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Acid-Sensitive Polyethylene Glycol Conjugates of Doxorubicin: Preparation, In Vitro Efficacy and Intracellular Distribution

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Abstract—Coupling anticancer drugs to synthetic polymers is a promising approach of enhancing the antitumor efficacy and reducing the side-effects of these agents. Doxorubicin maleimide derivatives containing an amide or acid-sensitive hydrazone linker were therefore coupled to α-methoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 20000 Da), α,ω-bis-thiopropionic acid amide poly(ethylene glycol) (MW 20000 Da) or α-*tert*-butoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 70000 Da) and the resulting polyethylene glycol (PEG) conjugates isolated through size-exclusion chromatography. The polymer drug derivatives were designed as to release doxorubicin inside the tumor cell by acid-cleavage of the hydrazone bond after uptake of the conjugate by endocytosis. The acid-sensitive PEG conjugates containing the carboxylic hydrazone bonds exhibited in vitro activity against human BXF T24 bladder carcinoma and LXFL 529L lung cancer cells with IC_{70} values in the range 0.02–1.5 μm (cell culture assay: propidium iodide fluorescence or colony forming assay). In contrast, PEG doxorubicin conjugates containing an amide bond between the drug and the polymer showed no in vitro activity. Fluorescence microscopy studies in LXFL 529 lung cancer cells revealed that free doxorubicin accumulates in the cell nucleus whereas doxorubicin of the acid-sensitive PEG doxorubicin conjugates is primarily localized in the cytoplasm. Nevertheless, the acid-sensitive PEG doxorubicin conjugates retain their ability to bind to calf thymus DNA as shown by fluorescence and visible spectroscopy studies. Results regarding the effect of an acid-sensitive PEG conjugate of molecular weight 20000 in the chorioallantoic membrane (CAM) assay indicate that this conjugate is significantly less embryotoxic than free doxorubicin although antiangiogenic effects were not observed.

Introduction

The attachment of antitumor drugs to synthetic polymers is a promising strategy of modifying their biodistribution, of reducing drug toxicity and thus improving the therapeutic efficacy of anticancer agents.[1] Polyethylene glycols (PEGs) are non-ionic, water-soluble synthetic polymers which are potential drug carriers due to their synthetic diversity and recognized biocompatability.[2] In recent years, a number of reports have appeared on the synthesis and biological activity of high-molecular weight polyethylene glycol conjugates (MW 20–40 kDa) with antitumor agents such as paclitaxel and camptothecins.[3,4] These conjugates were designed to increase the water-solubility and plasma half-life of the drug while slowly releasing the parent compound through hydrolysis of the chemical link between the drug and the polymer backbone.

In our recent work on serum protein conjugates an acid-sensitive hydrazone linker was incorporated between the drug and the thiolated protein carrier allowing the protein-bound drug to be released in the acidic environment of endosomes and/or lysosomes after cellular uptake of the conjugate by endocytosis.[5–9] Acid-sensitive anthracycline and chlorambucil conjugates with serum albumin and transferrin exhibit high antiproliferative activity in vitro, and selected conjugates, such as acid-sensitive doxorubicin albumin conjugates, show superior antitumor

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efficacy in a number of animal tumor models when compared to the parent compound.[10,11]

As part of our research program on anticancer polymer conjugates we have now developed doxorubicin conjugates with polyethylene glycols of molecular weight 20000 and 70000 which contain an amide or an acid-sensitive hydrazone linker thus allowing a direct comparison with analogous transferrin and albumin doxorubicin conjugates. In this paper we report on the preparation of doxorubicin polyethylene glycol conjugates, on their antiproliferative activity in vitro and on the effect of selected conjugates in the chorioallantoic membrane (CAM) assay. In addition, we investigated the cellular uptake of the conjugates with fluorescence microscopy and followed their interaction with calf thymus DNA using UV/VIS- and fluorescence spectrophotometry. In this way we wanted to obtain a first insight into the biological activity and mechanistic characteristics of the newly synthesized doxorubicin PEG conjugates.

Results and Discussion

Preparation and characterization

Polyethylene glycol conjugates of doxorubicin were prepared by reacting maleimide derivatives of doxorubicin (Hyd_1, Hyd_2, $Amid_1$)[12] with α-methoxy-poly-(ethylene glycol)-thiopropionic acid amide (MW 20000 Da), α,ω-bis-thiopropionic acid amide poly(ethylene glycol) (MW 20000 Da) or α-*tert*-butoxy-poly(ethylene glycol)-thiopropionic acid amide (MW 70000 Da) 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 doxorubicin conjugates

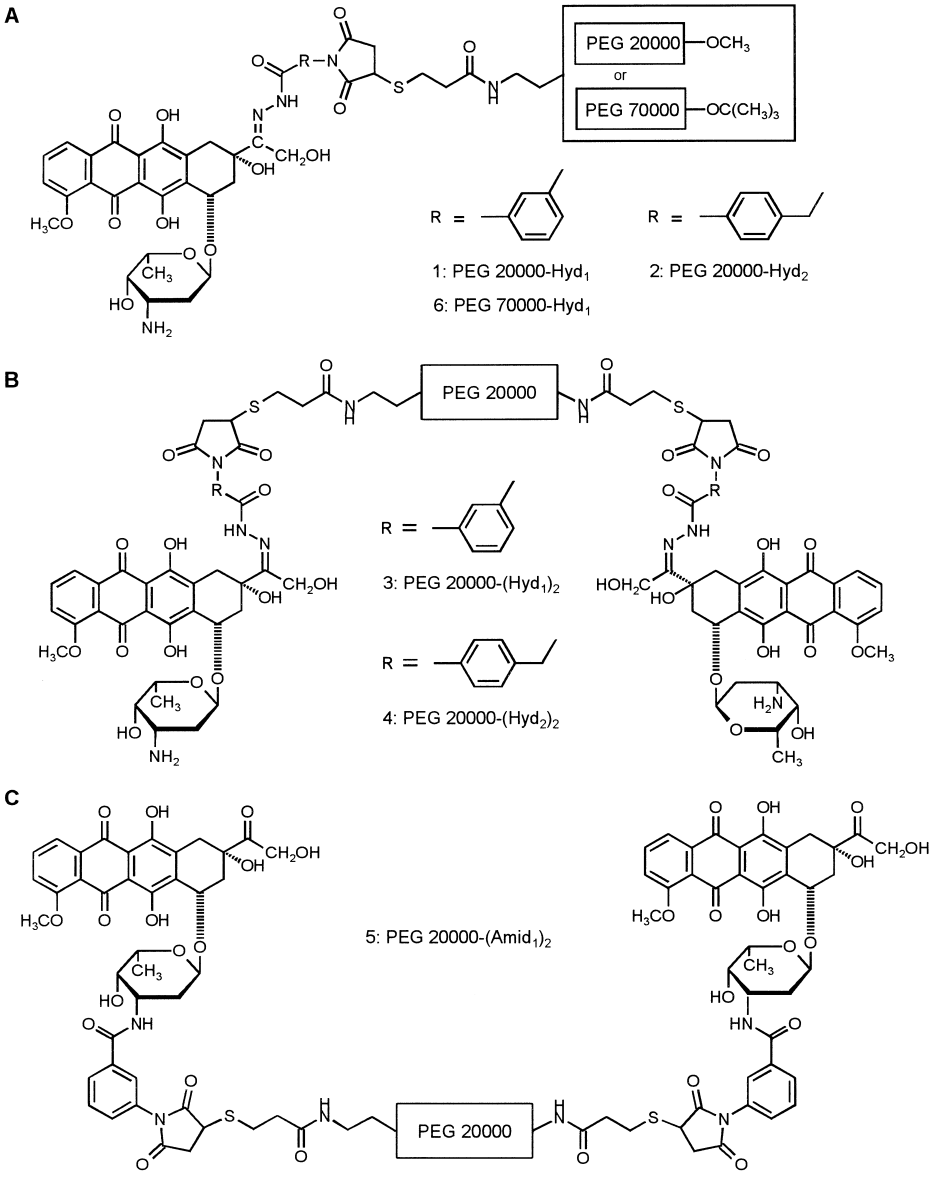

Figure 1. Structures of doxorubicin polyethylene glycol conjugates.

were isolated through size-exclusion chromatography over Sephadex® G-25 in phosphate buffer or over LH20® in methanol (see Experimental).

The purity of the samples was determined with an analytical HPLC-size exclusion column (Nucleogel® aqua-OH 40-8). A typical chromatogram, recorded at $\lambda = 495$ nm, is shown in Figure 2. Retention times of the doxorubicin PEG conjugates are between 7 and 10 min on this column; free doxorubicin elutes as a broad peak at approximately 45 min. UV/VIS-spectra of the doxorubicin PEG conjugates in phosphate buffer showed the typical absorption maxima at $\lambda = 495$, 480, 252, and 234 nm. Selected vacuum-dried samples (**1** and **2**), which were obtained over Sephadex® LH20 in methanol, were investigated with ^{1}H NMR spectroscopy in $CDCl_3$ (400 and 600 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, i.e. 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 typical strong singlet signal of the maleimide double bond (~7.2 ppm) was no longer present in the spectra indicating that the HS-group has reacted with the maleimide group. Assignment of the proton signals of the sugar ring of doxorubicin was not possible, however, due to a very broad signal of -CH_2-groups of the polymer despite our decoupling attempts.

pH-Dependent stability studies

The three maleimide derivatives of doxorubicin (Hyd_1, Hyd_2, $Amid_1$) differ in the site (3′-amino or 13-keto position) and stability (benzamide or benzoyl and phenylacetyl hydrazone bond) of the chemical link between doxorubicin and the spacer molecule. Previous stability studies demonstrated an acid-sensitive character of the carboxylic hydrazone bond.[6,7] In order to confirm this observation, pH-dependent stability of the doxorubicin PEG conjugates was studied at pH-values of 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 doxorubicin release. The results of these studies are shown in Table 1.

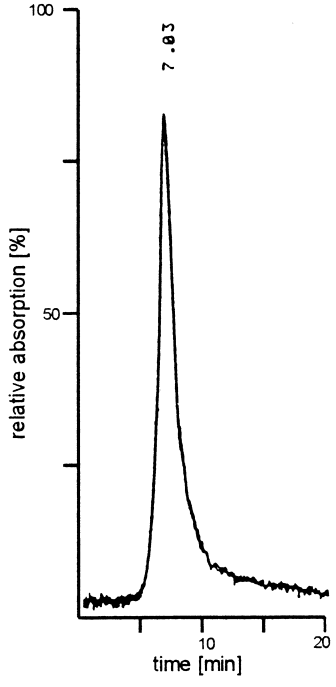

Figure 2. Chromatogram of PEG 20000-Hyd_2; [flow = 1.0 ml/min. $\lambda = 495$ nm, c = 30 μM].

Whereas the amide derivative **5** showed no release of doxorubicin at either pH 5.0 or 7.4, the carboxylic hydrazone derivatives **1**–**4** and **6** showed good stability at pH 7.4 (less than 10% release of doxorubicin after 48 h) but a marked release at pH 5.0. Acid-lability of the benzoyl hydrazone linker (Hyd_1) was more pronounced in comparison to the phenylacetyl hydrazone linker (Hyd_2). Furthermore, release of doxorubicin was slower for PEG 70000-Hyd_1 than for PEG 20000-Hyd_1 at pH 5.0.

Biological data

The doxorubicin PEG conjugates and free doxorubicin were subsequently tested for biological activity in two human tumor cell lines (T24 bladder carcinoma and LXFL 529 lung cancer cells) using the propidium iodide fluorescence assay and in a human bladder carcinoma xenograft (BXF 1299) using a clonogenic assay. Respective IC_{70} values are summarized in Table 2. Unbound polyethylene glycols had only marginal influence on cell growth in both cell lines (concentration range: 0.001–10 μM; data not shown). The conjugates **1** and **3** which contain a benzoyl hydrazone bond are more active than those containing a phenylacetyl hydrazone bond (**2** and **4**). In contrast, the amide conjugate **5** showed no activity at the concentrations tested (0.001–10 μM). PEG 70000-Hyd_1 was approximately 20-fold less active than PEG 20000-Hyd_1. A comparison of the in vitro activity of the previously synthesized transferrin conjugates T-Doxo-Hyd_1 and T-Doxo-Hyd_2 with analogously constructed PEG doxorubicin conjugates in the BXF 1299 xenograft demonstrates that the IC_{70} values lie in a very similar range (see Table 2). Of all compounds tested, free doxorubicin is the most active one exhibiting IC_{70} values which are 5- to 200-fold lower than those for the acid-sensitive doxorubicin PEG conjugates.

Table 1. Stability studies of doxorubicin PEG conjugates at pH 5.0 and 7.4

Doxorubicin PEG conjugate	t_{50} [h][a] at pH 5.0	Stability at pH 7.4[b]
1: PEG 20000-Hyd_1	~2	< 10%
2: PEG 20000-Hyd_2	~30	< 10%
3: PEG 20000-$(Hyd_1)_2$	~2	< 10%
4: PEG 20000-$(Hyd_2)_2$	~6	< 10%
5: PEG 20000-$(Amid)_2$	> 72	< 5%
6: PEG 70000-Hyd_1	~27	< 10%

[a] Time after which the peak area of the conjugate is 50% of its initial value.
[b] Decrease in peak area of the conjugate after 48h.

Table 2. In vitro antitumor activity of doxorubicin, of PEG and transferrin doxorubicin conjugates in two human tumor cell lines (T24, LXFL 529) and in the BXF 1299 xenograft

Compound	IC_{70} value in T24 [μM][a]	IC_{70} value in LXFL 529 [μM][a]	IC_{70} value in BXF 1299 [μM][b]
Doxorubicin	0.004	< 0.001	0.15
1: PEG 20000-Hyd_1	0.03	0.04	0.30
2: PEG 20000-Hyd_2	0.34	0.09	0.50
3: PEG 20000-$(Hyd_1)_2$	0.06	0.02	0.25
4: PEG 20000-$(Hyd_2)_2$	0.39	0.26	1.50
5: PEG 20000-$(Amid)_2$	> 10	> 10	Not determined
6: PEG 70000-Hyd_1	0.60	0.12	Not determined
T-Doxo-Hyd_1	Not determined	Not determined	0.5
T-Doxo-Hyd_2	Not determined	Not determined	1.8

[a] Propidium iodide fluorescence assay: the fluorescence assay was performed according to the method Dengler et al.[17] (also described by us in 6 and 8). After 6 days of continuous drug exposure nonviable cells were stained by addition of propidium iodide, and fluorescence (FU_1) was measured (exitation 530 nm, emission 620 nm). Microplates were then kept at −18°C for 24 h, which resulted in total cell kill. After thawing of the plates a second fluorescence measurement (FU_2) was carried out, and the amount of viable cells was calculated by FU_2-FU_1.
[b] Clonogenic assay: the assay was performed as a two-layer soft agar assay using a cell suspension derived from the human tumor xenograft BXF 1299 as described in detail in Fiebig et al.[18] Drug effects were evaluated after 15 days of drug exposure.

CAM-assay[13,14]

Doxorubicin and PEG 20000-Hyd_2 were tested in the CAM-assay for their antiangiogenic effect compared to standard controls (laminarin sulphate, suramin), see Table 3. At the concentration of 20 μg/pellet for free doxorubicin all embryos died within one day (data not shown) so that the maximum drug concentration of 10 μg/pellet was used in the experiments. PEG 20000-Hyd_2 is less toxic because it did not show any embryotoxic effect at the concentration of 10 μg doxorubicin equivalents/pellet. Free doxorubicin exhibited a very low antiangiogenic effect (score 0.4), and PEG 20000-Hyd_2 is inactive according to the score rating in the CAM assay. In conclusion, the data indicates that PEG 20000-Hyd_2 is better tolerated than free doxorubicin.

Fluorescence microscopy studies

In order to investigate the cellular uptake and intracellular distribution of doxorubicin and the acid-sensitive doxorubicin PEG conjugates, LXFL 529 cells were incubated with our drug formulations for 24 h and doxorubicin was subsequently detected by fluorescence microscopy. Fluorescence microscopy is a suitable technique to study the intracellular fate of the autofluorescent drug doxorubicin and to assess any qualitative differences between anthracycline derivatives regarding their intracellular distribution. Results are depicted in Figure 3 for doxorubicin and PEG 20000-Hyd_2 as a representative example. As shown in Figure 3A, doxorubicin is confined to the cell nucleus after 24 h. In contrast, when cells were incubated with PEG 20000-Hyd_2 for 24 h, decreased fluorescence was detected in the nucleus but was observed primarily in the cytoplasm (Fig. 3B). PEG 70000-Hyd_1 displayed a similar cellular distribution pattern (data not shown). The differences in the intracellular distribution are in accordance with our earlier work on acid-sensitive transferrin-doxorubicin conjugates in this cell line,[6] in which LXFL 529 cells treated with the acid-sensitive transferrin-doxorubicin conjugate (T-Doxo-Hyd_1) were devoid of nuclear fluorescence after 24 h.

Table 3. Antiangiogenic and embryotoxic effects of doxorubicin, PEG 20000-Hyd_2, suramin, laminarin sulphate, and agarose in the CAM-assay

Compound	Antiangiogenic effect (score-value)	Embryotoxic effect (loss of embryos in %)
Doxorubicin[a]	0.4 (±0.15)	31%
PEG 20000-Hyd_2[b]	0	No effect
Suramin[c]	0.5 ± 0.2	No effect
laminarin sulphate[a]	1.05 (±0.05)	No effect
Agarose	0	No effect

Test concentrations: [a] 10 μg/pellet; [b] 10 μg doxorubicin equivalents/pellet; [c] 50 μg/pellet. Score values: 0: no effect; 0.5: very weak effect, no capillary free area or area with a reduced density of capillaries around the pellet not larger than the area of the pellet; 1: medium effect, small capillary free area or area with significantly reduced density of capillaries, effects not larger than twice the size of the pellet. 2: strong effect, capillary free area around the pellet at least twice the size of the pellet.

Interactions with calf thymus DNA

In light of the results from our fluorescence microscopy studies it was of interest to establish whether the anthracycline chromophore of the doxorubicin PEG conjugates retains its ability to bind DNA through intercalation. Free doxorubicin and the conjugates **1**, **2** and **5** were therefore reacted with calf thymus DNA under physiological conditions and the reaction analyzed by fluorescence spectroscopy and visible spectrophotometry. It is well known that DNA-intercalation of doxorubicin results in a dramatic quenching (> 90%) of the intrinsic fluorescence of the anthracycline chromophore.[15,16] A similar fluorescence quenching was observed when **1** (∼90% quenching) or **2** (∼75% quenching) was incubated with saturating amounts of calf thymus DNA as shown in Figure 4(B and C) (Fig. 4A shows the results for free doxorubicin under the same conditions, quenching > 90%). In contrast, quenching is significantly less marked in the case of **5**, which is the in vitro inactive amide conjugate (quenching ∼20%, see Fig. 4D), and implies that DNA-intercalation is less efficient. These results were confirmed by

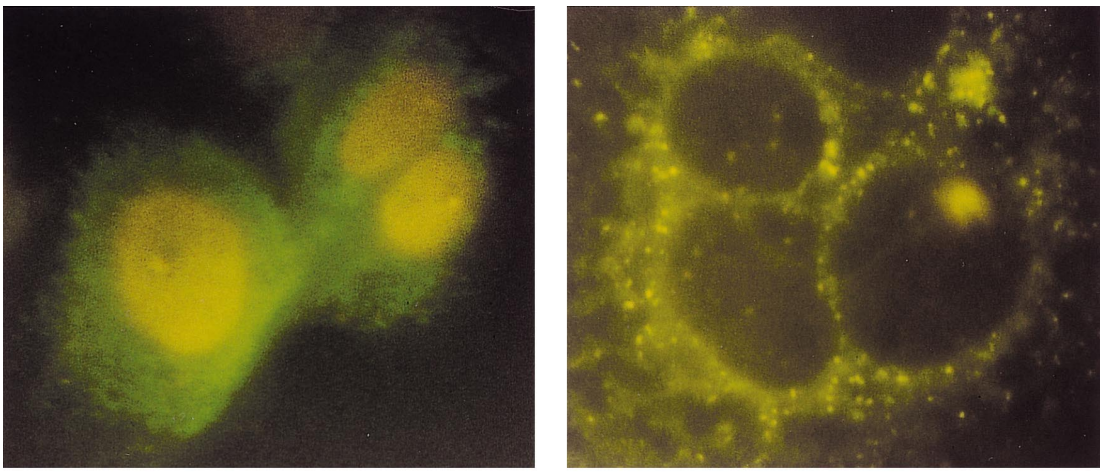

Figure 3. Fluorescence microscopy photographs of the cellular distribution of doxorubicin (A) and PEG 20000-Hyd_2 (B) after 24 h; LXFL 529 lung cancer cells were incubated with drug concentrations of $c \approx 1$ μM for 24 h. Fluorescence was excited at 450–490 nm and detected at a wavelength greater than 540 nm.

spectrophotometry in the visible region demonstrating that reaction of doxorubicin, **1** or **2** with DNA produced a marked change in the initial spectra without DNA which consisted in a considerable decrease and red shift of the main visible band (450–550 nm), data not shown. Smaller spectral perturbations were observed for **5**.

Summary

The purpose of the present study was to obtain a first picture of the biological activity and mode of action of doxorubicin PEG conjugates. From our data we conclude that the acid-sensitive properties of the link between PEG and doxorubicin are important for retaining the antitumor activity of doxorubicin. In principle, the order of antitumor activity of the conjugates correlates with their acid-lability. Fluorescence microscopy studies in LXFL 529 lung cancer cells show that free doxorubicin accumulates in the cell nucleus whereas doxorubicin of the acid-sensitive PEG conjugates is primarily localized in the cytoplasm. These results indicate that the conjugates might exert their cytotoxicity by a different mode of action other than intercalation with DNA. However, the acid-sensitive PEG doxorubicin conjugates retain their ability to bind to calf thymus DNA as shown by fluorescence and visible spectroscopy studies. Thus, in order to understand the mode of action of acid-sensitive anthracycline PEG conjugates in more detail, we are at present carrying out laser scanning confocal fluorescence microscopy studies as well as incubation studies with RNA.

Although the IC_{70} values of the conjugates are higher than for free doxorubicin, they nonetheless lie in a range which is relevant for further preclinical evaluation. From our experience, the benefit of antitumor macromolecular prodrugs can primarily be proven in suitable animal models due to the great difference in the biodistribution between the free drug and the drug polymer conjugate.[10,11] Our preliminary data in nude mice show that PEG 20000-$(Hyd_2)_2$ (**3**) and PEG 70000-Hyd_1 (**6**) can be administered at far higher doses than the maximum tolerated dose of free doxorubicin ($2\times16-24$ mg/kg as compared to 2×8 mg/kg). This is in accordance with our experience with analogous transferrin and albumin conjugates of doxorubicin.[6,7,10,11] Our data with PEG 20000-Hyd_2 in the CAM assay show that this conjugate is significantly less embryotoxic than free doxorubicin although antiangiogenic effects were not observed. In vivo experiments evaluating the antitumor efficacy of acid-sensitive doxorubicin PEG conjugates in xenograft models are in progress.

Experimental

Chemicals, materials and spectroscopy

^1H NMR and ^{13}C NMR: Bruker 400 or 600 MHz AMX (internal standard: TMS); 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, 30% v/v MeOH-pH 7.0. A Lambda 1000 UV–visible monitor from Bischoff (at $\lambda = 495$ nm), an autosampler Merck Hitachi AS400 and an Integrator Merck Hitachi D2500 were used; doxorubicin was a gift from Pharmacia and Upjohn, FRG. Organic solvents: HPLC grade (Merck). Other organic or inorganic compounds: Merck AG, FRG. Hyd_1, Hyd_2, $Amid_1$ were prepared previously.[12] 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, trypsin versene/EDTA) and fetal calf serum (FCS) were purchased from

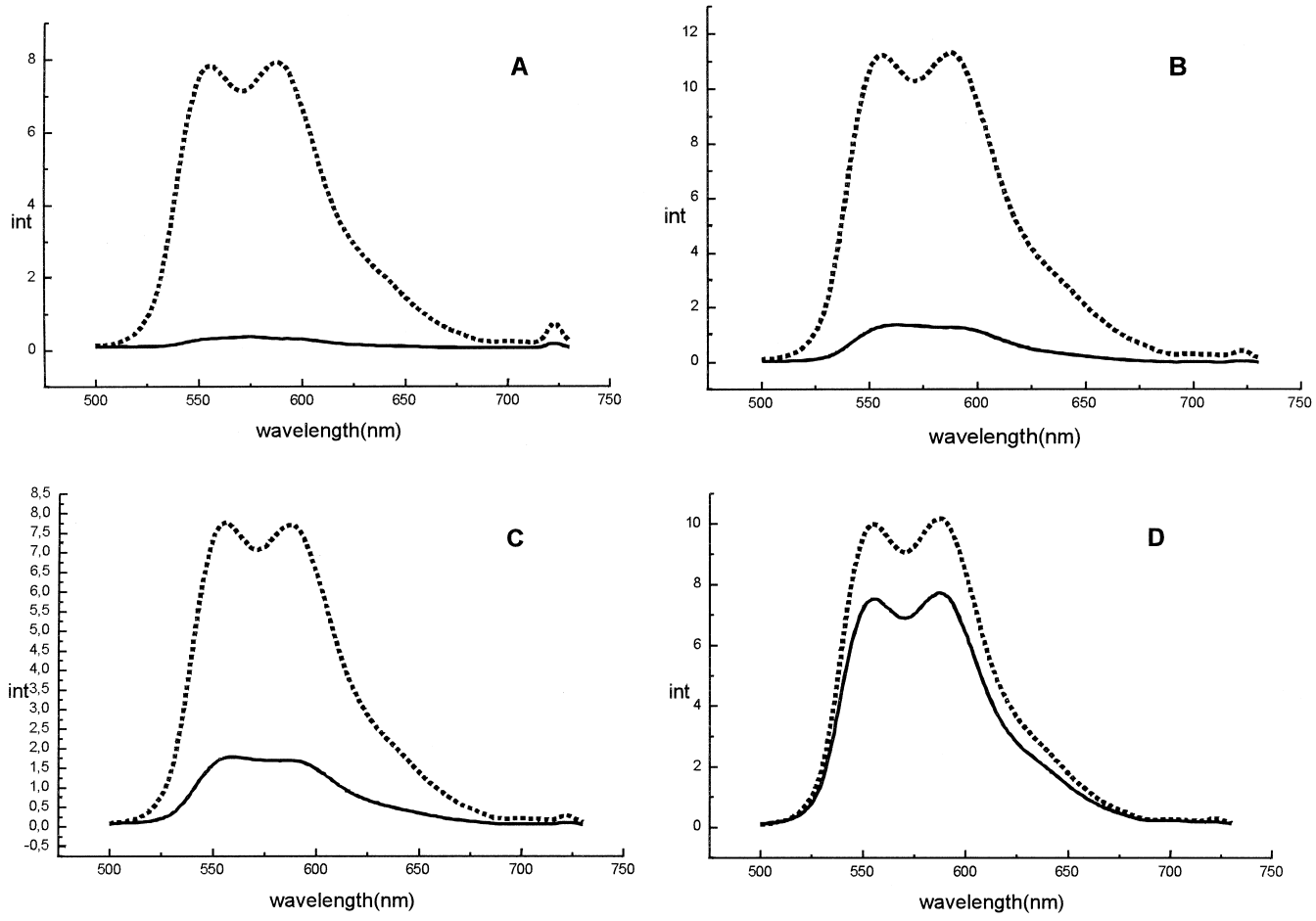

Figure 4. Fluorescence spectra of doxorubicin (A), of **2** (B), **1** (C) and **5** (D) ($c \approx 1$ μM) in the absence (- - -) and in the presence (——) of 0.6 mg/mL *calf thymus* DNA.

Bio Whittaker (Serva, Heidelberg, FRG). Propidium iodide was purchased from Aldrich-Sigma-Chemie, FRG. All culture flasks were obtained from Greiner Labortechnik (Frickenhausen, FRG).

Methods for the preparation of polyethylene glycol conjugates of doxorubicin. FPLC for preparation of conjugates: P-500 pump, LCC 501 Controller (Pharmacia) and LKB 2151 UV-monitor (at $\lambda = 280$ nm); buffer: 0.004 M sodium phosphate, 0.15 M NaCl, pH 7.4. All reactions were performed at room temperature unless otherwise stated. Data for one representative experiment is given:

Preparation of PEG 20000-(Hyd_1)$_2$. Eight milligrams (0.01 mmol) of Hyd_1 were 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 doxorubicin in the conjugate was adjusted to $c = 300 \pm 20$ μM using the ε-value for doxorubicin in physiological buffer ($\varepsilon_{495} = 10650$ M^{-1} cm^{-1}).[6,7] The conjugate was stored at −80°C. Doxorubicin polyethylene glycol conjugates for NMR studies were chromatographed over Sephadex® LH20 Gel (100×10 mm, loop size: 2 mL, flow: 1.0 mL/min, retention time: 3–6 min, eluent: 100% methanol HPLC grade).

pH dependent stability studies with the PEG doxorubicin conjugates at pH 5.0 and 7.4. Fifty microlitres of the stock solutions of the conjugates ($c = 300 \pm 20$ μM) in phosphate buffer were 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 $\lambda = 495$ nm every 2–4 h over a period of 72 h on an analytical HPLC column (Nucleogel® aqua-OH 40-8, 300×7.7 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.

Fluorescence spectra. Fluorescence spectra were carried out with a Jasco FP-750 spectrofluorimeter working at room temperature. Fluorescence was excited at 480 nm

and detected at wavelengths greater than 500 nm. Doxorubicin or conjugates were diluted with buffer (8 mM phosphate, 185 mM NaCl, 1 mM EDTA, pH 7.4) to a concentration of 1.0 μM. Calf thymus DNA (Sigma) was then added to a final concentration of $r \sim 0.01$, where r is the ratio between the molar concentration of doxorubicin and the DNA basepairs. Fluorescence spectra were recorded after incubating the samples for a few minutes.

Fluorescence microscopy. LXFL 529 lung carcinoma cells were grown in RPMI 1640 cell culture medium with 10% FCS to which were added 100 U/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL L-glutamine. After subcultivation, cells were washed twice in PBS, resuspended in medium to a final concentration of 2×10^4 cells/cm^2 and allowed to adhere on sterile TC chamber slides with glass bottom (NUNC, Denmark) for 24 h. Cells were incubated with drug concentrations of $c \approx 1$ μM for 24 h. Slides were then washed twice in PBS and covered with 24×50 mm coverslips. Cell preparations were studied by epi-illumination with the use of interference optics. An Olympus microscope fitted with an HBO-100 mercury-arc lamp was used. The microscope contained a 2-type filter complex for fluorescein detection that consisted of a 450–490 nm excitation filter, a 510 nm dichroic mirror, and a 540 nm barrier filter that allows wavelength greater than 540 nm to pass. The microscope was connected to an Olympus camera containing a 35 mm ASA 400 Elitechrome film. Fluorescence microscopy studies were repeated twice for doxorubicin and each conjugate.

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 doxorubicin in the stock solution of the conjugates was 300 μM.

Propidium iodide fluorescence assay. The fluorescence assay was performed according to the method of Dengler et al.[17] Briefly, cells were harvested from exponential phase cultures growing in RPMI culture medium by trypsinization, counted and plated in 96-well flat-bottomed microtitre 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).

Clonogenic assay. The assay was performed as a two-layer soft agar assay using a cell suspension derived from the human tumor xenograft BXF 1299 as described in detail in Fiebig et al.[18]

In vivo test for antiangiogenic activity. Test compounds were dissolved in a 2.5% agarose-solution (final concentrations: 1–5 mg/mL) at approximately 60°C. For preparing the pellets 10 μL of the respective warm solution were dropped on circular teflon supports of 3 mm in diameter and then cooled instantly to room temperature. The fertilized hen eggs were incubated for 65–70 h at 37°C at a relative humidity of 80%, positioned horizontally and rotated several times before being opened on the snub side. Prior to this 10 mL of albumin were aspirated from a hole on the pointed side. At two thirds of the height (from the pointed side) the eggs were traced with a scalpel and the shells removed with forceps. The aperture was covered with keep-fresh film, and the eggs were incubated at 37°C at a relative humidity of 80% for 75 h. When the formed chorioallantoic membrane had reached a diameter of approximately 2 cm, one pellet per egg was placed on it. The eggs were incubated for one further day and then evaluated under the stereo microscope. For every test compound 15–20 eggs were used. Each experiment was performed in duplicate (for details see refs 13 and 14).

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