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SHORT COMMUNICATION

In vitro and in vivo study of an albumin-binding prodrug of doxorubicin that is cleaved by cathepsin B

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Abstract
Purpose This study was designed to evaluate the in vitro and in vivo antitumor activity of an albumin-binding prodrug of doxorubicin **1** which incorporates a maleimide moiety and a *para*-aminobenzyloxycarbonyl (PABC) spacer coupled to the dipeptide Phe-Lys that is cleaved by cathepsin B.
Methods Cleavage of the albumin conjugate was studied with cathepsin B and in homogenates of MDA-MB 435 tumors. For in vivo studies, nude mice were injected with (a) glucose buffer, (b) doxorubicin (2 × 8 mg/kg, i.v, on days 10 and 17), or (c) compound **1** (3 × 24 mg/kg doxorubicin equivalent, on days 10, 17 and 24).
Results Prodrug **1** once bound to albumin was effectively cleaved by cathepsin B and in tumor homogenates releasing doxorubicin. A cytotoxicity assay of the albumin conjugate of **1** in two human tumor cell lines showed that doxorubicin was ~6 times more active than the conjugate. In contrast, in an in vivo study, the prodrug exhibited superior antitumor activity (*T/C* 15%) compared to doxorubicin (*T/C* 49%) in an equitoxic comparison.
Conclusions The cathepsin B cleavable spacer Phe-Lys-PABC incorporated in an albumin-binding prodrug is an effective way to increase the therapeutic index of doxorubicin.

Keywords Albumin-binding prodrug · Albumin · Cathepsin B · 1,6-Self-immolative spacer · Doxorubicin

Introduction

Doxorubicin, an anthracycline isolated from streptomyces strains, is one of the most efficacious anticancer drugs for the treatment of leukemia and a broad range of solid tumors such as breast and ovarian carcinoma, sarcoma and many other solid tumors [1].

The clinical application of this anthracycline is, however, limited by its dose-related side effects that include bone marrow toxicity, gastrointestinal disorders, stomatitis, alopecia, acute and cumulative cardiotoxicity as well as extravasation [2]. To circumvent these limitations and to improve the chemotherapeutic potency of doxorubicin, liposomes [3], nanoparticles [4], macromolecular prodrugs and antibody conjugates [5] have been developed with anthracyclines.

In our group we have investigated a passive targeting approach in which maleimide-bearing prodrugs of doxorubicin exploit endogenous albumin as a drug carrier and improve the therapeutic index of the anthracycline. This macromolecular prodrug concept is based on two features: (1) rapid and selective binding of a maleimide functionalized prodrug to the cysteine-34 position of endogenous albumin after intravenous administration, and (2) release of the albumin-bound drugs predominantly at the tumor site due to the incorporation of a cleavable bond between the drug and the carrier [6–10]. Albumin demonstrates prefer-

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ential tumor uptake in various tumor animal models [10, 11] due to the enhanced permeability and retention of macromolecules for solid tumors [12].

A pivotal result of our research was an acid-sensitive albumin-binding prodrug of doxorubicin, i.e. the (6-maleimidocaproyl)hydrazone derivative of doxorubicin (INNO-206, formerly DOXO-EMCH), that reacted ideally with the cysteine-34 of human serum albumin (HSA) [7, 9]. INNO-206 is a prodrug of doxorubicin in which doxorubicin is derivatized at its C-13 keto-position with a thiol-binding spacer molecule, i.e. 6-maleimidocaproic acid hydrazide. It contains an acid-sensitive hydrazone linker that allows doxorubicin to be released either extracellularly in the slightly acidic environment often present in tumor tissue or intracellularly in acidic endosomal or lysosomal compartments after cellular uptake of the albumin conjugate by the tumor cell. INNO-206 was superior to free doxorubicin in several tumor models and is under clinical development [9].

Additionally, a series of albumin-binding prodrugs of doxorubicin with enzymatically cleavable linkers have been developed in the past 5 years [8, 13–16]. A current focus in our group is the development of doxorubicin prodrugs that incorporate a peptide linker that serves as a substrate for the tumor-associated protease, cathepsin B, which is overexpressed in several solid tumors [16]. We have recently developed a doxorubicin prodrug that incorporates the peptide linker Ala-Leu-Ala-Leu. Cleavage of the prodrug in its albumin-bound form by cathepsin B as well as in human tumor homogenates primarily released H-Leu-Ala-Leu-doxorubicin, H-Leu-doxorubicin and small amounts of doxorubicin. In addition, the in vivo antitumor efficacy of the prodrug was only comparable to that of free doxorubicin in the M-3366 mamma carcinoma xenograft model [16].

A possible explanation is that direct coupling of the protease substrate to the amino position of doxorubicin impairs cleavage by the protease. There are only a few examples of doxorubicin prodrugs with a protease substrate that are directly cleaved at the 3′-amino position such as in PK1 in which doxorubicin is conjugated with a *N*-(2-hydroxypropyl)methacrylamide copolymer through a Gly-Phe-Leu-Gly peptide [17]. One possibility of reducing steric interaction between the drug peptide substrate and the respective protease is to integrate a self-immolative spacer between the drug and the protease substrate. The self-immolative spacer hydrolytically decomposes upon deacylation, spontaneously releasing the free drug [18, 19]. Therefore, to liberate doxorubicin effectively at the tumor site we decided to study an albumin-binding prodrug that contains the *para*-aminobenzyloxycarbonyl (PABC) spacer between doxorubicin and the lysosomally cleavable dipeptide Phe-Lys that is specifically recognized by cathepsin B [18]. Dubowchik et al. [18] described a maleimide containing prodrug of doxorubicin **1** [EMC-Phe-Lys-PABC-Doxo (EMC = 6-maleimidocaproic acid)] that meets these properties (Fig. 1). **1** was used for the synthesis of monoclonal antibody conjugates with doxorubicin that showed cytotoxic activity against tumor cell lines and a rapid and almost quantitative drug release in the presence of cathepsin B while demonstrating excellent stability in human plasma [18, 20].

The goal of the present work was to investigate the albumin-binding properties of the prodrug **1** and to ascertain whether cathepsin B as well as tumor homogenates can cleave the prodrug once bound to albumin. In a next step, we wanted to perform in vitro and in vivo studies of **1** in order to study its cytotoxicity and to assess its efficacy in a tumor bearing model in a comparison to doxorubicin.

Materials and methods

Preparation of **1**

Prodrug **1** was synthesized in a modified procedure of the work of Dubowchik et al. [18] by replacing the methoxytrityl

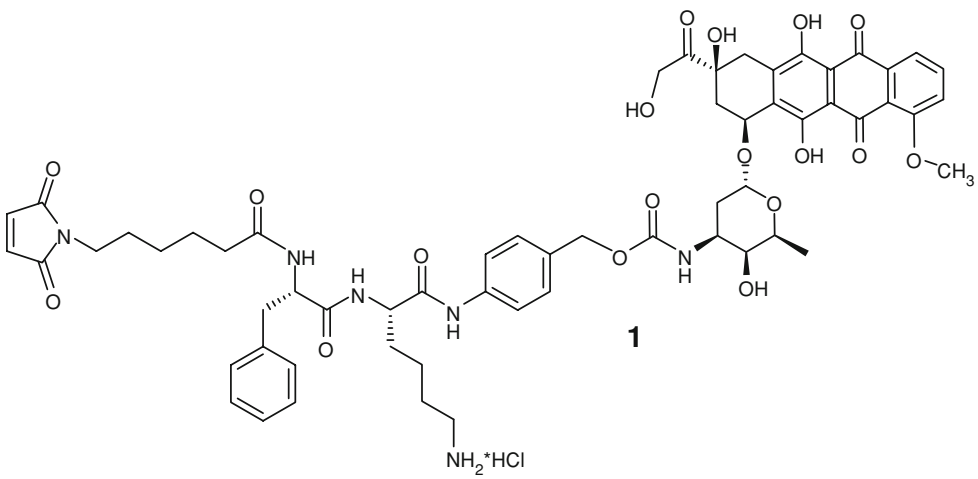


Fig. 1 Chemical structure of prodrug **1**

group in the original work with the methyltrityl (Mtt) group which was finally cleaved with 0.5% trifluoroacetic acid (TFA) in dichloromethane [21, 22]. The product was purifed by HPLC (25 cm Nucleosil C-18 column (100-7, 250 × 21 mm) with a pre-column (100-7, 50 × 21 mm) from Macherey-Nagel; flow rate: 10 mL/min) to yield **1** after lyophilization with diluted hydrochloric acid as a red solid. HPLC analysis [column: symmetry 300 (250 × 4.6 mm) from Waters, flow rate: 1 mL/min, mobile phase A: 65% H_2O, 35% acetonitrile, 0.1% TFA; mobile phase B: 30% H_2O, 70% acetonitrile, 0.1% TFA, gradient: 0–15 min 100% mobile phase A; 15–35 min increase to 100% mobile phase B; 35–45 min 100% mobile phase B; 45–50 min decrease to 100% mobile phase A; 50–60 min 100% mobile phase A] 25.8 min, >97% of peak area, 495 nm. $C_{60}H_{68}N_6O_{18}$, ESI–MS: calcd $[M + H]^+$ 1161.46, found 1161.44.

Preparation of the albumin conjugate **HSA-1**

1 (1.78 mg) was dissolved in 5% glucose solution (500 µL, pH 3.5–5.0, Braun) and DMF (250 µL). **1** (750 µL, 2 mM) was subsequently incubated with human serum albumin (HSA as a 5% octalbumin® solution containing ~60% free thiol groups, 4.25 mL) under slight stirring at 37°C for 90 min after which no free **1** was detectable by HPLC. The albumin conjugate **HSA-1** was obtained after subsequent size-exclusion chromatography (Sephadex G25, 50 mM sodium acetate buffer, pH 5.0) followed by concentrating the solution with CENTRIPREP® YM-10-concentrators (Amicon, FRG) (4°C and 4,500 rpm) to 2.4 mL. The content of anthracycline in the sample was determined using the ε-value for doxorubicin [λ_{495} (pH 7.4) = 10,650 M^{-1} cm^{-1}] yielding a concentration of doxorubicin in the conjugate **HSA-1** of 330 µM. HPLC analysis [column: symmetry 300 (250 × 4.6 mm) from Waters, flow rate: 1 mL/min, mobile phase A: 85% H_2O, 15% acetonitrile, 0.1% TFA; mobile phase B: 20% H_2O, 80% acetonitrile, 0.1% TFA; gradient: 0–10 min increase to 20% mobile phase B; 10–35 min increase to 100% mobile phase B; 35–40 min decrease to 100% mobile phase A; 40–60 min 100% mobile phase A] 20.5 min, >98% of peak area, 495 nm. Samples were kept frozen at −80°C and thawed prior to use.

Cleavage studies of **HSA-1** with cathepsin B and tumor homogenates

Cleavage studies with cathepsin B

Four hundred microliters of the **HSA-1** stock solution (330 µM) were mixed with 10 µL cathepsin B (0.4 µg/mL, 23.8 U/mg, from Calbiochem, Bad Soden, FRG) and 40 µL of buffer (50 mM sodium acetate buffer, pH 5.0) containing L-cysteine (8 mM). The mixture was incubated at 37°C and aliquots (60 µL) were taken over 24 h and analyzed by HPLC at 495 nm using the method described above for **HSA-1**.

Table 1 IC_{50} values of doxorubicin and **HSA-1** in two carcinoma cell lines

Substance	IC_{50} value in MDA-MB-231 LN (µM ± SD)*	IC_{50} value in AsPC1 LN (µM ± SD)$^+$
Doxorubicin	0.18 ± 0.05	0.26 ± 0.09
HSA-1	1.16 ± 0.41	1.70 ± 0.35

* P value = 0.0023, $^+$ P value = 0.014

Cleavage studies with tumor homogenate

Two hundred and eighty microliters of the **HSA-1** stock solution (330 µM) were mixed with homogenates of MDA-MB 435 tumors (140 µL, 400 mg tumor/800 µL sodium acetate buffer, pH 5) and incubated at 37°C. Aliquots (60 µL) were taken over 28 h and analyzed by HPLC at 495 nm using the method described above for **HSA-1**. MDA-MB 435 cell lines were obtained from the National Cancer Institute (USA).

Binding to endogenous albumin in human plasma

Human blood plasma (EDTA-stabilized) was taken from healthy volunteers. **1** was added to plasma preincubated at 37°C at a final concentration of 340 µM, and the samples were incubated at 37°C. Aliquots (60 µL) were taken at various time points (1, 3, and 5 min) and analyzed by HPLC at 495 nm using the HPLC conditions described above for the analysis of **1**.

In vitro cellular experiments for the determination of IC_{50} values

In vitro cytotoxicity of the albumin-bound form of **1** (**HSA-1**) and doxorubicin was determined using a luciferase assay in the pancreatic tumor cell line AsPC1 LN and mamma carcinoma cell line MDA-MB-231 LN. AsPC1 LN and MDA-MB-231 LN cell lines were obtained from AsPC1 (ATCC no. CRL-1682, LGC Promochem, FRG) and MDA-MB 231 (ATCC no. HTB26, LGC Promochem, FRG) and tranduced with a retrovirus encoding a luciferase-aminoglycoside phosphotransferase (neomycin resistance) fusion gene (LN), via a VSV-G (BD Clontech) pseudotyped retrovirus according to the instructions from the manufacturer. Selection of the cells, and assays for luciferase activity were carried out as described previously [23]. For IC_{50} measurements, 0.2×10^4 cells were plated per well in a 96-well plate. After 24 h, the drugs were added as triplicates at different concentrations (1 nM–10 µM), and the cells were

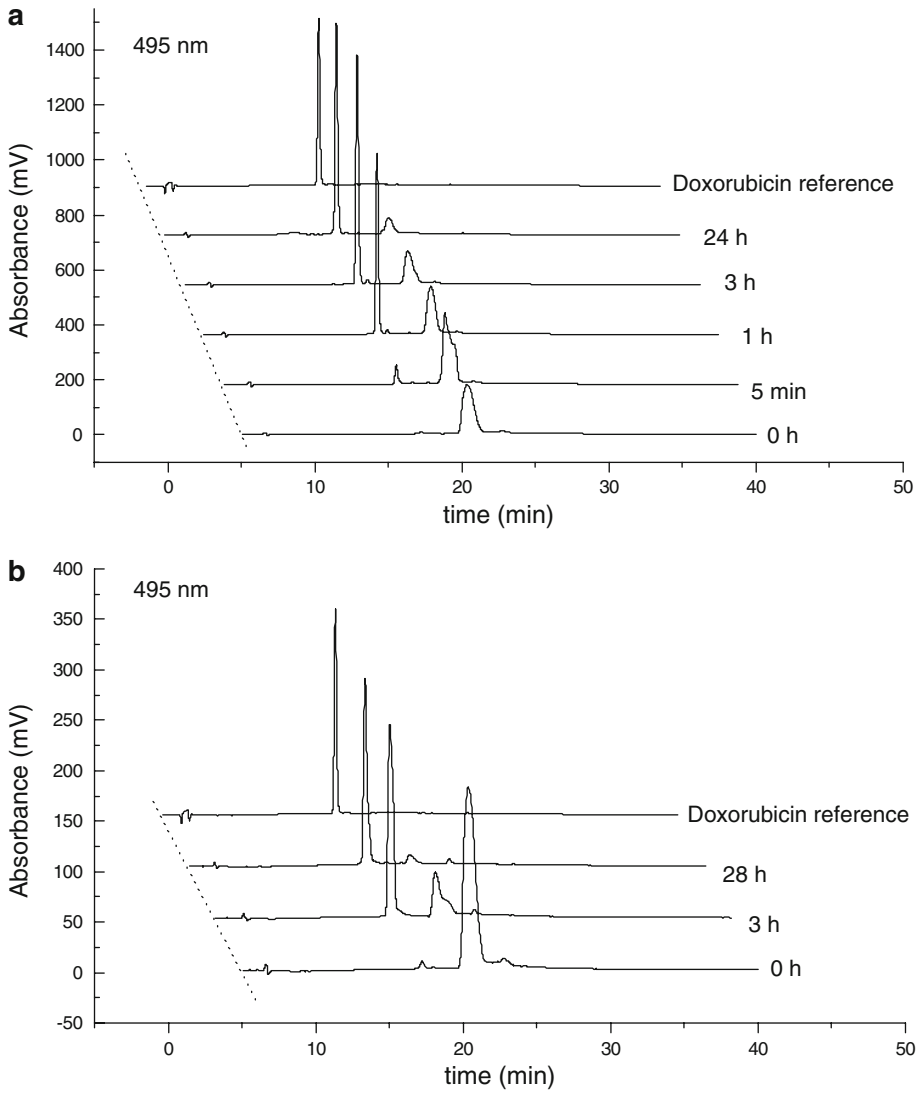


Fig. 2 Chromatograms of incubation studies of **HSA-1** (330 μM) with activated cathepsin B (10 μL; 0.095 U) (**a**); with homogenates of MDA-MB 435 tumors (**b**); Studies were performed at pH 5.0, 37°C and monitored at 495 nm. Doxorubicin is shown as a reference. While the albumin conjugate **HSA-1** elutes at 20.5 min, the cleavage product eluting at 16.7 min was identified by HPLC to be the free anticancer agent doxorubicin

incubated for another 72 h. Cells were lysed in 100 μL of luciferase assay buffer (25 mM TRIS-phosphate pH 7.8; 2 mM EDTA; 2 mM DTT; 0.1% Triton X-100) and 10 μL was assayed for luciferase activity (Promega E4550). IC_{50} values were determined using GraphPad prism software. Statistical analyses were done using Student's *t* test.

In vivo activity of **1**

For the in vivo testing of **1** in comparison with doxorubicin in the xenograft model MDA-MB 435, female NMRI: nu/nu mice (inhouse breeding) were used. The mice were held in individually ventilated cages under sterile and standardized environmental conditions (25 ± 2°C room temperature, 50 ± 10% relative humidity, 12 h light–dark rhythm). They received autoclaved food and bedding (ssniff, Soest, Germany) and acidified (pH 4.0) drinking water ad libitum. All animal experiments were performed under the auspices of the German Animal Protection Law and with approval from the local responsible authorities.

MDA-MB 435 tumor cells (10^7 cells) from in vitro culture were transplanted subcutaneously (s.c.) into the left flank region of mice on day 0. Mice were randomly distributed to the experimental groups (6 mice per group). When the tumors were grown to a palpable size (90–130 mm^2), treatment was initiated. The mice were treated intravenously with either 5% D-glucose buffer pH 5.8, doxorubicin (2 × 8 mg/kg, on days 10 and 17) or **1** (3 × 24 mg/kg doxorubicin equivalent, administered as a solution in 5% D-glucose buffer pH 5.8, on days 10, 17, and 24). The volume of administration was 0.2 mL/20 g body weight.

Tumor size was measured twice weekly with a caliper-like instrument in two dimensions. Individual tumor volumes *(V)* were calculated by the formula $V = (\text{length} \times [\text{width}]^2)/2$ and related to the values on the first day of treatment (relative tumor volume, RTV). At each measurement

day, treated/control values *(T/C)* were calculated as percentage for each experimental group; the optimum (lowest) values obtained within 4 weeks after treatment were used for evaluating the efficacy of the compounds, and optimum *T/C*-values are presented in Fig. 3. Statistical analysis was performed with the *U* test (Mann and Whitney) with a significance level of $P < 0.05$. The body weight of mice was determined every 3–4 days.

Results and discussion

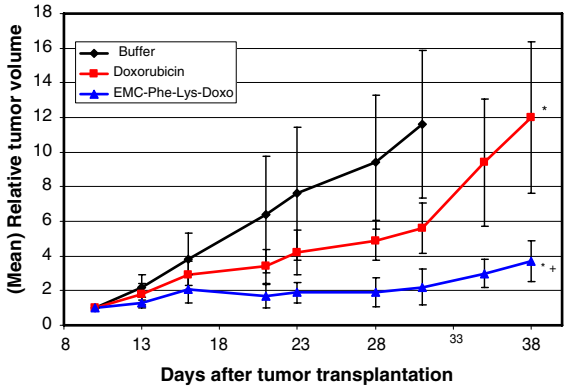
The maleimide-bearing prodrug **1** with the anticancer agent doxorubicin incorporates a 1,6-self-immolative spacer coupled to the dipeptide Phe-Lys that acts as a substrate for cathepsin B. It was synthesized in a modified procedure of the work of Dubowchik et al. [18] by replacing the methoxytrityl group in the original work with the Mtt group for the protection of the side chain amino group of lysine. Mtt was finally cleaved with 0.5% TFA in dichloromethane to furnish the prodrug **1** (see "Materials and methods").

Following the synthesis of the prodrug, we wanted to ascertain whether cathepsin B can cleave the prodrug once bound to albumin. In analogy to our previous work on the preparation of drug albumin conjugates [7], **1** was quantitatively and selectively bound through its maleimide moiety to the cysteine-34 group of HSA, and the albumin-bound prodrug **HSA-1** could be conveniently purified with size-exclusion chromatography (see "Materials and methods").

In addition, incubation of **1** with human plasma at 37°C demonstrated quantitative binding to endogenous albumin within 5 min (data not shown) in analogy to our previous experience with albumin-binding prodrugs [6, 8, 13, 15].

The cleavage properties of **HSA-1** were evaluated using enzymatically active cathepsin B and in tumor homogenates at pH 5.0. Reverse phase HPLC showed that incubation with cathepsin B (Fig. 2a) as well as with homogenates of MDA-MB 435 tumors (Fig. 2b) resulted in an efficient and almost complete cleavage over a period of 24 and 28 h, respectively. The cleavage product was identified by HPLC to be the free anticancer agent doxorubicin.

Our second aim was to assess the in vitro and in vivo antitumor efficacy of **1** in a comparison with free doxorubicin. Cytotoxicity with **HSA-1** was performed with two human tumor cell lines: MDA-MB-231 LN (breast carcinoma) and AsPC1 LN (pancreatic carcinoma) both transfected with the luciferase gene. IC_{50} values of **HSA-1** and doxorubicin are shown in Table 1 (dose-response curves are shown in Supplementary Fig. 1 in the supporting information). The albumin-conjugate showed cytotoxic activity against the cell lines in the low molecular range. However, the cell lines were found to be ~6 times less sensitive towards **HSA-1** than doxorubicin. In contrast, prodrug **1**



Substance	Treatment [on day]	Dose [mg/kg/inj.]	Toxic deaths [on day]	BWC (%) [on day]	optimum T/C [on day]
Glucose-phosphate-buffer			0	+7 [31]	
Doxorubicin	10, 17	8	0	-1 [38]	49 [31]*
1	10,17,24	24	1 (24)	-10 38]	15 [31]*+

* Significant to buffer (doxorubicin to buffer: *P* value = 0.025; **1** to buffer: *P* value = 0.006), + Significant to doxorubicin (*P* value = 0.004).

Fig. 3 Therapeutic efficacy of doxorubicin and **1** against the xenograft mode MDA-MB 435 (6 mice per group)

was significantly more active in vivo over doxorubicin in an equitoxic comparison: the in vivo antitumor efficacy of **1** and doxorubicin was evaluated in a nude mice model (MDA-MB 435). The standard and maximum tolerated dose (MTD) of doxorubicin (2 × 8 mg/kg) was compared to **1** at 3 × 24 mg/kg doxorubicin equivalents which is a good estimate of the MTD of albumin-binding prodrugs of doxorubicin in nude mice models [7, 8, 14, 15, 21]. Doxorubicin (2 × 8 mg/kg) is the MTD in nude mice models and higher doses led to unacceptable toxicity and mortality [24, 25].

Whereas doxorubicin was moderately active (*T/C* 49%) at its optimal dose of 2 × 8 mg/kg, **1** exhibited superior antitumor activity (*T/C* 15%) at the dose of 3 × 24 mg/kg doxorubicin equivalents, and mice treated with **1** showed a clear antitumor response (Fig. 3). At the end of the experiment (day 38), tumor size in the doxorubicin treated group had increased by a factor of ~11, whereas in the group treated with **1** tumor size had only increased three-fold. One death occurred in the group treated with **1** and body weight loss was higher (−10%) than for the doxorubicin treated group (−1%) (see Fig. 3 and Supplementary Fig. 2 in the supporting information) although this difference could also be attributed to the more pronounced increase in tumor volume during the course of the experiment.

Besides doxorubicin, prodrugs with the Phe-Lys-PABC spacer have been realized with camptothecin [26, 27] and paclitaxel [28] for the development of drug immunoconjugates.

Additionally, we have recently started a research program aimed at the development of dual-acting prodrugs

incorporating two active substances independently bound through a 1,6-self-immolative Phe-Lys-PABC spacer to a branched maleimide-containing linker [21, 22].

Conclusion

Employed as an albumin-binding prodrug, integrating a self-immolative Phe-Lys-PABC spacer between the anticancer drug doxorubicin and the albumin-binding maleimide group efficiently increased the therapeutic index of the drug. Above all, an in vivo study showed that the prodrug exhibited significantly superior antitumor activity compared to doxorubicin.

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