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Multivalency: Key Feature in Overcoming Drug Resistance with a Cleavable Cell-Penetrating Peptide-Doxorubicin Conjugate

Marco Lelle[1,2,3] · Christoph Freidel[1] · Stefka Kaloyanova[1] · Klaus Müllen[1] · Kalina Peneva[1,2]

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Abstract Multivalency is often used in biological systems, to increase affinity and specificity through avidity. This inspired us to prepare a synthetic bioconjugate that mimics natural multivalent systems. It is composed of doxorubicin and two octaarginine cell-penetrating peptides, to strengthen the electrostatic interactions between the negatively charged glycosaminoglycans of the plasma membrane and the guanidinium groups of the arginine residues. The multivalent conjugate has improved cellular uptake and cytotoxicity, compared to a peptide-drug conjugate with only one polyarginine and as a result it can overcome drug resistance in Kelly-ADR cells. The synthetic approach and the multivalent structure reported here can be used further as model systems, to gain insight into the biological interaction of cell-penetrating peptides with artificial membranes or for the preparation of more complex multimers.

Keywords Cell-penetrating peptide · Multivalency · Drug-peptide conjugate · MDR · Doxorubicin · Drug resistance

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✉ Kalina Peneva
kalina.peneva@uni-jena.de

[1] Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

[2] Institute of Organic and Macromolecular Chemistry, Jena Center of Soft Matter, Friedrich Schiller University Jena, Lessingstr. 8, 07743 Jena, Germany

[3] Institute of Physiology II, University Hospital Jena, Kollegiengasse 9, 07743 Jena, Germany

Introduction

The anthracycline doxorubicin ranks among the most efficient anticancer agents and is widely applied against breast cancer, soft tissue sarcomas, non-Hodgkin's lymphoma, leukaemia as well as childhood solid tumors (Gewirtz 1999; Nadas and Sun 2006; Minotti et al. 2004). Nevertheless, there are a few types of cancer where doxorubicin is non-effective (e.g. melanoma, colon cancer) (Weiss 1992). Moreover, numerous severe adverse effects restrict the therapeutic efficacy of the drug, like the onset of drug resistance in cancer cells and the poor tumor selectivity (Minotti et al. 2004; Nadas and Sun 2006; Meyer-Losic et al. 2006; Kratz et al. 1998; Weiss 1992). Nowadays, drug resistance is the main contributor to the failure of many anticancer agents including anthracyclines (Szakacs et al. 2014). The various cellular mechanisms that cancer cells use to resist chemotherapeutics are known to lead to a phenomenon called multidrug resistance (MDR). This term describes a process in which cancer cells become resistant to cytostatic or cytotoxic actions of multiple, structurally dissimilar and functionally divergent, drugs commonly used in cancer chemotherapy (Gottesman et al. 2002; Ambudkar et al. 1999). MDR in cancer is attributed to the presence of membrane proteins, which belong to the ATP-binding cassette transporter superfamily (Leslie et al. 2005; Dean et al. 2001). These membrane-associated transporters such as P-glycoprotein are also a common feature of noncancerous cells and are involved in tissue detoxification as well as protection (Ambudkar et al. 1999). The protective function of these proteins is mediated through an energy-dependent excretion of cytotoxic substances and their harmful metabolites from the cytoplasm (Ambudkar et al. 1999; Leslie et al. 2005). Thereby, the intracellular accumulation of antitumor drugs like doxorubicin can be markedly reduced, which makes chemotherapeutics less effective

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(Minotti et al. 2004; Nadas and Sun 2006; Gottesman et al. 2002). Furthermore, it has been demonstrated for many different tumor cells that they substantially overexpress membrane-associated transporter proteins and that the drug efflux is even increased after exposure to anticancer agents (Gottesman et al. 2002; Leslie et al. 2005). To overcome this limitation and bypass these transporter proteins, doxorubicin has been modified with different peptides such as penetratin, TAT or other arginine-rich amino acid sequences (Mazel et al. 2001; Liang and Yang 2005; Meyer-Losic et al. 2006; Aroui et al. 2009; Lelle et al. 2017). These small peptide sequences (typically less than 30 residues) belong to the so called cell-penetrating peptides and are also known as protein transduction domains (Zorko and Langel 2005; Jones and Sayers 2012; Milletti 2012; Tabujew et al. 2015). One of their key properties is the ability to efficiently deliver diverse cargoes such as drugs across the cell membrane (Miklan et al. 2009; Vargas et al. 2014; Dubikovskaya et al. 2008; Böhme and Beck-Sickinger 2015). The uptake mechanisms related to these cationic peptide sequences typically involve direct translocation across the cellular membrane as well as endocytosis and are based on electrostatic interactions (Jones and Sayers 2012; Duchardt et al. 2007; Ziegler and Seelig 2008). Whereas doxorubicin is quickly taken up by passive diffusion (Siegfried et al. 1985). Thereby, small hydrophobic drugs are particularly prone to be a substrate to membrane-associated efflux pumps, due to their lipophilic nature, which favors the residence in the cell membrane, where the transporter proteins reside (Vargas et al. 2014; Gatlik-Landwojtowicz et al. 2006; Rottenberg and Borst 2012).

In this study we describe the synthesis and characterization of a multivalent cleavable cell-penetrating peptide-drug conjugate that can overcome drug resistance in doxorubicin-resistant cancer cells. In biological systems, multivalent interactions are often employed to increase the affinity and specificity (Fasting et al. 2012). This inspired us to prepare a synthetic bioconjugate that mimics natural multivalent systems. It is composed of doxorubicin and two octaarginine cell-penetrating peptides, to strengthen the electrostatic interactions between the negatively charged plasma membrane and the amino acid side chains. Thus, cellular uptake and cytotoxicity, respectively, can be improved, compared to a peptide-drug conjugate with only one polyarginine, which we used as a reference. The introduced bioconjugates exhibit not only the capability to circumvent membrane-associated transporter proteins and to overcome drug resistance accordingly, but are also well-suited to target cancer cells mediated through high expression levels of glycosaminoglycans (Nakase et al. 2012).

Multivalent formulations of doxorubicin, like liposomes, organic or inorganic particles, have been applied to overcome multidrug resistance as well (Iyer et al. 2013; Kunjachan et al. 2012). Although these nanoformulations have been widely used, they exhibit certain disadvantages, e.g. application of undefined molecular structures, drug loading inconsistencies or lack of stability (Gu et al. 2012; Furedi et al. 2017; Wu et al. 2011; Riganti et al. 2011). In addition, a recent in vitro study demonstrated that the ability of the carrier to overcome resistance is much less prominent as reported and it can be cell type- and carrier-dependent (Kunjachan et al. 2012). The multivalent cell-penetrating peptide-doxorubicin conjugate reported here is capable of overcoming drug resistance without the aforementioned limitations, since the precise structure with two peptides per drug molecule can only be cleaved within the reductive environment of the cancer cell.

Materials and Methods

General Information

Solvents and reagents were purchased from commercial sources (Acros Organics, Alfa Aesar, AppliChem, Deutero, Thermo Fisher Scientific, Fluka, Merck, Sigma-Aldrich) and used without further purification. Doxorubicin hydrochloride was ordered from Ontario Chemicals, Inc. (Guelph, Ontario, Canada) and custom peptide synthesis of Ac-CRRRRRRRR-NH_2 was carried out from Genosphere Biotechnologies (Paris, France). The CellTiter-Glo luminescent cell viability assay was bought from Promega (Mannheim, Germany). Silica gel (0.063–0.200 mm) for column chromatography as well as thin layer chromatography sheets (ALUGRAM SIL G/UV_{254}) were ordered from Macherey-Nagel (Düren, Germany) and applied with suitable solvent systems.

Synthesis of 2-(2-Pyridyldithio)ethylamine Hydrochloride (2)

2,2′-Dithiopyridine (25 g, 113.47 mmol) was dissolved in 150 mL methanol and degassed in an ultrasonic bath for 30 min. Afterwards, 2-mercaptoethylamine hydrochloride (2.15 g, 18.91 mmol, **1**) was slowly added within 1 h to the colorless solution. The flask was sealed with a rubber septum and the reaction mixture was stirred overnight at ambient temperature under argon. Subsequently, the yellow solution was precipitated twice in cold diethyl ether and the product was obtained by filtration as colorless solid (4.21 g, 18.91 mmol, quantitative yield). *m/z* (MALDI-TOF) 187.00 $[M+H]^{+;}$ ^{1}H NMR (300 MHz, DMSO-d^6, 298 K) δ (ppm) = 8.56–8.46 (m, 1H), 8.30 (s, br, 3H), 7.88–7.80 (m, 1H), 7.76 (d, 1H, J = 8.1 Hz), 7.34–7.25 (m, 1H), 3.17–3.01 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d^6, 298 K) δ (ppm) = 158.09, 149.80, 137.89, 121.59, 120.00, 37.65, 34.74.

Synthesis of (*Boc*-Aminooxy)acetic Acid *N*-Hydroxysuccinimide Ester (4)

A slurry of *N*-hydroxysuccinimide (2.79 g, 24.28 mmol) as well as (*Boc*-aminooxy)acetic acid (4.42 g, 23.12 mmol, **3**) was prepared in 55 mL dry dichloromethane (DCM). Subsequently, *N,N′*-diisopropylcarbodiimide (3.06 g, 3.76 mL, 24.88 mmol) was added and the clear solution was stirred for 2 h under argon. Afterwards, another volume *N,N′*-diisopropylcarbodiimide (244 mg, 300 µL, 1.99 mmol) was added and the reaction mixture was stirred for further 2 h at room temperature. The precipitated urea was removed by filtration and washed with a small amount of DCM. After that, the obtained solution was diluted with 200 mL DCM and washed four times with water. The organic layer was dried with magnesium sulfate and the solvent was removed *in vacuo*, to obtain the product as a colorless solid (6.13 g, 21.27 mmol, 92%). *m/z* (MALDI-TOF) 357.04 $[M+3Na]^{+;}$ ^{1}H NMR (300 MHz, DMSO-d^6, 298 K) δ (ppm) = 10.36 (s, 1H), 4.82 (s, 2H), 2.84 (s, 4H), 1.42 (s, 9 H); ^{13}C NMR (75 MHz, DMSO-d^6, 298 K) δ (ppm) = 169.95, 165.14, 156.57, 80.60, 69.90, 27.93, 25.47.

Synthesis of *tert*-Butyl(2-oxo-2-((2-(pyridinyldithio) ethyl)amino)ethoxy)carbamate (5)

The active ester **4** (3.88 g, 13.47 mmol) and 2-(2-pyridyldithio)ethylamine hydrochloride (3.00 g, 13.47 mmol) were dissolved in 60 mL dry *N,N*-dimethylformamide (DMF). Afterwards, *N,N*-diisopropylethylamine (DIPEA) (6.96 g, 9.16 mL, 53.87 mmol) was added and the reaction mixture was stirred at room temperature under argon for 3 h. Subsequently, the reaction mixture was diluted with 250 mL ethyl acetate and washed three times with water. The organic layer was dried with magnesium sulfate and the solvent was removed *in vacuo*. The residue was purified by silica gel column chromatography (ethyl acetate—hexane 2:1), whereby the product was obtained as colorless oil (3.53 g, 9.83 mmol, 73%). m/z (FD) 357.8 $[M]^{+;}$ ^{1}H NMR (300 MHz, DMSO-d^6, 298 K) δ (ppm) = 10.29 (s, 1H), 8.52–8.40 (m, 1H), 8.23 (t, 1 H, J = 5.7 Hz), 7.91–7.70 (m, 2H), 7.30–7.17 (m, 1H), 4.16 (s, 2H), 3.43 (q, 2 H, J = 6.5 Hz), 2.93 (t, 2 H, J = 6.5 Hz), 1.40 (s, 9 H); ^{13}C NMR (75 MHz, DMSO-d^6, 298 K) δ (ppm) = 168.05, 158.98, 156.78, 149.62, 137.77, 121.20, 119.31, 80.60, 74.68, 37.37, 37.25, 27.92.

Synthesis of the 2-Pyridyl Disulfide-Carrying Doxorubicin Derivative (7)

tert-Butyl(2-oxo-2-((2-(pyridinyldithio)ethyl)amino)ethoxy)carbamate (1.87 g, 5.14 mmol, **5**) was dissolved in 30 mL dry DCM and subsequently diluted with 30 mL trifluoroacetic acid (TFA). The obtained reaction mixture was stirred for 1 h at room temperature. Afterwards, solvent and reagent were removed under reduced pressure and the residue was dried for further 6 h in a high vacuum. Doxorubicin hydrochloride (1.49 g, 2.57 mmol) was dissolved in 175 mL sodium acetate buffer (0.4 M, pH 4.8) and added to the oily residue dissolved in 175 mL DMF. The solution was stirred for 48 h and subsequently purified by RP-HPLC. The solvent of the collected fractions was evaporated and the obtained residue was dissolved in methanol and precipitated in diethyl ether, to yield the product as red solid (1.76 g, 2.08 mmol, 81%). *m/z* (MALDI-TOF) 807.98 $[M+Na]^{+}$; RP-HPLC t_R = 15.56 min (480 nm), A—25 mM triethylammonium acetate (TEAA) buffer (pH 7), B—acetonitrile (ACN), 0 min 75% A, 45 min 30% A; ^{1}H NMR (700 MHz, D_2O, 298 K) δ (ppm) = 8.21 (d, 1H, J = 4.2 Hz), 7.67–7.58 (m, 2H), 7.50 (d, 1H, J = 8.4 Hz), 7.46 (d, 1H, J = 7.0 Hz), 7.32 (d, 1H, J = 7.0 Hz), 7.13 (t, 1 H, J = 5.6 Hz), 5.41 (s, 1H), 4.83 (s, 1H), 4.61 (dd, 2H, J = 16.1 Hz, J = 22.4 Hz), 4.51 (dd, 2H, J = 12.6 Hz, J = 38.5 Hz), 4.23 (q, 1H, J = 6.3 Hz), 3.86 (s, 3H), 3.77 (s, 1H), 3.60 (t, 1H, J = 8.4 Hz), 3.42–3.31 (m, 2H), 3.13–3.02 (m, 1H), 2.94–2.79 (m, 3H), 2.40–2.25 (m, 2H), 1.99 (d, 2H, J = 6.3 Hz), 1.90 (s, 3H), 1.31 (d, 3H, J = 6.3 Hz).

Synthesis of the Monovalent Peptide-Drug Conjugate (8)

The 2-pyridyl disulfide-carrying doxorubicin derivative (300 mg, 355 µmol, **7**) and the peptide (1.15 g, 496 µmol, Ac-CRRRRRRRR-NH_2) were dissolved in 100 mL Dulbecco's phosphate-buffered saline (DPBS) and DMF (4:1). The solution was stirred for 2 h at room temperature in argon atmosphere and subsequently purified by RP-HPLC. Afterwards, the solvent of the isolated fractions was removed in vacuo and the obtained residue was dissolved in methanol and precipitated in diethyl ether, to yield the product as red solid (679 mg, 259 µmol, 73%). *m/z* (MALDI-TOF) 2086.18 $[M+H]^{+}$; RP-HPLC t_R = 15.21 min (480 nm), A—25 mM TEAA buffer (pH 7), B—ACN, 0 min 100% A, 35 min 35% A; ^{1}H NMR (700 MHz, D_2O, 298 K) δ (ppm) = 7.98 (d, 1 H, J = 7.7 Hz), 7.90 (t, 1H, J = 7.7 Hz), 7.62 (d, 1H, J = 7.7 Hz), 5.59–5.55 (m, 1H), 5.14 (t, 1H, J = 5.6 Hz), 4.53 (dd, 2H, J = 16.1 Hz, J = 49.4 Hz), 4.46 (s, 2H), 4.41 (t, 1H, J = 6.3 Hz), 4.35–4.24 (m, 9H), 4.26 (q, 1H, J = 6.3 Hz), 4.06 (s, 3H), 3.85–3.83 (m, 1H), 3.70–3.64 (m, 1H), 3.50–3.41 (m, 1H), 3.29–3.23 (m, 1H), 3.23–3.10 (m, 16H), 3.09-3.00 (m, 1H), 2.97–2.91 (m, 1H), 2.90–2.85 (m, 1H), 2.80–2.74 (m, 1H), 2.72–2.65 (m, 1H), 2.58–2.49 (m, 2H), 2.36–2.30 (m, 1H), 2.08–2.02 (m, 2H), 2.01 (s, 3H), 1.91 (s, 24H), 1.87–1.51 (m, 32H), 1.30 (d, 3H, J = 6.3 Hz).

Synthesis of (*S*)-*tert*-Butyl(1,5-dioxo-1,5-*bis*((2-(2pyridinyldithio)ethyl)amino)pentan-2-yl)carbamate (10)

The protected glutamic acid derivative **9** (500 mg, 2.02 mmol) was dissolved in 20 mL *N,N*-dimethylformamide together with the coupling reagent *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (1.22 g, 4.04 mmol) and **2** (901 mg, 4.04 mmol). After that, DIPEA (1.72 mL, 10.11 mmol) was added and the reaction mixture was stirred in an argon atmosphere for 5 h at room temperature. Subsequently, 350 mL of ethyl acetate was added and the solution was washed three times with brine. The organic layer was dried with magnesium sulfate and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (ethyl acetate—methanol 15:1), to get the product as yellow oil (1.02 g, 1.73 mmol, 86%). *m/z* (MALDI-TOF) 605.99 $[M+Na]^{+;}$ ^{1}H NMR (300 MHz, DMSO-d^6, 298 K) δ (ppm) = 8.51–8.43 (m, 2H), 8.07 (t, 1H, *J* = 6.3 Hz), 8.04 (t, 1H, *J* = 6.3 Hz), 7.87–7.72 (m, 4H), 7.28–7.20 (m, 2H), 6.88 (d, 1H, *J* = 7.8 Hz), 3.92–3.74 (m, 1H), 3.44–3.24 (m, 4H), 2.88 (t, 4H, *J* = 6.3 Hz), 2.10 (t, 2H, *J* = 7.8 Hz), 1.92–1.61 (m, 2H), 1.36 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d^6, 298 K) δ (ppm) = 171.85, 171.66, 159.09, 159.01, 155.20, 149.63, 149.57, 137.76, 121.16, 119.33, 119.23, 78.06, 54.07, 37.78, 37.67, 37.45, 31.82, 28.15, 27.79.

Synthesis of (*S*)-*tert*-Butyl((1,5-dioxo-1,5-*bis*((2-(2pyridinyldithio)ethyl)amino)pentan-2-yl)amino)-2-oxoethoxycarbamate (11)

The modified glutamic acid **10** (250 mg, 428.2 µmol) was gradually dissolved in 10 mL dry DCM and the same amount of TFA was added. The reaction mixture was stirred for 1 h at room temperature followed by removal of solvent and reagent in vacuum. Afterwards, the obtained oil was dissolved in 5 mL dry DMF and consecutively DIPEA (387.4 mg, 509.8 µL, 3 mmol) as well as **4** (135.8 mg, 471 µmol) were added. The solution was stirred for 3 h under argon at room temperature and was subsequently diluted with 250 mL ethyl acetate. The organic layer was washed three times with brine, dried over magnesium sulfate and the solvent was removed under reduced pressure. After that, the residue was purified by silica gel column chromatography (ethyl acetate—methanol 15:1), whereby the product was obtained as yellow oil (187.2 mg, 284.9 µmol, 78%). *m/z* (MALDI-TOF) 695.22 $[M+K]^{+;}$ ^{1}H NMR (300 MHz, DMSO-d^6, 298 K) δ (ppm) = 10.30 (s, 1H), 8.51–8.40 (m, 2H), 8.24 (t, 1H, *J* = 5.7 Hz), 8.12 (d, 1H, *J* = 8.0 Hz), 8.05 (t, 1H, *J* = 5.7 Hz), 7.88–7.70 (m, 4H), 7.28–7.19 (m, 2H), 4.33–4.09 (m, 1H), 3.43–3.24 (m, 4H), 2.89 (t, 2H, *J* = 7.0 Hz), 2.87 (t, 2H, *J* = 7.0 Hz), 2.09 (t, 2H, *J* = 8.0 Hz), 1.97–1.69 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d^6, 298 K) δ (ppm) = 171.40, 170.88, 167.91, 159.08, 159.04, 156.86, 149.58, 137.76, 121.17, 119.25, 80.61, 74.64, 51.80, 37.84, 37.78, 37.43, 37.27, 31.59, 28.04, 27.91.

Synthesis of the *bis*(2-pyridyl disulfide)-Carrying Doxorubicin Derivative (12)

Initially, a solution of **11** (450 mg, 685.1 µmol) in 10 mL dry DCM was prepared. To this solution 10 mL of TFA was added and the reaction mixture was stirred for 1 h at room temperature. Afterwards, solvent and reagent were removed in vacuo. Doxorubicin hydrochloride (397.3 mg, 685.1 µmol) was dissolved in 80 mL DMF/0.4 M sodium acetate buffer (1:1, pH 4.8) and added to the oily residue. The solution was stirred for 48 h at room temperature and was subsequently purified by RP-HPLC. After that, the solvent of the isolated fractions was removed in vacuum, to obtain the product as red solid (579.1 mg, 507.0 µmol, 74%). *m/z* (MALDI-TOF) 1104.09 $[M+Na]^+$; RP-HPLC t_R = 28.88 min (480 nm), A—25 mM TEAA buffer (pH 7), B—ACN, 0 min 100% A, 40 min 30% A; ^{1}H NMR (850 MHz, DMSO-d^6, 298 K) δ (ppm) = 8.42–8.37 (m, 2H), 8.24 (t, 1H, *J* = 5.7 Hz), 8.03 (d, 1H, *J* = 8.2 Hz), 8.00 (t, 1H, *J* = 5.7 Hz), 7.93–7.87 (m, 2H), 7.79–7.74 (m, 2H), 7.70 (d, 1H, *J* = 8.0 Hz), 7.65 (d, 1H, *J* = 8.0 Hz), 7.63 (d, 1H, *J* = 8.0 Hz), 7.21–7.16 (m, 2H), 5.26–5.38 (m, 1H), 5.23–5.17 (m, 1H), 5.10–5.02 (m, 1H), 4.95–4.90 (m, 1H), 4.57–4.27 (m, 5H), 4.25–4.18 (m, 1H), 4.07 (q, 1H, *J* = 6.6 Hz), 3.98 (s, 3H), 3.35–3.30 (m, 2H), 3.30–3.27 (m, 1H), 3.23–3.17 (m, 2H), 3.07–2.99 (m, 2H), 2.87–2.81 (m, 1H), 2.84 (t, 2H, *J* = 6.7 Hz), 2.76 (t, 2H, *J* = 6.8 Hz), 2.48–2.43 (m, 1H), 2.12–2.07 (m, 1H), 2.06–1.99 (m, 2H), 1.91–1.82 (m, 1H), 1.84 (s, 3H), 1.75–1.67 (m, 1H), 1.63–1.58 (m, 1H), 1.49–1.44 (m, 1H), 1.15 (d, 3H, *J* = 6.6 Hz).

Synthesis of the Multivalent Peptide-Drug Conjugate (13)

The *bis*(2-pyridyl disulfide)-carrying doxorubicin derivative (52.8 mg, 46.2 µmol, **12**) and the cell-penetrating peptide (225.5 mg, 97 µmol, Ac-CRRRRRRRR-NH_2) were dissolved in 20 mL DPBS and DMF (3:1). The solution was stirred for 3 h under argon at room temperature and was subsequently purified by RP-HPLC. After that, the solvent of the isolated fractions was removed in vacuo and the obtained residue was dissolved in methanol and precipitated in diethyl ether, to yield the product as red solid (134.6 mg, 31.9 µmol, 69%). *m/z* (MALDI-TOF) 3683.79 $[M+H]^+$; RP-HPLC t_R = 18.57 min (480 nm), A—25 mM TEAA buffer (pH 7), B—ACN, 0 min 100% A, 40 min 30% A; ^{1}H NMR (850 MHz, D_2O, 298 K) δ (ppm) = 7.94 (d, 1H, *J* = 7.7 Hz), 7.85 (t, 1H, *J* = 7.7 Hz), 7.59 (d, 1H, *J* = 7.7 Hz), 5.53–5.50

(m, 1H), 5.11–5.06 (m, 1H), 4.60–4.51 (m, 3H), 4.47–4.42 (m, 3H), 4.30–4.17 (m, 18H), 4.01 (s, 3H), 3.80–3.76 (m, 1H), 3.64–3.61 (m, 1H), 3.39–3.25 (m, 4H), 3.16–3.06 (m, 34H), 2.98–2.94 (m, 2H), 2.84–2.78 (m, 2H), 2.72–2.61 (m, 4H), 2.44–2.39 (m, 1H), 2.37–2.31 (m, 1H), 2.23–2.17 (m, 2H), 2.02–1.93 (m, 9H), 1.86–1.82 (m, 1H), 1.84 (s, 51H), 1.81–1.65 (m, 32H), 1.63–1.49 (m, 32H), 1.24 (d, 3H, $J = 6.5$ Hz).

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX 300, Bruker WS 700 and Bruker WB 850 (Bruker Avance III) spectrometer. Chemical shifts are displayed in parts per million (ppm), relative to the residual solvent signals of DMSO-d^6 ($\delta_H = 2.50$, $\delta_C = 39.52$) and D_2O ($\delta_H = 4.79$) (Gottlieb et al. 1997) The corresponding coupling constants (J) are represented in Hz.

Mass Spectrometry

Field desorption (FD) mass spectrometry was conducted on a VG Instruments ZAB 2-SE-FPD (8 kV) spectrometer. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was carried out on a Bruker Reflex II TOF spectrometer equipped with a 337 nm nitrogen laser. 2,5-Dihydroxybenzoic acid as well as α-cyano-4-hydroxycinnamic acid (peptidic sample) were applied as matrix.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was performed on a Jasco LC-2000Plus system (Groß-Umstadt, Germany), equipped with a diode array detector (MD-2015) and appropriate solvent delivery pumps (PU-2086). Analytical RP-HPLC was conducted on a Jasco ReproSil 100 C18 column (250 × 4.6 mm), with 5 µm particle size as stationary phase and a flow rate of 1 mL/min. Purification of the substances was carried out on a ReproSil 100 C18 column from Jasco (250 × 20 mm), at a flow rate of 15 mL/min and 5 µm silica as stationary phase. The applied eluents were 25 mM triethylammonium acetate buffer at pH 7 (A) and acetonitrile (B), with suitable linear gradients. All compounds were detected at the characteristic absorbance of doxorubicin (480 nm).

Glutathione-Mediated Drug Release

A 1 mM solution of **8** in Dulbecco's phosphate-buffered saline was incubated with a tenfold excess of glutathione at 37 °C in an Eppendorf Thermomixer compact (300 rpm). The disulfide cleavage of the peptide-drug conjugate was monitored by RP-HPLC with a linear gradient (0 min 100% A, 35 min 35% A) for 24 h.

Cell Cultures

MCF-7 cells (human breast adenocarcinoma cell line, ATCC HTB-22) were cultured in Dulbecco´s modified Eagle medium supplemented with 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin antibiotics. The human neuroblastoma cell line Kelly-WT was obtained from ATCC and authenticated using STR genotyping. Kelly-WT cells were grown in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin antibiotics. The anthracycline-resistant Kelly-ADR cells were maintained like its drug-sensitive counterpart and were generated by continuous exposure of Kelly-WT cells to increasing doses of doxorubicin. Repeated selection rounds resulted in Kelly-ADR cells with tenfold enhanced resistance to doxorubicin and a concomitant increase in the IC_{50} value from 0.1 to 1 µg/mL. These cells were taken for further experiments. All of the above-mentioned cells were grown at 37 °C and 5% CO_2 in a humidified incubator and were subcultured every 2–3 days using 0.25% trypsin.

Cell Viability Assay

The antiproliferative effect of **8, 13**, doxorubicin and the cysteine-carrying octaarginine cell-penetrating peptide on MCF-7, Kelly-WT as well as Kelly-ADR cells in vitro was examined with the luciferase-based CellTiter-Glo cell proliferation assay according to the manufacturer's instructions. Briefly, the cells were seeded into 96-well plates at a density of 1×10^3 cells per well in 100 µL of medium and incubated for 24 h to adhere. Subsequently, the medium was removed and the drugs as well as the peptide were added at various concentrations from 0.05 to 20 µM. The cells were incubated for 72 h with the substances and after that the cell viability was determined by quantitation of ATP with the CellTiter-Glo luminescent cell viability assay on a Tecan plate reader. Viability of cells treated with doxorubicin or drug derivatives was compared to untreated controls and corrected for effects of the medium. The applied concentrations of the utilized compounds were transformed into a logarithmic scale prior to analysis and the obtained data from the survival curves were expressed as IC_{50} values in µM. Each cell viability experiment was performed in independent triplicates.

Fluorescence Microscopy

The intracellular localization of the multivalent cleavable cell-penetrating peptide-drug conjugate was studied in

Kelly-WT and doxorubicin-resistant Kelly-ADR live cells at 37 °C and 5% CO_2 via fluorescence confocal laser scanning microscopy and compared to the native drug. Microscopy images were obtained with a 63x/1.2 water-immersion objective on a TCS SP5 microscope (Leica) and an incubation chamber for live cell imaging (37 °C, 5% CO_2). The anthracycline derivative **13** as well as doxorubicin were applied at a concentration of 10 µM to 1×10^4 cells per well (µ-Slide 8 Well, ibiTreat, Ibidi) for 24 and 72 h, respectively. Excitation of the drugs was performed with an argon laser at $\lambda ex = 488$ nm (power set to 20%) and the emission range was set to $\lambda em = 550–660$ nm. The fluorescence signal was detected by hybrid detectors (HyD) with fixed gain values set to 100. Counterstaining of the nuclei was accomplished by incubation with 5 µM DRAQ5 (Thermo Fisher Scientific) 5 min prior to microscopy.

Results and Discussion

Preparation of the Monovalent Peptide-Drug Conjugate

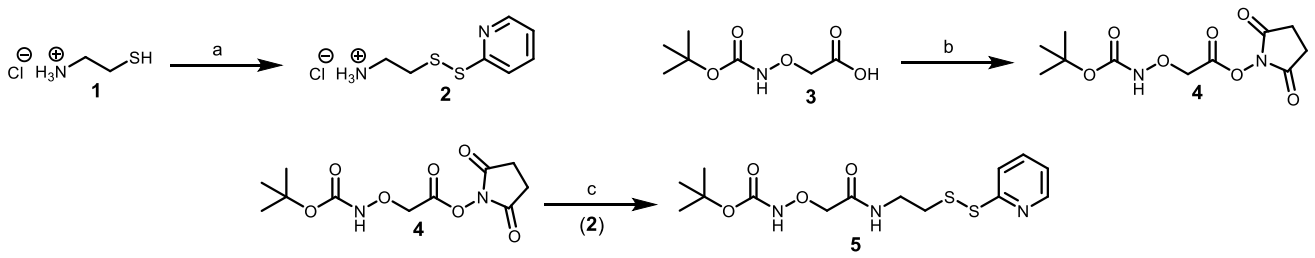
The synthesis and characterization of the monovalent cleavable cell-penetrating peptide-drug conjugate (**8**) we have reported previously (Lelle et al. 2014). Briefly, **8** was obtained via a heterobifunctional cross-linking reagent (**5**), which contains a thiol-reactive 2-pyridyl disulfide functionality as well as a protected carbonyl-reactive aminooxy group. In order to synthesize this cross-linker molecule, 2-mercaptoethylamine hydrochloride was transferred into its corresponding 2-pyridyl disulfide **2** with an excess of 2,2′-dithiopyridine and the *N*-hydroxysuccinimide ester **4** of (*Boc*-aminooxy)acetic acid was prepared. Afterwards, the active ester **4** was reacted with 2-(2-pyridyldithio)ethylamine hydrochloride (Scheme 1). Our former strategy for the synthesis of the linker molecule involved a coupling reagent-based approach, where the free amine of **2** was directly coupled to the carboxylic acid of **3**. Herein, the active ester was synthesized, to allow further modifications, which are necessary for the preparation of the multivalent bioconjugate **11**. Nonetheless, both methods are straightforward and give the cross-linking reagent in high yield.

The two orthogonal groups present in **5** are capable of reacting with thiol-containing biomolecules like cysteine residues of peptides as well as carbonyl groups. The aliphatic ketone of doxorubicin is such an easily accessible carbonyl. After the removal of the protective group from **5** under strong acidic conditions, the condensation between the cross-linker and the drug was carried out (Scheme 2). This reaction is typically acid-catalyzed and yields the oxime exclusively in *E* configuration, as already described for oxime-based bioconjugates of doxorubicin (Orban et al. 2011; Szabo et al. 2009). Afterwards, the sulfhydryl-reactive group carrying drug derivative was coupled to the N-terminal cysteine residue of the cationic octaarginine cell-penetrating peptide (Ac-CRRRRRRRR-NH_2). The conversion to the monovalent peptide-drug conjugate was achieved rapidly and the product was isolated by RP-HPLC.

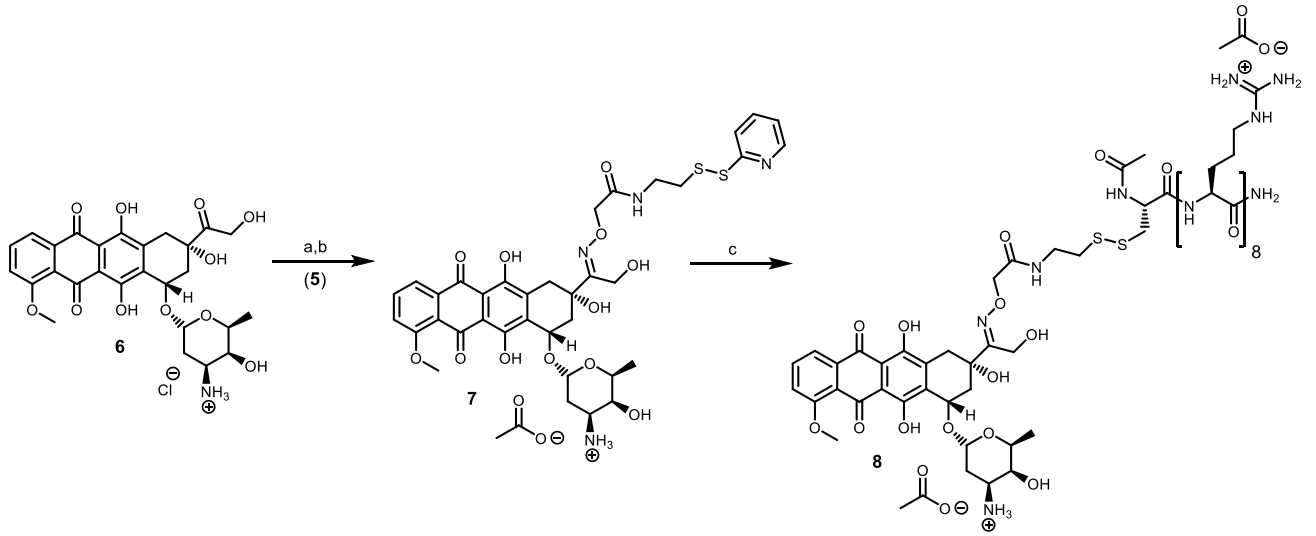
Preparation of the Multivalent Peptide-Drug Conjugate

For the synthesis of the multivalent peptide-drug conjugate carrying two cell-penetrating peptides **13**, a different cross-linking reagent with two 2-pyridyl disulfides was prepared. Previously we have reported an amino acid-based heterofunctional linker molecule, which exhibits the desired criteria to fulfill the necessary requirements based on glutamic acid. (Lelle et al. 2015). This amino acid derivative bears two 2-pyridyl disulfides (**11**), which are capable of intercalating into reduced disulfides, and is equipped with an aminooxy functionality to react with the aliphatic ketone of doxorubicin. Therefore, we have utilized this trifunctional amino acid scaffold to build up a multivalent peptide-drug conjugate with two octaarginine cell-penetrating peptides.

Initially, the amino-protected glutamic acid derivative **9** was modified with two molecules of **2** using *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate as coupling reagent. The *Boc* protective group was subsequently removed and the free amine of the amino acid was



Scheme 1 Synthesis of the heterobifunctional cross-linking reagent. Reagents and conditions: *a* 2,2′-dithiodipyridine (6 equiv), methanol, argon, overnight, r.t., quantitative yield. *b* *N*-hydroxysuccinimide (1.05 equiv), *N,N′*-diisopropylcarbodiimide (1.15 equiv), DCM, argon, 4 h, r.t., 92%. *c* **2** (1 equiv), DIPEA (4 equiv), DMF, argon, 3 h, r.t., 73%



Scheme 2 Synthesis of the monovalent peptide-drug conjugate. Reagents and conditions: *a* **5**, DCM/TFA (1:1), 1 h, r.t., quantitative yield. *b* Doxorubicin hydrochloride (**6**, 0.5 equiv), DMF/0.4 M sodium acetate buffer (1:1), pH 4.8, 48 h, r.t., 81%. *c* Ac-CRRRRRRRR-NH_2 (1.4 equiv), DMF/DPBS (1:4), argon, 2 h, rt, 73%

reacted afterwards with the active ester **4**, to prepare the heterobifunctional cross-linker **11** (Scheme 3).

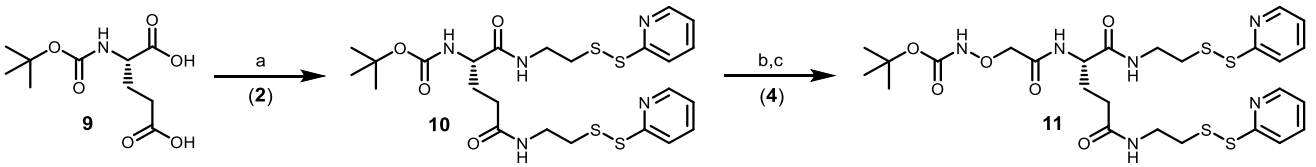
The glutamic acid-based heterofunctional cross-linking reagent was deprotected at first, to generate the carbonyl-reactive aminooxy group. The deprotection of the aminooxy functionality was always performed directly before the reaction with doxorubicin, to avoid decomposition and side reactions, due to the high reactivity of the alpha effect nucleophile. After the *Boc* protecting group was successfully removed in quantitative yield, the condensation with the drug was carried out, to get an anthracycline derivative with two 2-pyridyl disulfides (Scheme 4). These mixed disulfides were modified with the cysteine-carrying polyarginine cell-penetrating peptide, to yield the multivalent peptide-drug conjugate.

Glutathione-Dependent Drug Delivery

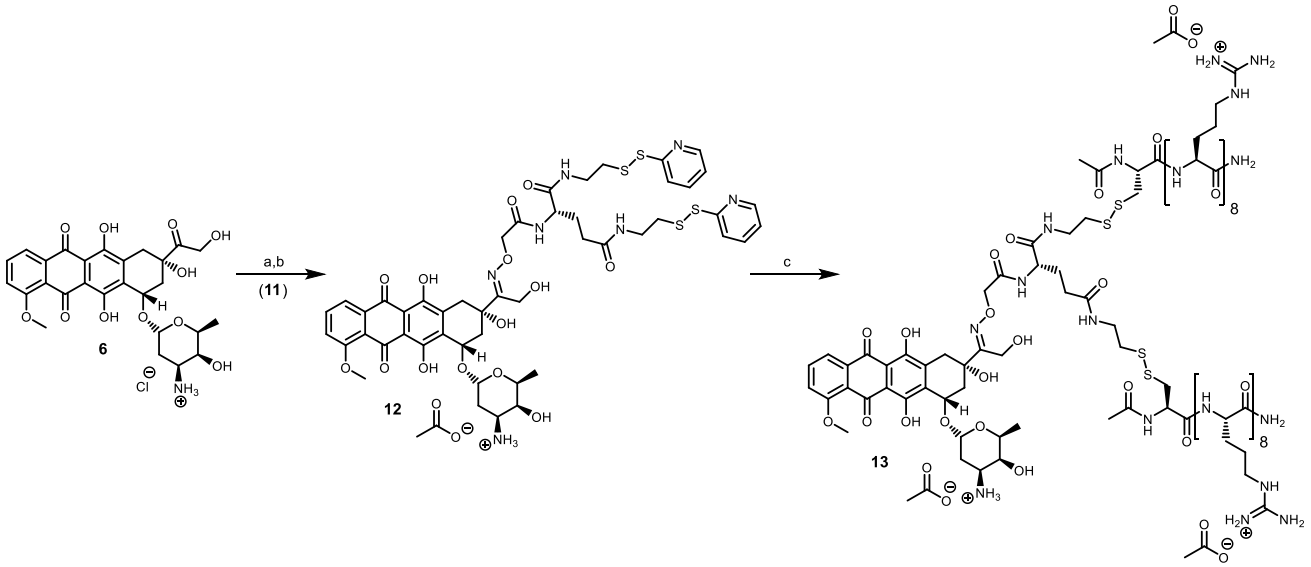
Following cellular internalization, peptide-drug conjugates like **13** have to be cleaved, in order to release their cytotoxic freight. The cleavage can take place via disulfide scission, which will occur in the reductive environment inside the cell. The tripeptide glutathione is an reducing agent omnipresent in mammalian cells and the most prevalent cellular thiol (Deneke and Fanburg 1989). The intracellular level of glutathione is characteristically in the millimolar range (0.5–10 mM), whereas micromolar concentrations are found in blood plasma (Meister and Anderson 1983). Moreover, the intracellular concentration of the reducing agent and glutathione-associated enzymes in drug-sensitive and in particular drug-resistant tumor cells is usually increased, which can mediate efficient drug release inside the cancer cells (Balendiran et al. 2004; Gamcsik et al. 1995; Tew 1994).

To study the release of doxorubicin from the conjugates, we have incubated a 1 mM solution of **8** with ten equivalents of glutathione in DPBS at 37 °C, following the protocol described previously. (Lelle et al. 2014). The cleavage of the peptide-drug conjugate was monitored for 24 h by RP-HPLC at the characteristic absorption maximum of doxorubicin (480 nm).

The degradation of the monovalent bioconjugate was highly efficient, which is illustrated by the chromatogram at 0 h. This corresponds to the point of time where **8** and the reducing agent were just put together and analyzed



Scheme 3 Synthesis of the *bis*(2-pyridyl disulfide)-carrying heterofunctional cross-linking reagent. Reagents and conditions: *a* **2** (2 equiv), *N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (2 equiv), DIPEA (5 equiv), DMF, argon, 5 h, r.t., 86%. *b* DCM/TFA (1:1), 1 h, r.t., quantitative yield. *c* **4** (1.1 equiv), DIPEA (7 equiv), DMF, argon, 3 h, r.t., 78%



Scheme 4 Synthesis of the multivalent peptide-drug conjugate. Reagents and conditions: *a* **11**, DCM/TFA (1:1), 1 h, r.t., quantitative yield. *b* Doxorubicin hydrochloride (**6**, 1 equiv), DMF/0.4 M sodium acetate buffer (1:1), pH 4.8, 48 h, r.t., 74%. *c* Ac-CRRRRRRRR-NH_2 (2.1 equiv), DMF/DPBS (1:3), argon, 3 h, rt, 69%

by RP-HPLC (Fig. 1). Even after such a short period of time, the peptide-drug conjugate was almost fully cleaved. Consequently, another peak was visible that matched to substance **a**, which is a glutathione-carrying doxorubicin derivative. Under the applied incubation conditions, **8** did not elute as a sharp peak, because the charged guanidinium groups of the amino acid side chains are highly influenced from various counter ions such as acetate, phosphate or the carboxylic acid of glutathione. Nevertheless, the peptide-drug conjugate could not be detected anymore after 1 h of incubation. Moreover, a substance evolved that corresponds to an anthracycline derivative with a residual linker molecule (**b**). The fraction of this compound increased over time and was after 3 h already the dominant species, whereas **a** became less and less due to further reduction by glutathione. Under physiological conditions a full degradation of **a** would occur, due to the constant glutathione level maintained in mammalian cells by enzymatic processes (Deneke and Fanburg 1989). As a result, the peptide drug conjugate will be entirely metabolized to **b**. Substance **b** can be considered as the cytotoxic drug metabolite, as previous scientific works have already shown that residual amino acids from linker molecules do not diminish the DNA-binding properties of the anthracycline (Orban et al. 2011; Schlage et al. 2011). As a consequence, cleavage product **b** released from the bioconjugate **8** can still successfully interact with DNA and thereby generate its cytotoxic effects. The release of the native anthracycline was a minor process during the degradation study, which can be seen in the chromatogram at 3 h for instance.

Previously we have also investigated the degradation of a peptide-drug conjugate, which is similar to the herein introduced bioconjugate **13**, since the anthracycline is connected via two disulfides to a peptide backbone in both cases (Lelle et al. 2015). This system has also demonstrated that peptide-drug conjugates derived from **12** can efficiently release the cytotoxic freight in the presence of an excess glutathione, although the degradation was slower and yielded more cleavage products, which was attributed to the two developing sulfhydryl groups.

Moreover, we have incubated the cell-penetrating peptide-doxorubicin conjugates at lower pH values, such as 5.0, to simulate the conditions present in endosomes and around cancer cells (Sorkin and von Zastrow 2002; Tannock and Rotin 1989). But also here, no significant amount of free doxorubicin could be detected by RP-HPLC after 48 h of incubation. The high hydrolytic stability of the oxime is crucial for our approach, to mediate exclusive drug delivery by disulfide scission, which can reduce harmful secondary effects due to an early release of the drug in the bloodstream (Kalia and Raines 2008).

Cell Viability Studies

After we could successfully show that elevated glutathione levels can cleave the disulfide-based bioconjugates, to deliver the anthracycline drug inside the tumor cells, the antiproliferative effect of **8** and **13** on different cell lines was investigated. The cytotoxicity data were obtained with the CellTiter-Glo luminescent cell viability assay, expressed as IC_{50} values in µM and compared to the effects of the native

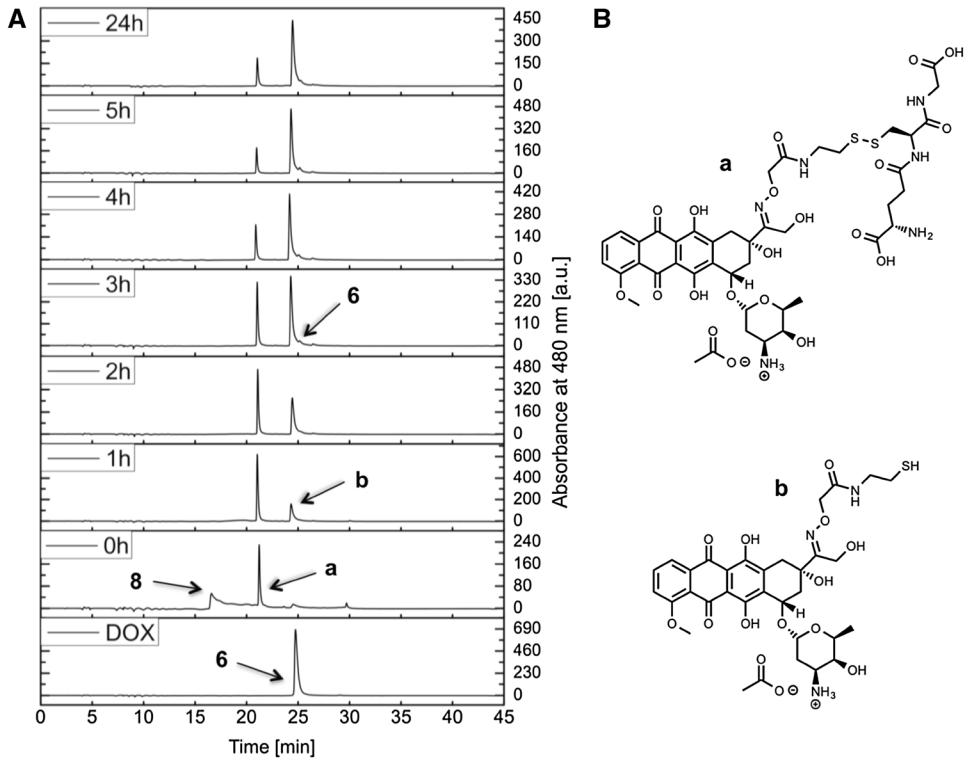


Fig. 1 Glutathione-mediated drug release by reduction of the monovalent cell-penetrating peptide-doxorubicin conjugate **8**. **A** RP-HPLC analysis of the degradation of **8** (1 mM) in the presence of an excess glutathione (10 mM) in DPBS at 37 °C. **B** Chemical structures of the cleavage products

drug as well as the cysteine-carrying octaarginine cell-penetrating peptide.

The in vitro cytotoxic effects of the applied compounds were examined with cell lines from tissues where doxorubicin is typically utilized. One of these cell lines is the MCF-7 breast cancer cell line (Nadas and Sun 2006; William Lown 1993). The antiproliferative effect of the native drug on this adenocarcinoma cells is very strong. However, the multivalent peptide-drug conjugate was also very cytotoxic for MCF-7 breast cancer cells, as expressed by an IC_{50} value in the lower micromolar range like doxorubicin. In contrast, the IC_{50} value of the monovalent bioconjugate (**8**) was almost sevenfold higher compared to **13** (Table 1). The cell viability data for the peptide-drug conjugates support our hypothesis that the multivalent compound will have an increased cellular uptake and thereby exhibit stronger cytotoxic effects. The octaarginine cell-penetrating peptide alone was non-toxic to the applied adenocarcinoma cell line, which is a good indication that the antiproliferative effect of **8** and **13** is related to the anthracycline drug and not to the peptide.

Doxorubicin has played an undisputed role in the treatment of childhood solid tumors such as neuroblastoma (Minotti et al. 2004). For that reason, we have also used the wild-type human neuroblastoma cell line Kelly-WT and its adriamycin-resistant subline Kelly-ADR, to investigate the compounds. The drug-resistant counterpart of the cell line was created by continuous exposure of Kelly-WT cells to increasing doses of the anthracycline drug, which is in good agreement with the obtained IC_{50} values from the herein presented cytotoxicity study. Hence, the IC_{50} value of doxorubicin was almost 40-times higher for the anthracycline-resistant subline (Table 1). The peptide-drug conjugates **8** and **13** showed the opposite, but also here the trend was visible that the multivalent compound is equipped with a stronger antiproliferative effect compared to the monovalent bioconjugate. Whereas **8** and **13** were both less active than doxorubicin on Kelly-WT cells, the multivalent conjugate **13** was more cytotoxic to drug-resistant Kelly-ADR cells compared to the native anthracycline.

Table 1 In vitro cytotoxic effects of the peptide-drug conjugates (**8** and **13**), doxorubicin and the octaarginine cell-penetrating peptide in MCF-7, Kelly-WT and Kelly-ADR cells, determined with the CellTiter-Glo luminescent cell viability assay

Compound	MCF-7 IC_{50} (μM)	Kelly-WT IC_{50} (μM)	Kelly-ADR IC_{50} (μM)
8	24.36 ± 4.15	11.55 ± 1.03	7.04 ± 2.23
13	3.80 ± 1.95	1.40 ± 0.66	1.84 ± 0.57
Doxorubicin	0.90 ± 0.12	0.18 ± 0.08	6.57 ± 0.61
Octaarginine cell-penetrating peptide	≥100	≥100	≥100

The cells were incubated for 72 h with the compounds and the data are expressed as IC_{50} values in μM ($n \geq 3$, mean ± standard deviation)

This phenomenon can be attributed to the drug resistance of the neuroblastoma cell line towards the anthracycline, which renders doxorubicin less effective, since the drug is efficiently excreted from Kelly-ADR cells by membrane-associated transporter proteins (Ambudkar et al. 1999; Gottesman et al. 2002). Through its different uptake mechanisms, which are mainly based on endocytosis and direct translocation, the multivalent bioconjugate can presumably circumvent the drug efflux pumps, to evolve a greater cytotoxicity (Jones and Sayers 2012; Vargas et al. 2014; Madani et al. 2011). This effect is perhaps not so pronounced for the monovalent peptide-drug conjugate, because the uptake is decreased due to the presence of a signle cell-penetrating peptide. In contrast, doxorubicin enters the cell by passive diffusion through the cell membrane and is thereby an easy target for the membrane-associated transporter proteins (Siegfried et al. 1985). In addition, the cytotoxic effects of the peptide-drug conjugates can be strengthened by the fact that polyarginine cell-penetrating peptides are known to rapidly distribute into the nucleus after cellular internalization (Duchardt et al. 2007). This feature is highly desired for substances like anthracycline-based drugs, which develop their cytotoxicity around DNA.

Intracellular Trafficking

We investigated the native drug and **13** by fluorescence confocal laser scanning microscopy at various points of time (Fig. 2), to get insight of their uptake and if the subcellular distribution of the multivalent compound has an influence on

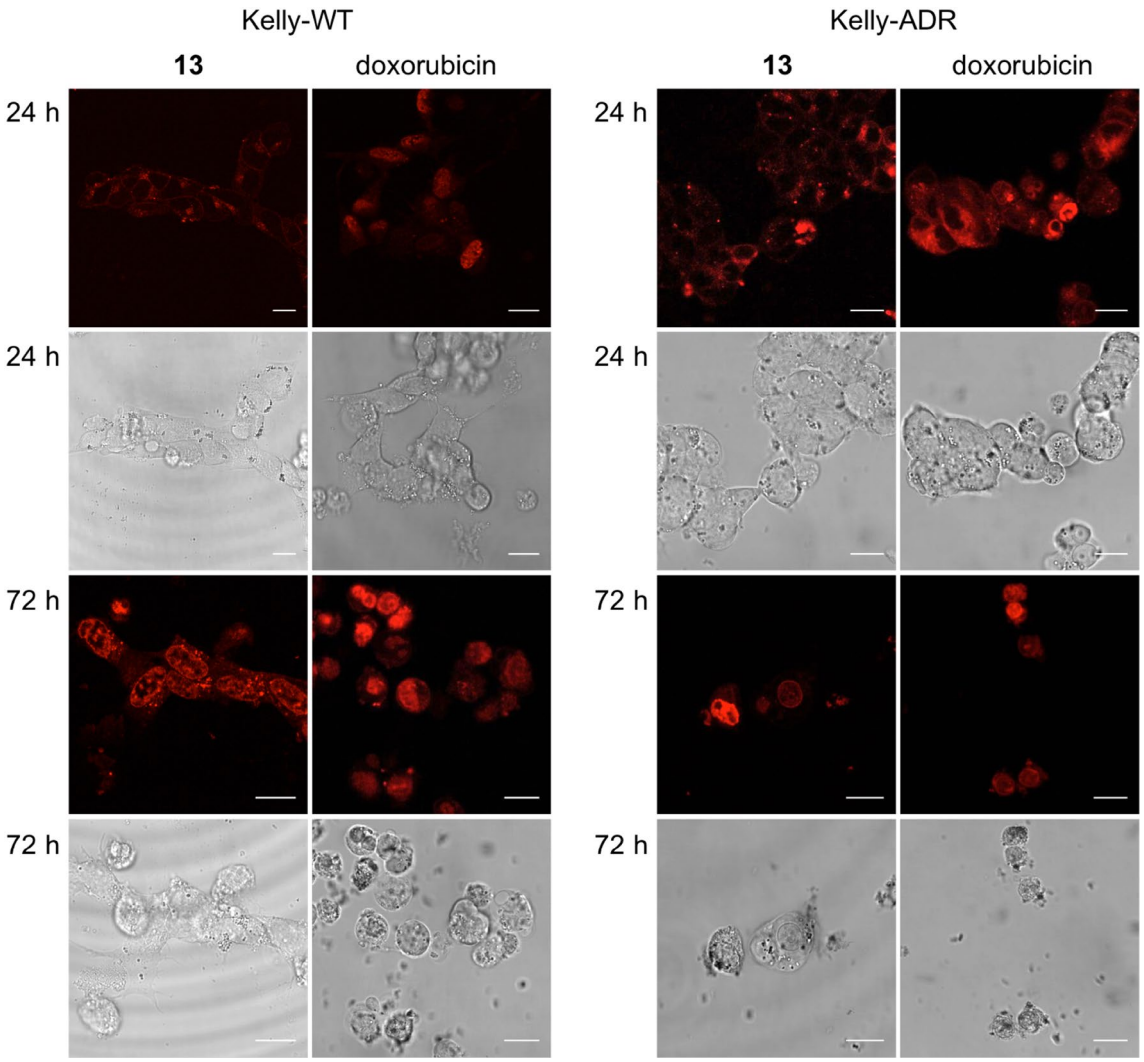


Fig. 2 Investigation of the subcellular distribution of the peptide-drug conjugate **13** and doxorubicin in Kelly-WT and Kelly-ADR cells by fluorescence confocal laser scanning microscopy, 24 and 72 h after the incubation with 10 µM of the drug at 37 °C. Fluorescence related to the fluorophore part of the anthracycline is illustrated by *red color* and the associated brightfield images are depicted below. *Scale bars* represent 15 µm. (Color figure online)

the cytotoxicity accordingly. After 24 h of incubation with the drugs, anthracycline-associated fluorescence (depicted in red) was visible in both adriamycin-sensitive Kelly-WT and adriamycin-resistant Kelly-ADR cells. The cell uptake of doxorubicin at that point of time was higher compared to **13** for wild-type as well as drug-resistant cells. This is attributed to the different uptake mechanisms of the compounds, which are not equally fast. Typically, the passive diffusion of doxorubicin across the cell membrane is a process faster than endocytosis. In Kelly-WT cells doxorubicin was found already in the nucleus, whereas Kelly-ADR cells exhibited no drug in the nuclei. By contrast, the uptake of **13** was equal in both types of cells.

Longer incubation (72 h) of the cells with the multivalent peptide-drug conjugate has shown a similar result. This observation corresponds to the data from the cell viability assay, where **13** was approximately equally toxic to drug-sensitive as well as resistant neuroblastoma cells (Table 1). Compared to the results after 24 h, the uptake of the bioconjugate increased, which was not the case for the native drug in Kelly-ADR cells. A reasonable explanation for this phenomenon is that doxorubicin got excreted from the drug-resistant cells by the membrane-associated transporter proteins. This supports the obtained IC_{50} values and our hypothesis that the multivalent peptide-drug conjugate can bypass efflux pumps, to overcome drug resistance in cancer cells.

To get deeper insights, whether the anthracycline derivative can enter the nucleus after the glutathione-dependent cleavage of the peptide-drug conjugate, Kelly-WT and Kelly-ADR cells were incubated with **13** and analyzed via fluorescence confocal laser scanning microscopy while the nuclei of the tumor cells were simultaneously stained with DRAQ5 (Fig. 3). The nuclear staining is depicted in blue, whereas colocalization with the drug (red color) appears violet. The obtained microscopy images highlight that the cleaved cell-penetrating peptide-doxorubicin conjugate delivers the anthracycline drug to its target in both adriamycin-sensitive Kelly-WT and adriamycin-resistant Kelly-ADR cells, which is a crucial prerequisite for the overcoming of drug resistance.

Conclusions

Multivalency is a powerful strategy found in many biological systems, utilized for achieving high-affinity molecular recognition and specificity through avidity. Multivalent peptides based on a synthetic scaffold like multiple antigen peptides have already found application as immunogens that can induce an immune response. In this work we hypothesize that multiple cell-penetrating peptides can possess stronger interaction with the cellular membrane and as consequence lead to a better cellular uptake of a chemotherapeutic in drug-resistant cell lines. For this purpose we designed and prepared a multivalent drug-peptide conjugate by attaching cell-penetrating peptides to an anthracycline drug using a low-molecular weight cleavable linker. The fluorescent chemotherapeutic drug was used to reveal the translocation of the conjugate through the cell membrane and its intracellular distribution. We investigated the cytotoxicity of the multivalent construct in a drug-resistant cell line, which confirmed our hypothesis

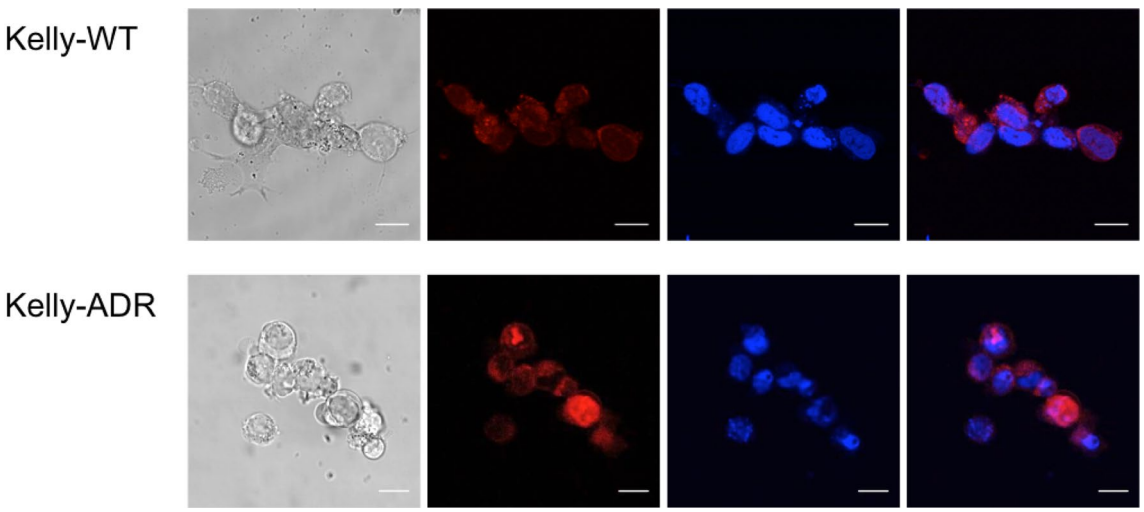


Fig. 3 Examination of the nuclear localization of the anthracycline drug in Kelly-WT and Kelly-ADR cells by fluorescence confocal laser scanning microscopy. Images were taken 72 h after the incubation with 10 µM of the peptide-drug conjugate **13** at 37 °C. Drug-associated fluorescence is illustrated by *red color*, whereas the nuclear stain DRAQ5 is depicted in *blue*. The corresponding bright-field images are represented by the *left panels* and the *right panels* display the overlay of the anthracycline-associated fluorescence and the nuclear counterstain. Colocalization is visualized by *violet color*. *Scale bars* represent 15 µm. (Color figure online)

that multimerization can enhance the efficacy of drug-peptide conjugates. Additionally, the herein presented synthetic approach and the multivalent structure can be further employed as model systems, to gain deeper insights into membrane-associated biological processes or for the preparation of more complex multimers that mimic natural multivalent systems.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Research Involving Human and Animal Rights This article does not contain any studies with animals and human participants performed by any of the authors.

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