Bioorganic & Medicinal Chemistry Letters 19 (2009) 995–1000

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Development of dual-acting prodrugs for circumventing multidrug resistance

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ARTICLE INFO

Article history:
Received 9 October 2008
Revised 17 November 2008
Accepted 18 November 2008
Available online 24 November 2008

Keywords:
Multidrug resistance
Albumin-binding prodrug
Doxorubicin

ABSTRACT

We have developed a novel dual-acting maleimide-bearing prodrug that incorporates the anticancer agent doxorubicin and an inhibitor of the P-glycoprotein efflux pump that is over-expressed in multidrug resistant tumor cells. Additionally, the prodrug contains a 1,6-self-immolative spacer coupled to the dipeptide Phe-Lys that acts as a substrate for cathepsin B. The prodrug, once bound through its maleimide moiety to the cysteine-34 group of human serum albumin, was cleaved by cathepsin B and in tumor homogenates demonstrating a release of the anticancer agent doxorubicin and the inhibitor.

Intrinsic or acquired multidrug resistance (MDR) is a major problem in the treatment of malignant diseases. A number of biochemical mechanisms have been described that are responsible for the multidrug resistance phenotype that include changes in the cellular target of the respective drug, alterations in enzymatic activation and detoxification mechanisms, defective apoptotic pathways, membrane changes as well as elimination of the drug from the tumor cell through the action of drug efflux pumps.[1] A frequent cause of drug resistance results from an elevated expression of the cell-membrane transporters that function by increasing the efflux of cytotoxic drugs from cancer cells and allows them to survive by lowering the intracellular concentrations of the drugs. The cell-membrane efflux pumps include P-glycoprotein (P-gp), multiple resistance protein (MRP) and breast cancer resistance protein (BCRP) which belong to the ATP-binding cassette (ABC) transporter family.[2–4]

The best-studied efflux pump is the P-glycoprotein (ABCB1), energy-dependent, transmembrane efflux pump, which is a 170-kDa cell-membrane protein encoded by the MDR1 gene. Its biochemistry and pharmacology has been intensely studied.[5,6] The cytotoxic drugs that are most frequently associated with MDR and which are substrates for the P-glycoprotein are hydrophobic as well as amphiphilic compounds including taxanes, vinca alkaloids, anthracyclines, epipodophyllotoxins, actinomycin D, and mitomycin C.[7,8]

A great number of studies have evaluated the expression of MDR1 mRNA or P-gp demonstrating an over-expression in leukemia and solid tumors.[9] For example, data from studies comprising breast cancer patients treated with cytotoxic agents showed a significant increase in the proportion of patients with tumors expressing P-gp subsequent to therapy.[9] In addition, patients with P-gp expressing tumors were threefold less likely to achieve an objective response after treatment compared with patients whose tumors were MDR1/P-gp negative.[10] The extent of P-gp expression correlated with in vitro resistance to doxorubicin and paclitaxel in functional assays.[11]

Consequently, there has been a concerted effort over the past 20 years to develop specific P-gp modulators that circumvent MDR.[12] MDR modulators of the first generation in combination with standard chemotherapy regimens demonstrated initial success in clinical trials against retinoblastoma[13] and acute myeloid leukemia.[14] However, due to the lack of specificity and selectivity of 1.generation MDR modulators high doses were required resulting in adverse effects, for example, cardiotoxicity and immunosuppression.[5,6,15] Furthermore, the MDR modulators were substrates for metabolic enzyme systems, for example, the P450 cytochrome system (P450 3A4), resulting in unpredictable modification of the pharmacokinetic profile of the co-administered anticancer agents with a concomitant dose reduction of the latter.[2]

MDR modulators of the second generation were analogues of the 1.generation modulators.[16] Although these drugs were better tolerated, they still altered the pharmacokinetic profile of the co-administered anticancer drugs and required a dose reduction of 60% or more.[17–21]

3.generation MDR modulators demonstrated high selectivity for P-gp with inhibitory activities in the low nanomolar range. Several clinical studies have been performed and demonstrated that the pharmacokinetic profile of co-administered drugs was not altered.[2,22–25] Although some objective responses were achieved, combination therapy with these MDR modulators caused unacceptable toxicity, for example, severe neurotoxicity.[26]

In summary, although other resistance mechanisms play a role in solid tumors due to the heterogeneity and genomic instability of

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doi:10.1016/j.bmcl.2008.11.063

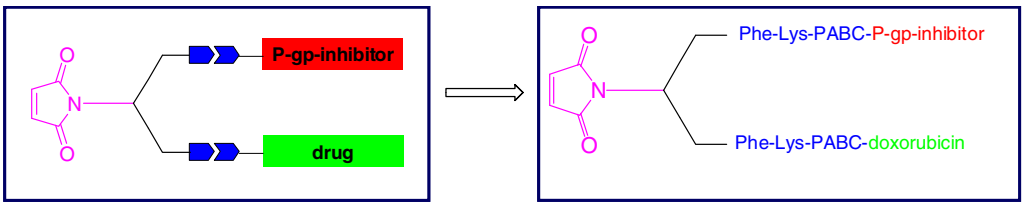


Figure 1. General design of albumin-binding dual-acting prodrugs.

the tumor cell population, it is feasible that P-gp is responsible for multidrug resistance in a defined subpopulation of cancer patients and thus represents a molecular target for circumventing MDR. Therapeutic strategies that combine a MDR modulator with anticancer agents have the following inherent disadvantages[27]: (a) MDR modulators often alter the pharmacokinetic profile of the co-administered anticancer drugs resulting in a considerable dose reduction, (b) systemic toxicity of the MDR modulators, (c) sufficient concentrations of the MDR modulator and the anticancer drug in resistant tumor cell are not achieved simultaneously since the therapy schedule for two pharmacokinetically unrelated drugs remains unpredictable and needs to be assessed empirically, (d) a lack of tumor targeting for the MDR modulators as well as anticancer agents.

The goal of this work was to develop dual-acting prodrugs that avoid these disadvantages and prove more efficacious in the potential treatment of cancer patients with intrinsic or acquired resistance caused by an over-expression of the P-glycoprotein. Such prodrugs consist of an anticancer agent, a P-glycoprotein inhibitor and a thiol-binding moiety that allows coupling to a suitable carrier (Fig. 1). Dual-acting prodrugs for circumventing P-gp mediated MDR have not been described to date and could have the advantage that an anticancer agent and an inhibitor of P-gp are transported simultaneously on a molecular level to resistant cancer cells that express P-gp, thus preventing the efflux of the anticancer drug with a concomitant increase in tumor cell death.

For the design of the prodrug (see Fig. 1), we selected to introduce the dipeptide Phe-Lys that is cleaved by cathepsin B, a cysteine protease that is over-expressed in solid tumors. Recently, we described the synthesis of maleimide bifunctional linkers that can be applied in the synthesis of dual-acting prodrugs with enzymatically cleavable linkers.[28] The linkers contain two arms with the 1,6-self-immolative *para*-aminobenzyloxycarbonyl (PABC) spacer and the dipeptide Phe-Lys. In addition, they contain a maleimide group for conjugation with a suitable polymer or protein carrier. In our previous work, we have investigated a passive targeting approach in which maleimide-bearing prodrugs bind selectively to the cysteine-34 position of circulating albumin after intravenous administration. This macromolecular prodrug concept is based on two features: (i) rapid and selective binding of a maleimide functionalized prodrug to the cysteine-34 position of endogenous albumin after intravenous administration, and (ii) release of the albumin-bound drugs predominantly at the tumor site due to the incorporation of an acid-sensitive or an enzymatically cleavable bond between the drug and the carrier.[29–31]

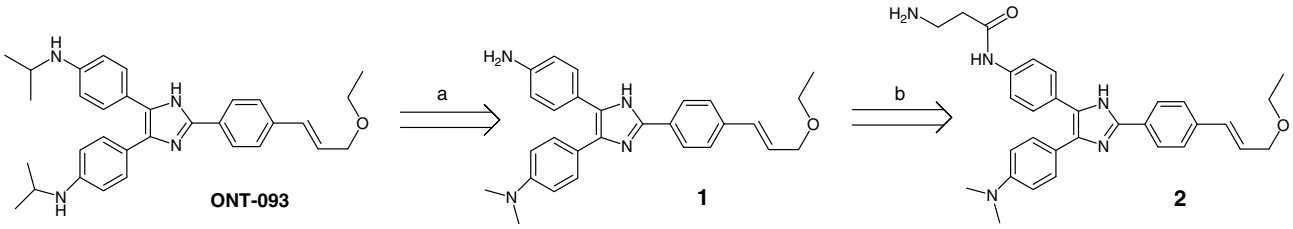
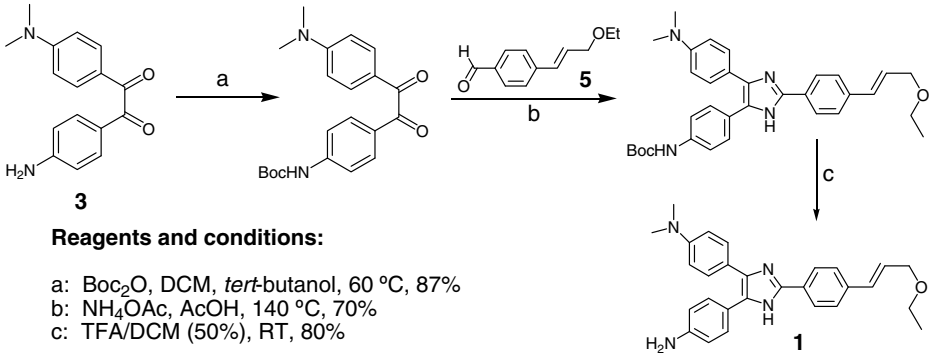


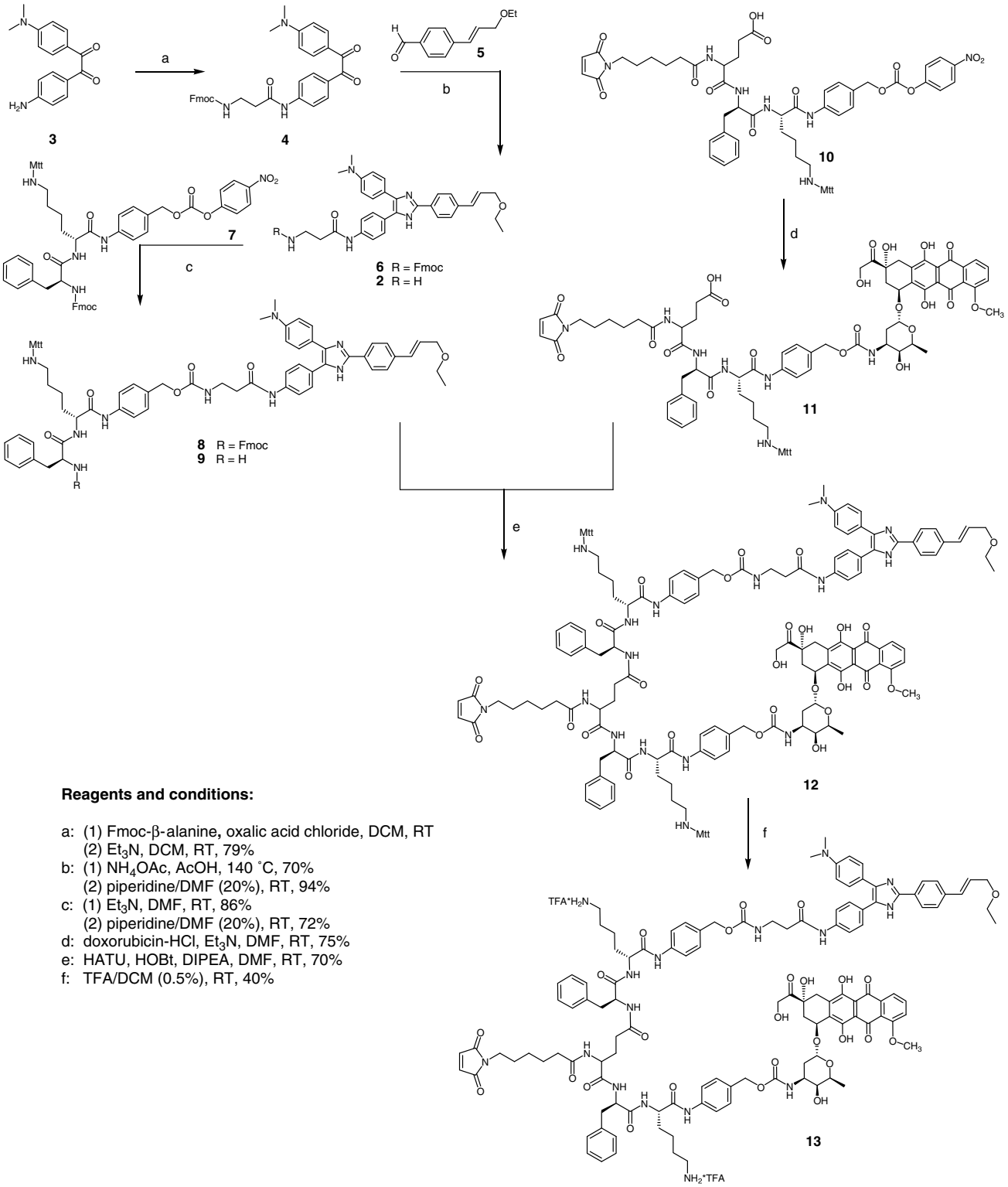
Figure 2. Structure of ONT-093 and its chemical modifications.



Scheme 1. Synthesis of ONT-093 derivative 1.

Albumin demonstrates preferential tumor uptake in various tumor animal models[32] due to the enhanced permeability and retention of macromolecules for solid tumors.[33] In situ binding of maleimide-based prodrugs to the cysteine-34 position of circulating albumin is meanwhile a preclinically and clinically validated technology of increasing the therapeutic index of anticancer agents. Besides an acid-sensitive prodrug of doxorubicin, the (6-maleimidocaproyl)hydrazone derivative of doxorubicin [INNO-206, previously DOXO-EMCH] that has been studied clinically[34], a series of albumin-binding prodrugs with enzymatically cleavable linkers have been developed in the past 5 years.[29,33–39]

In our first example of the synthesis of such dual-acting prodrugs, we focused on its potential use for the treatment of resistant breast cancer. An anticancer agent of choice for such an



Reagents and conditions:

a: (1) Fmoc-β-alanine, oxalic acid chloride, DCM, RT
(2) Et_3N, DCM, RT, 79%
b: (1) NH_4OAc, AcOH, 140 °C, 70%
(2) piperidine/DMF (20%), RT, 94%
c: (1) Et_3N, DMF, RT, 86%
(2) piperidine/DMF (20%), RT, 72%
d: doxorubicin-HCl, Et_3N, DMF, RT, 75%
e: HATU, HOBt, DIPEA, DMF, RT, 70%
f: TFA/DCM (0.5%), RT, 40%

Scheme 2. Synthesis of the prodrug **13**.

approach is doxorubicin since an anthracycline-based regimen is routinely used to treat patients with breast cancer. In addition, its chemical structure allows the design of prodrugs with enzymatically cleavable bonds through its amino group.[40] Furthermore, we selected the third generation MDR modulator, ONT-093 (Fig. 2) as an inhibitor of P-gp because its structure–activity relationships have been intensely studied and it possesses suitable functional groups for chemical modification.[41] ONT-093 reversed resistance to doxorubicin and paclitaxel in six different cell lines expressing P-gp in the low nanomolar range with an average EC_{50} value of 32 nM. Although ONT-093 shows high specificity for P-gp, it is not a P-gp transport substrate.[42]

The synthesis of ONT-093 derivatives is based on the formation of an imidazole ring. 2,4,5-Trisubstituted imidazoles can be simply obtained in high yield from 1,2-diketone and aldehyde in the presence of ammonium acetate, either under microwave irradiation[43] or by heating at high temperatures.[41,44] Since the modulator ONT-093 has no suitable functional groups for direct derivatization, a structural modification is required. Zhang et al. could show that the replacement of the isopropyl amino groups in ONT-093 with dimethyl amino groups or other substituents has only a very weak effect on the activity of the substance.[41] Aided with theses results, we first modified this inhibitor and replaced one isopropyl amino group with a primary amino group, and replaced the other isopropyl amino group with a tertiary amine to prevent any competition reaction thus obtaining **1** (Fig. 2). According to the literature[41], the imidazole ring in **1** (Fig. 2) should be obtained from the coupling reaction (Scheme 1) of **3** and **5** in the presence of ammonium acetate in acetic acid at 140 °C. Unfortunately, the reaction gave in our hands only by-products that may be attributed to the side reactions of the free amino group in reactant **3**. We could overcome this problem and obtained the desired product **1** by protecting the free aromatic amino group in **3** followed by coupling of this Boc-protected 1,2-diketone with the aldehyde **5** in the presence of ammonium acetate and acetic acid by heating at 140 °C and subsequent treatment with trifluoroacetic acid (TFA).[45]

A non-expected very low reactivity of the aromatic amino group in **1** and **3** was observed, when reacting these with amino acids which did not form an amide bond in the presence of various standard amide-coupling reagents or under reaction conditions that were applied for aromatic amines.[46] The chemical behavior of the aromatic amino group led us to investigate the introduction of an aliphatic amino group. Thus, the dione **3** was reacted with Fmoc-ß-alanine chloride[47] to give **4** (Scheme 2). The desired imidazole

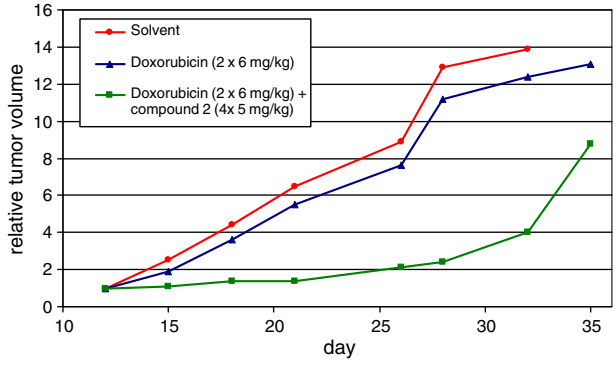


Figure 3. Curves depicting tumor growth inhibition of subcutaneously growing MT-3/ADR-xenografts under therapy with doxorubicin and a combination of compound **2** and doxorubicin. Female NMRI-nu mice (three/group) were injected with 10^7 MT-3/ADR cells on day 0. Compound **2** (5 mg/kg) was administered i.p. as a solution in PEG 400/D-glucose buffer pH 5 (7:3) on days 12, 13, 19, and 20. The treatment with doxorubicin (6 mg/kg, i.v.) was performed on days 13 and 20 without and after the administration of compound **2**.

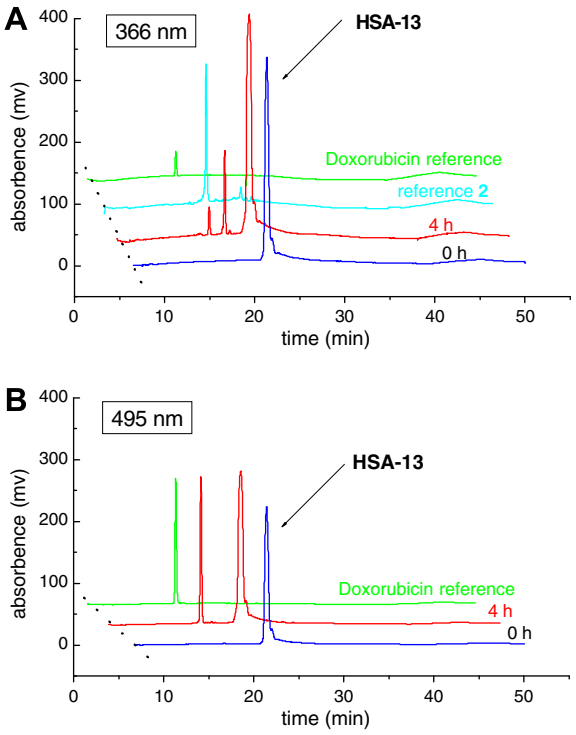


Figure 4. (A and B) Chromatograms of incubation studies of **HSA-13** (385 μM) with activated cathepsin B (10 μL; 0.095 U) at 37 °C and monitored at 366 nm (A) and 495 nm (B). Doxorubicin and **2** are shown as references.

product **6** was obtained from the reaction of 1,2-dione **4** and aldehyde **5** in the presence of ammonium acetate and acetic acid at 140 °C. Standard Fmoc-cleavage using piperidine in DMF (20%) furnished **2** as the derivative of the P-gp-inhibitor ONT-093.[48]

In an orientating in vivo study, we first wanted to assess the efficacy of the ONT-093 derivative **2** in combination with doxorubicin in the MT-3/ADR Adriablastin resistant mamma carcinoma xenograft model that is based on a cell line that expresses P-glycoprotein.[49] Female nude mice with subcutaneously growing MT-3/ADR-xenografts were randomly distributed to the experimental groups (three/group) and injected with (a) saline (on days 13 and 20), (b) doxorubicin (6 mg/kg, i.v, on days 13 and 20), or (c) compound **2** (5 mg/kg, administered i.p. as a solution in 70% PEG 400/30% D-glucose buffer pH 5, on days 12, 13, 19, and 20) and doxorubicin (6 mg/kg, i.v, on days 13 and 20 after the administration of **2**).

Whereas doxorubicin (2 × 6 mg/kg) was inactive, a combination of doxorubicin (2 × 6 mg/kg) and **2** (4 × 5 mg/kg) exhibited superior antitumor activity, and mice treated with this combination showed a clear antitumor response (Fig. 3). However, the combination was accompanied with considerable body weight loss (−23%). These preliminary in vivo results support our development of a more active and less toxic dual-acting prodrug that contains both doxorubicin and **2** for circumventing MDR.

For developing such a prodrug we used the recently developed building block **7** and the bifunctional maleimide spacer **10** that allow the derivatization with two different pharmaceutically active compounds independently bound by enzymatically cleavable linkers[26]—see Scheme 2. The introduction of the anticancer agent doxorubicin in one arm of the linker **10** was performed through the reaction of its amino group with the carbamate group of **10** to give **11**.[50] The building unit for the P-gp inhibitor **9** was obtained from the reaction between **2** and the activated carbonate **7** with subsequent cleavage of the Fmoc group with diethylamine.[51] **9** was then introduced in the prodrug by reacting its free amino

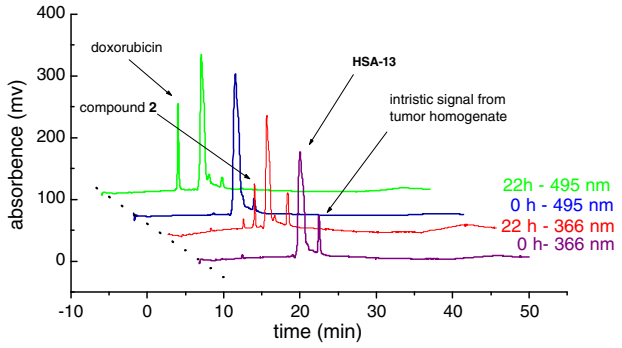


Figure 5. Chromatograms of incubation studies of **HSA-13** (385 μM) with a homogenate from doxorubicin resistant MT-3/ADR tumors at 366 and 495 nm.

group with the carboxylic acid group in **11** in the presence of *O*-(azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxybenzotriazole (HOBt) to give **12**. Finally, cleavage of both Mtt protecting groups in **12** with 0.5% TFA in DCM furnished the prodrug **13**. Purification by HPLC and subsequent lyophilization yielded a red powder.[52] The prodrug **13** was characterized by analytical HPLC and mass spectra. It showed good water solubility of ~6.5 mg/mL in glucose buffer (5% D-(+)-glucose, pH 5.8) when diluted with 10% PEG 400.

In a next step, we wanted to assess the albumin-binding properties of the prodrug **13** bound to human serum albumin (HSA). In analogy to our previous work on the preparation of drug albumin conjugates[27,28], the albumin conjugate of **13** was prepared by reacting the maleimide moiety of **13** to the cysteine-34 group of HSA and purifying the albumin-bound prodrug **HSA-13** with size-exclusion chromatography.[53] The cleavage properties of **HSA-13** were evaluated using enzymatically active cathepsin B at pH 5.0 and in tumor homogenates.[53] Reverse phase HPLC showed that the incubation with cathepsin B (Fig. 4) as well as with homogenates of doxorubicin resistant MT-3/ADR tumors (Fig. 5) resulted in an efficient cleavage over a period of 22 h (λ = 366 nm for the inhibitor **2** and λ = 495 nm for doxorubicin). The cleavage products were identified by HPLC to be the free anticancer agent doxorubicin (eluting at 16.7 min) and the inhibitor **2** (eluting at 18.5 min).

In conclusion, we have demonstrated for the first time the synthesis of a dual-acting prodrug which consists of an anticancer agent and an inhibitor of P-gp independently bound by enzymatically cleavable linkers to a targeting protein-binding moiety. The prodrug in its albumin-bound form was cleaved specifically by cathepsin B and in tumor homogenates releasing the free drugs. These results form a good basis for evaluating the activity of the new prodrug in a P-gp expressing animal model. Preliminary toxicity studies with **13** in nude mice have shown that a dose of 24 mg/kg doxorubicin equivalents can be safely administered which is at least a threefold increase over the standard dose of doxorubicin (6–8 mg/kg) that is used in nude mice models.[54]

Acknowledgment

The support of the Fördergesellschaft der Klinik für Tumorbiologie is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.063. ESI-Mass spectrum of prodrug **13**. This material is available free of charge via the Internet.

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45. Synthesis of ONT-093 derivative **1**: Boc_2O (1 mL) was added under nitrogen atmosphere to a solution of **4** (0.19 mmol, 50 mg) in dry DCM (2 mL) and *tert*-butanol (2 mL), and the mixture was heated at 60 °C for 20 h (TLC: ethyl acetate/hexane, 1:1). The solvents were removed under reduced pressure. The raw product was precipitated with hexane (50 mL) as a red solid (60 mg, 87%). $C_{21}H_{24}N_2O_4$, ESI-MS: calcd $[M+H]^+$ 369.17, found 369.30. The raw product (1.0 equiv, 0.14 mmol, 50 mg) and aldehyde **5** (1.5 equiv, 0.21 mmol, 40 mg) were placed in acetic acid (4 mL). Ammonium acetate (10 equiv, 1.4 mmol, 108 mg) was taken up in acetic acid (2 mL) in a separate flask. Both flasks were heated at 140 °C. As soon as the solids dissolved, the ammonium acetate solution was added to the other mixture. The resulting solution was heated at 140 °C for 6 h (TLC, ethyl acetate/hexane, 1:1). The solvent was removed under reduced pressure. The residue was purified with FC eluting with ethyl acetate/hexane, 1:1 yielding a yellow solid (52 mg, 70%). $C_{33}H_{38}N_4O_3$, ESI-MS: calcd $[M+H]^+$ 539.29, found 539.10. The product (0.09 mmol, 50 mg) was then dissolved in a mixture of DCM and TFA (1:1; 4 mL) and stirred at room temperature for 2 h (TLC: $CHCl_3$/MeOH, 10:1). A red solid precipitated upon adding the solution to diethyl ether (50 mL). **1** was collected as a TFA-salt by suction filtration, washed with ether and dried to give 33 mg (80%). $C_{28}H_{30}N_4O$, ESI-MS: calcd $[M+H]^+$ 439.24, found 439.20.
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48. Synthesis of ONT-093 derivative **2**: Fmoc-β-alanine chloride was prepared from Fmoc-β-alanine (1 equiv, 27.3 mmol, 4.4 g) and oxalic acid chloride (0.8 equiv, 21.8 mmol, 1.85 mL) in dry DCM (50 mL). The solution of Fmoc-β-alanine chloride (21.8 mmol) was added slowly to a mixture of **3** (1 equiv, 16.4 mmol, 4.4 g) and anhydrous triethylamine (1.25 equiv, 20.5 mmol, 2.8 mL) in dry DCM (80 mL). The solution was stirred at rt for 1 h. Excess amount of the acid chloride was quenched with MeOH (10 mL). The residue was purified by FC eluting with $CHCl_3$/MeOH, 30:1 to give **4** as a yellow solid (7.3 g, 79%). $C_{34}H_{31}N_3O_5$, ESI-MS: calcd $[M+H]^+$ 562.23, found 562.0. 1,2-Diketone **4** (1.0 equiv, 9.8 mmol, 5.5 g) and aldehyde **5** (1.5 equiv, 14.7 mmol, 2.75 g) were placed in acetic acid (30 mL). Ammonium acetate (10 equiv, 98 mmol, 7.5 g) was taken up in acetic acid (7.5 mL) in a separate flask. Both flasks were heated at 140 °C. As soon as the solids dissolved, the ammonium acetate solution was added to the mixture of **4** and **5**. The resulting solution was heated at 140 °C for 3 h. Removal of the solvent under reduced pressure and purification of the residue with FC eluting with $CHCl_3$/MeOH, 20:1 gave **6** as a yellow solid (5.7 g, 80%). $C_{46}H_{45}N_5O_4$, ESI-MS: calcd $[M+H]^+$ 732.35, found 732.30. A solution of **6** (3 g) in piperidine/DMF (20%, 10 mL) was stirred at rt for 15 min. Product **2** was precipitated with ether (500 mL) as a yellow solid (1.9 g, 92%). $C_{31}H_{35}N_5O_2$, ESI-MS: calcd $[M+H]^+$ 510.28, found 510.30.
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50. Synthesis of EMC-Glu-Phe-Lys(Mtt)-PABC-doxorubicin **11**: Triethylamine (1 equiv, 1.31 mmol, 182 μL) was added to a solution of **10** (1 equiv, 1.31 mmol, 1.50 g) and doxorubicin-HCl (1 equiv, 1.31 mmol, 0.761 g) in dry DMF (20 mL), and the solution was stirred at room temperature for 18 h (TLC: $CHCl_3$/MeOH 7:1). The product was precipitated with ether (1 L) and it was purified by FC eluting with $CHCl_3$/MeOH 15:1 to give **11** as a red solid (1.52 g, 75%). $C_{85}H_{91}N_7O_{21}$; ESI-MS: calcd $[M+H]^+$1546.63, found 1546.00.
51. Synthesis of Phe-Lys(Mtt)-PABC-inhibitor **9**: A mixture of **2** (1 equiv, 2.56 mmol, 1.3 g), **7** (1.03 equiv, 2.63 mmol, 2.75 g) and anhydrous triethylamine (2 equiv, 5.12 mmol, 718 μL) in dry DMF (35 mL) was stirred at rt for 16 h. The product **8** was precipitated with ether yielding a yellow solid (3.1 g, 86%). **8** (3 g) was then dissolved in 20% piperidine/DMF (30 mL), and the solution was stirred at rt for 10 min. Product **9** was finally precipitated with ether (500 mL) as a yellow solid (1.8 g, 72%). $C_{74}H_{79}N_9O_6$, ESI-MS: calcd $[M+H]^+$1190.52, found 1190.30.
52. Synthesis of the prodrug **13**: Compound **11** (1 equiv, 0.84 mmol, 1.30 g), HATU (1.1 equiv, 0.93 mmol, 035 g) HOBt (1 equiv, 0.84 mmol, 0.10 g), DIPEA (2 equiv, 1.68 mmol, 260 μL), and **9** (1 equiv, 0.84 mmol, 1.00 g) were dissolved in dry DMF (10 mL), and the solution was stirred at rt for 3 h. The product was precipitated with ether (1 L) and was then purified by FC eluting with $CHCl_3$/MeOH, 15:1 to give **12** as an orange solid (1.60 g, 70%). $C_{159}H_{168}N_{16}O_{26}$, ESI-MS: calcd $[M+H]^+$ 2718.23, found 2718.24, and calcd $[M+Na]^+$ 2740.23, found 2740.26; HPLC analysis: (Nucleosil 100-5-C-18 (Macherey-Nagel); flow rate: 1 mL/min; mobile phase A: 70% H_2O, 30% acetonitrile, 0.1% TFA; mobile phase B: 20% H_2O, 80% acetonitrile, 0.1% TFA; λ = 366 nm; retention time 39.12 min) > 94% of peak area. **12** (300 mg) was dissolved in DCM (6 mL) and then treated with a solution of 1% TFA in DCM (6 mL), and stirred at rt for 24 h after which no **12** was detectable by HPLC. The product was then precipitated with DCM (120 mL), washed with ether (100 mL), centrifuged and then purified 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; mobile phase A: 70% H_2O, 30% acetonitrile, 0.1% TFA; mobile phase B: 20% H_2O, 80% acetonitrile, 0.1% TFA) to yield after lyophilization **13** as a red solid (97 mg, 40%). HPLC analysis (25 cm Nucleosil C-18 column (100-5, 250 × 4 mm) from Macherey–Nagel); flow rate: 1 mL/min; λ = 495 and 366 nm; retention time: 29.39 min) >99% of peak area. $C_{119}H_{136}N_{16}O_{26}$, ESI-MS: calcd $[M+H]^{2+}$ 1103.50, found 1103.51, and calcd $[M+3H]^{3+}$ 736.00, found 736.00. (see Supporting information of mass spectrum of **13**).
53. Cleavage studies of **HSA-13** with cathepsin B and with tumor homogenate*Preparation of the albumin conjugate **HSA-13***: **13** (1.6 mg) was dissolved in PEG 400 (Aldrich, 25 μL) and was then diluted with 5% Glucose solution (225 μL, pH 3.5-5, Braun). **13** (600 μL, 2600 μM) was subsequently incubated with HSA (5% octalbin, 5.4 mL) and ethanol (Lichrosolv, 3 mL) under slight stirring at 37 °C for 1 h. The albumin conjugate **HSA-13** was obtained after subsequent size-exclusion chromatography (Sephacryl S-100, 50 mM sodium acetate buffer, pH 5.0) followed by concentrating the solution with CENTRIPREP® YM-10-concentrators (Amicon, FRG) (4 °C and 4500 rpm) to 2.75 mL. The content of anthracycline in the sample was determined using the ε-value for doxorubicin [495 (pH 7.4) = 10650 $M^{-1}\,cm^{-1}$] yielding a concentration of doxorubicin in the conjugate **HSA-13** of 385 μM. Samples were kept frozen at −80 °C and thawed prior to use.*Cleavage studies with cathepsin B:* 500 μL of the **HSA-13** stock solution (385 μM) were mixed with 10 μL cathepsin B (from Calbiochem (Bad Soden, FRG), 0.4 μg/mL, 23.8 U/mg) and 50 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 removed at various time points and analyzed by HPLC.*Cleavage studies with tumor homogenate:* 340 μL of the **HSA-13** stock solution (385 μM) were mixed with homogenates of doxorubicin resistant MT-3/ADR tumors (170 μL, 400 mg tumor/800 μL sodium acetate buffer, pH 5) and incubated at 37 °C. Aliquots (60 μL) were removed at various time points and analyzed by HPLC. HPLC conditions were flow rate: 1 mL/min, λ = 495 nm and 366 nm; mobile phase A: 85% H_2O, 15% acetonitrile, 0.1% TFA, containing heptane-1-sulfonic acid (2 g/L); mobile phase B: 20% H_2O, 80% acetonitrile, 0.1% TFA, containing heptane-1-sulfonic acid (2 g/L); 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, injection volume: 50 μL; column: symmetry 300 (250 × 4.6 mm) from Waters.
54. Preliminary toxicity studies of **13**: Female nude mice were randomly distributed to the experimental groups (two/group) and injected with compound **13** (i.v, administered as a solution in 10% PEG 400/90% D-glucose buffer pH 5) at the following doses: (a) 33.6 mg/kg = 8 mg/kg doxorubicin equivalents, (b) 67.1 mg/kg = 16 mg/kg doxorubicin equivalents, or (c) 100.8 mg/kg = 24 mg/kg doxorubicin equivalents. The body weight change was observed daily for 13 days.