.045 mmol): R_f = 0.38 (THF/iso-hexane = 2:1); ^{1}H NMR (DMSO-d_6/TMS): δ = 11.69/10.99 (s, 1H, NH-N), 9.32 (d, 1H, NH), 8.12–7.28 (m, 23 arom. H), 7.22 (s, 2H, 10″-H/11″-H), 6.31 (s, 1H, 10-H), 5.85 (t, 1H, 13-H), 5.77/5.57 (d, 1H, 3′-H), 5.38 (d, 1H, 2′-H), 5.36 (d, 1H, 2-H), 4.94 (dd, 1H, 5-H), 4.92 (s, 1H, 1-OH), 4.70 (s, 1H, 7-OH), 4.13 (dd, 1H, 7-H), 4.03/3.80 (dd, 2H, 20α-H/20β-H), 3.63 (d, 1H, 3-H), 2.43 (s, 3H, 7″-H), 2.35 (m, 1H, 6α-H), 2.30 (s, 3H, 4-OCOCH$_3$), 2.11 (s, 3H, 10-OCOCH$_3$), 1.94/1.76 (m, 2H, 14-H), 1.85 (s, 3H, 18-H), 1.68 (m, 1H, 6β-H), 1.51 (s, 3H, 19-H), 1.04 (s, 3H, 16-H), 1.01 (s, 3H, 17-H); ^{13}C NMR (75.4 MHz, DMSO-d_6/TMS): δ = 202.22 (C-9), 169.73 (C-9″/C-12″), 169.62 (C-1′), 168.66 (10-OCOCH$_3$), 168.65 (4-OCOCH$_3$), 166.69 (3′-NHCOPh), 166.68 (2′-OCOPh), 165.09 (C-8″), 164.82 (C-1″), 150.15/146.25 (C-6″), 143.10 (C-12), 139.14 (C-11), 134.94 (C-10″/C-11″), 136.94/134.31/133.40/131.42/ 129.83/129.48/129.42/128.67/128.57/128.31/127.69/127.32/126.73 (C-arom.), 83.52 (C-5), 80.18 (C-4), 76.65 (C-1), 75.44 (C-2′), 74.41 (C-20), 71.07 (C-10), 70.32 (C-2), 66.51 (C-13), 66.50 (C-7), 57.31 (C-8), 53.73 (C-3′), 46.01 (C-3), 42.88 (C-15), 36.32 (C-6), 34.25 (C-14), 26.24 (C-17), 23.46 (4-OCOCH$_3$), 22.45 (C-16), 20.56 (10-OCOCH$_3$), 13.89 (C-18), 13.84/13.47 (C-7″), 9.69 (C-19); MS (ESI: 4.5 kV, 250 °C): m/z = 1235 (100) [$M^{+\bullet} + Na^+$], 1267 (40) [(M − 1) + Na^+ + CH_3OH], 1022 (30) [(M − 1) + Na^+ − $C_{11}H_7N_2O_3$]. Anal. ($C_{67}H_{64}N_4O_{18}$) M = 1213.24 g/mol.
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29. HPLC studies were performed on a reversed-phase RP 18 (Lichrosorb, RT 250-4, Merck); mobile phase: acetonitrile/0.004 M sodium phosphate (pH 6.0) = 60/40; Kontron 422 pump (flow 1 mL/min); UV/VIS detector Kontron 535 and integrator (at λ = 254 and 230 nm); Auto sampler Merck/Hitachi AS4000, injection volume 50 μL PEEK-Loop.
30. ε-values in THF: ε_{280} (paclitaxel) = 1180 ± 90 M^{-1} cm^{-1}, ε_{280} (1) = 2880 ± 120 M^{-1} cm^{-1}, ε_{309} (**3a**) = 18,400 ± 250 M^{-1} cm^{-1}, ε_{309} (**3b**) = 18,930 ± 250 M^{-1} cm^{-1}, ε_{303} (**3a**) = 21,940 ± 240 M^{-1} cm^{-1}.
31. Data for one representative experiment is given: *Preparation of PEG20000-(**3a**)$_2$*: 8.2 mg (7 μmol) of **3a** were dissolved in 1 mL dimethylformamide and added to 40 mg (2 μmol) PEG-20000(SH)$_2$ dissolved in 1 mL buffer (0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4). The mixture was homogenized and stirred at 30 °C for 30 min. The solvent was removed in vacuo, the residue dissolved in 2 mL methanol and chromatographed over Sephadex® LH20 Gel (100 mm × 10 mm, loop size: 2 mL, flow: 1.0 mL/min, retention time: 15–16 min, eluent: 100% methanol HPLC grade, λ = 280 nm). Methanol was removed in vacuo, the residue dissolved in 200 μL CH_2Cl_2 and the product was precipitated with diethylether. The colorless solid was dried in vacuo. The purity of the samples was analyzed through a semi-analytical FPLC-column (Sephadex® LH 20, flow: 0.1 mL/min of 100% Methanol HPLC-grade, retention time: 15–17 min, λ = 280 nm).
32. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
33. *FPLC studies:* Studies at pH 4.0 and pH 7.4 were performed with the PEG paclitaxel conjugates. 50 μL of the stock

solutions of the conjugates (*c* 1100±100 μM) in methanol were diluted 1:20 with a 65:35 mixture of buffer pH 4.0 (0.001 M sodium acetate adjusted to pH 4.0 with acetic 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 = 280 nm on a FPLC column (Sephadex G25), mobile phase: methanol : buffer (0.15 M NaCl, 0.004 M sodium phosphate, pH 7.4) = 30:70 every 3 h for 24 h and after 48 h.

34. Kratz, F.; Beyer, U.; Schütte, M. T. *Crit. Rev. Ther. Drug Carrier Sys.* **1999**, *16*, 245.

35. *Stability studies of paclitaxel and PEG20000-(**3c**)$_2$ in MCF-7 cell-conditioned medium*: A stock solution of paclitaxel or PEG20000-(**3c**)$_2$ in MeOH (2400 μM) was added to MCF-7 cell-conditioned RPMI medium (after 48 h cell exposure) to a final concentration of 480 μM and the samples incubated at 37 °C for 18 h. At 0 and 18 h, a 100 μL sample was extracted with 100 μL CH_2Cl_2 and 20 μL of the organic phase were added to 480 μL mobile phase [acetonitrile/buffer pH 6.0 (0.004 M sodium phosphate adjusted to pH 6.0) = 40:60] and 50 μL sample analyzed at λ = 230 nm on an analytical HPLC column (Lichrosorb, RT 250-4, RP-column, mobile phase: acetonitrile/buffer pH 6.0 (0.004 M sodium phosphate adjusted to pH 6.0) = 40:60. A UV/VIS detector Kontron 535 and integrator as well as an auto sampler Merck Hitachi AS4000 were used.

36. HPLC stability studies with **1** at pH 4.0 and 7.4: 50 μL of a stock solutions of **1** (*c* 6500±100 μM) in methanol were diluted 1:50 with a 65:35 mixture of buffer pH 4.0 (0.001 M sodium acetate adjusted to pH 4.0 with acetic acid) or pH 7.4 (0.15 M NaCl, 0.004 M sodium phosphate) and acetonitrile. The solution was incubated at room temperature and analyzed by HPLC every 3 h over a period of 24 h according to ref 36.

37. *Propidium iodide fluorescence assay:* The fluorescence assay was basically performed according to the method of Dengler et al.[38] Briefly, cells were harvested from exponential phase cultures growing in RPMI 1640 culture medium supplemented with 10% FCS and 1% gentamycin (100 U/mL) by trypsinization, counted and plated in 96-well flat-bottomed microtitre plates (140 μL cell suspension/well, 8.0×10^4 cells/mL). After a 24 h recovery in order to allow cells to resume exponential growth, 10 μL culture medium (six control wells per plate) or culture medium containing drug was added to the wells. Stock solutions of all compounds were prepared in DMSO (10 mM) and diluted with cell culture medium to final concentrations of 0.003–30 μM. Each drug concentration was plated in triplicate. After 4 days of continuous drug exposure, medium or medium with drug was removed and 200 μL of an aqueous propidium iodide solution (6 μg/mL) was added that intercalates the DNA of dead cells. Microtiter plates were then kept at −20 °C for 24 h resulting in death of all cells. Fluorescence (FU_1) was measured using a Millipore Cytofluor 4000 microplate reader (excitation 530 nm, emission 620 nm). Growth inhibition was expressed as treated/control×100 (%T/C).

38. Dengler, W. A.; Schulte, J.; Berger, D. P.; Mertelsmann, R.; Fiebig, H. H. *Anti-Cancer Drugs* **1995**, *6*, 522.