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Novel Doxorubicin–Monoclonal Anti-carcinoembryonic Antigen Antibody Immunoconjugate Activity *in vitro*

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Abstract—Doxorubicin was modified with five different heterobifunctional reagents to produce drug analogs containing 3'-*N*-amide or C-13 hydrazone linkage with maleimide. Synthesis and characterization of two new reagents, 4-maleimidobenzohydrazide trifluoroacetate salt (**13**) and *N*-(4-maleimidobenzoyl)-6-aminocaprohydrazide trifluoroacetate salt (**14**) are described here. All Dox maleimido derivatives were conjugated to thiolated anti-carcinoembryonic antigen monoclonal antibody, 11-285-14, via a Michael addition reaction. Antibody-directed cytotoxicity was demonstrated with the MTT assay using combinations of antigen-positive and antigen-negative cell lines. The immunoconjugates prepared from Dox 3'-*N*-amide analogs are not active *in vitro*, however, Dox(hydrazone-linked) immunoconjugates are selectively toxic to the CEA positive cell line.

Introduction

Recent developments in conjugation chemistry focus on novel methods for producing drug–monoclonal antibody (MAb) immunoconjugates for site-specific drug delivery with preservation of both drug toxicity and antibody binding. Efforts to investigate the usefulness of antibody mediated targeting (AMT) have led to the development of a variety of cross-linking reagents to produce immunoconjugates in which the linkage can be cleaved by the target cells. The development and efficacy of AMT have recently been reviewed.[1,2]

Doxorubicin (Dox, **1**; Chart 1) plays an important role in cancer treatment and may be the most utilized antitumor drug worldwide.[3] However, its efficacy is impeded by toxicity, including myelosuppression, mucositis and alopecia. The greatest concern is the unique cumulative cardiac damage, which is the major obstacle to the use of Dox in cancer treatment.[4] Thus, Dox is the logical choice for conjugation with MAbs in targeted chemotherapy. Carcinoembryonic antigen (CEA) is the best known tumor associated antigen which often exists in elevated amounts in various human cancers and in the blood of cancer patients and is associated with the common solid tumors that cause high mortality.[5] Thus, CEA highly specific MAb, 11-285-14, was selected as a carrier of Dox. Moreover, recent investigations in our laboratory have demonstrated that this particular MAb is internalized in all high CEA-expressing cell lines such as LS174T. This is an essential requirement to achieve drug targeting and potential *in situ* release of the drug.[6]

Recently, the development of hydrazone linkers has been shown to allow release of the unmodified Dox under mild acidic conditions and to produce complete tumor regression *in vivo*.[7–9] Deconjugation via a chemical process in the intracellular compartment, lysosome, to release the free drugs will become the major approach to design Dox immunoconjugates for improving therapeutic efficacy.

Below, we report the *in vitro* cytotoxicity results obtained with Dox 3'-*N*-amide derivatives (**2a–4a**), Dox C-13 hydrazone derivatives (**5a**, **6a**) and their immunoconjugates (**2b–6b**). *In vitro* testing indicates that the Dox 3'-*N*-amide linked immunoconjugates are not active. However, preliminary evaluation of Dox C-13 hydrazone linked immunoconjugates suggests selective toxicity for a high CEA-expressing cell line.

Results and Discussion

This paper reports on our effort to use MAb as a tool in targeted chemotherapy. We also describe the methods for linking Dox to MAbs via an acylhydrazone and arylhydrazone bond at the 13-keto position of Dox. Our newly developed heterobifunctional linkers (**9**, **10**, **13** and **14**) are regioselectively reacted with the 3'-*N*-amine or C-13 keto moiety of Dox. All linkers are easy to prepare and stable during handling. Using these linkers, we obtained immunoconjugates with different linkages (amide or hydrazone linkage) and different linker arms (phenyl, *N*-benzoylaminopentyl or *N*-benzoylaminodecyl spacer arm), and then compared the cytotoxic effect from these immunoconjugates *in vitro*.

CEA highly specific MAb, 11-285-14, has been well characterized immunocytochemically and confirmed to

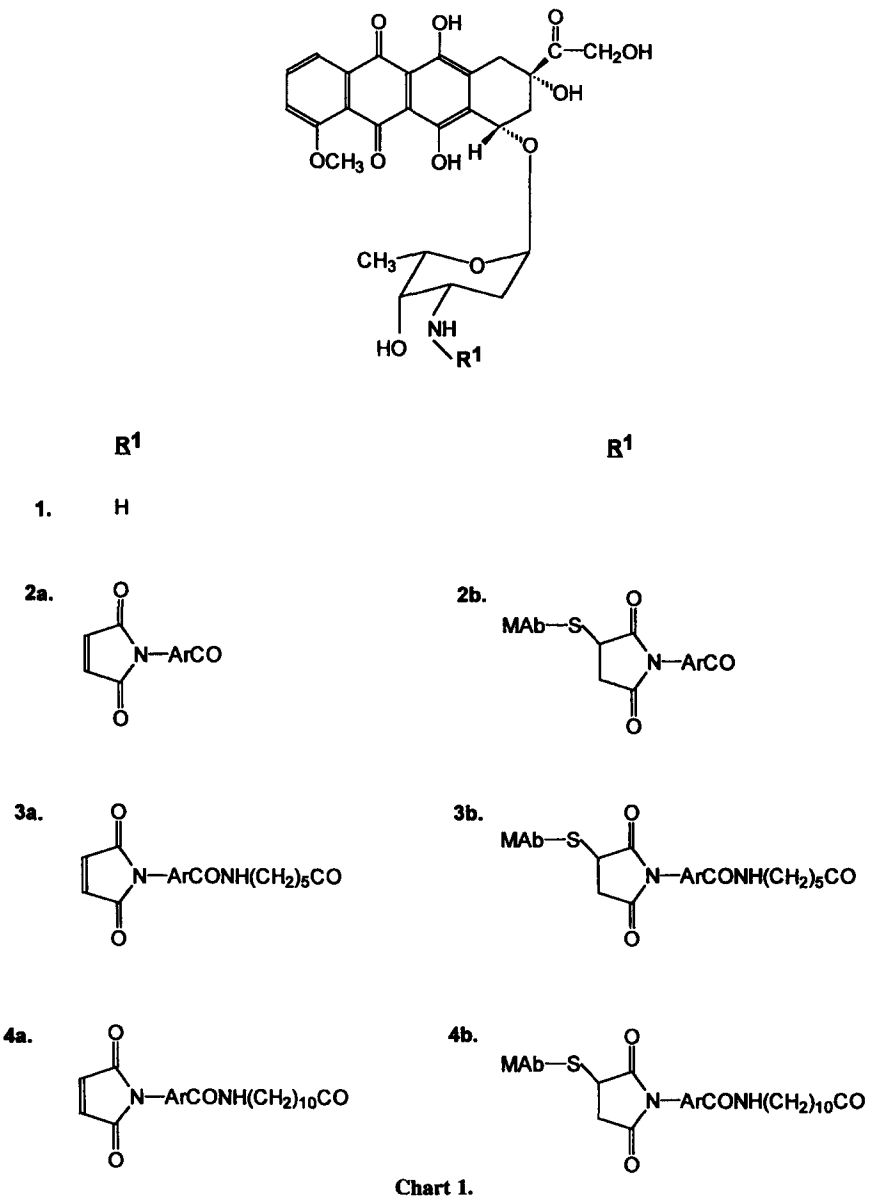


Chart 1.

be reactive only with CEA and non-reactive with normal cross-reacting antigens (NCA).[10-12] The potential of targeted chemotherapy using vindesine 11-285-14 conjugates had previously been demonstrated *in vitro*; the efficacy and specificity have been correlated with CEA density in different cancer cell lines.[13] These conjugates have shown their efficacy *in vivo* with xenografts in a nude mouse model.[14] Preliminary results with Dox–11-285-14 conjugates have also shown efficacy *in vitro*.[15] Therefore, we further investigated the efficacy of Dox–11-285-14 conjugates using novel heterobifunctional linkers.

Dox has been coupled to an antibody via the amino group of the sugar moiety (daunosamine) via either the carbonyl group at C-13 or the C-14 aglycon side chain. Modifications of the amino sugar of anthracyclines had been shown to decrease the cytotoxic activity of the drug.[16] Our experimental results indicated that all Dox 3'-*N*-amide derivatives were less toxic than the unconjugated drug against the CEA positive cell line, LS174T. The binding of Dox to DNA by intercalation is one of the most accepted mechanisms postulated for its cytotoxicity. Structure–activity studies with anthracyclines have shown that the sugar residue is an important contributor to cytotoxicity.[4,16]

It has been suggested that anthracycline immunoconjugates show no significant cytotoxicity if the drugs are not deconjugated from the antibody carrier at the target site.[17] The attachment of Dox to the antibody with acid-sensitive linkage and the release of Dox from immunoconjugates after internalization inside the lysosome compartment enable the drug to retain pharmacological activity. The very poor cytotoxicity of Dox 3'-*N*-amide immunoconjugates is probably due to a reduced intracellular release of the active drug from the conjugates. The amide linkage has been shown to be too stable to produce an effective conjugate.[18] However, the bioconjugates prepared from Dox or Daunorubicin

3'-*N*-amide analogs that contain a phenyl isothiocyanate group have shown efficacy *in vitro*.[19] Moreover, there have been a few reports in the literature where drug derivatives were inactive *in vitro* but after being linked to an antibody, showed increased cytotoxicity of the immunoconjugates.[20,21] *In vitro* testing results from Dox 3'-*N*-amide derivatives and immunoconjugates were matched with each other; no activity was obtained from any drug derivative nor its conjugate. If the drug derivatives do not exhibit any cytotoxicity, one might expect that the immunoconjugates should not show any activity when tested *in vitro*.

Synthesis of Dox immunoconjugates

Synthesis of the immunoconjugates was achieved by thiolating the MAbs with 2-iminothiolane (2-IT) and reacting the thiolated MAbs with Dox derivatives which contained maleimide. The Dox derivatives and their corresponding immunoconjugates are listed in Charts 1 and 2. Dox:MAb ratios of 1.75–2.63 were achieved when 10 equivalents of Dox derivatives were mixed with MAb containing 6.26–7.14 thiol groups. Final protein yields following conjugation of drug to MAb are 32–60%.

Cytotoxicity of Dox derivatives and immunoconjugates on human colon adenocarcinoma cell lines

Dox derivatives and immunoconjugates were tested *in vitro* for cytotoxicity using the MTT assay with antigen positive (LS174T) and antigen negative (COLO-320DM) cell lines. All Dox 3'-*N*-amide derivatives did not show significant cytotoxic effects on the LS174T cell line when compared to Dox (Fig.1). Derivative **2a** showed the highest cytotoxicity and caused 57% cell mortality at 40 μM while **3a** caused 50% cell death at 40 μM. Compound **4a** was much less cytotoxic and showed 30% inhibition of cell growth at 40 μM. However,

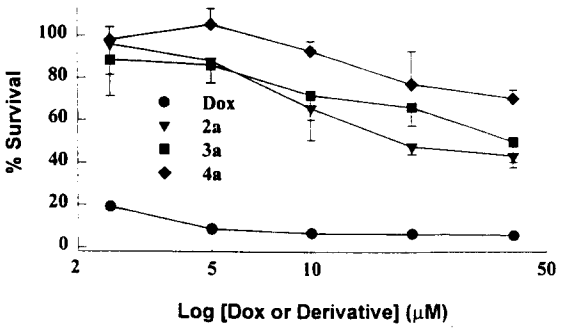
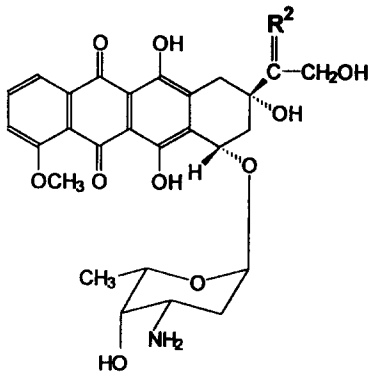


Figure 1. Dose–response curves for Dox and Dox 3'*N*-amide derivatives on LS174T cells (mean ± SD).



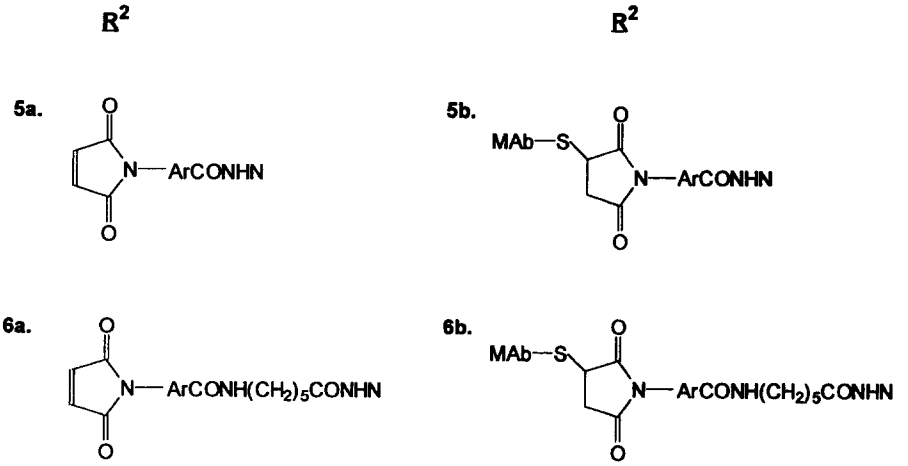


Chart 2.

the Dox hydrazone derivatives **5a** and **6a** showed similar cytotoxic effects when compared to Dox on both the LS174T and COLO320DM cell lines (Figs 2 and 3). The LS174T cell line showed higher sensitivity to Dox and Dox hydrazone derivatives than the COLO320DM cell line.

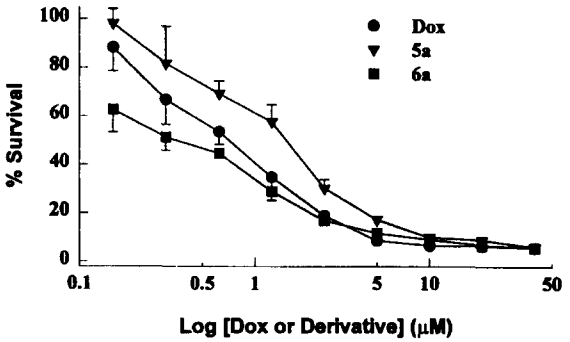


Figure 2. Dose–response curves for Dox and Dox C-13 hydrazone derivatives on LS174T cells (mean ± SD).

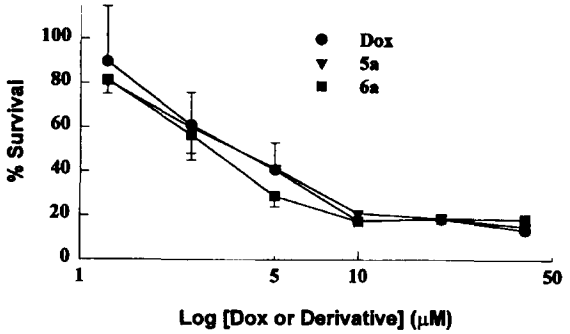


Figure 3. Dose-response curves for Dox and Dox C-13 hydrazone derivatives on COLO320DM cells (mean ± SD).

Immunoconjugates synthesized from Dox 3'-*N*-amide derivatives were not sufficiently cytotoxic to produce 50% inhibition of growth of the LS174T cell line (Fig. 4). These immunoconjugates exhibited only very low cytotoxicity even at 10 μM. However, the immunoconjugates prepared from Dox C-13 hydrazone derivatives were sufficiently cytotoxic to achieve 50% inhibition of LS174T cell growth at ~8 μM (Fig. 5) and had no appreciable cytotoxic effects on the COLO-320DM cell line (Fig. 6).

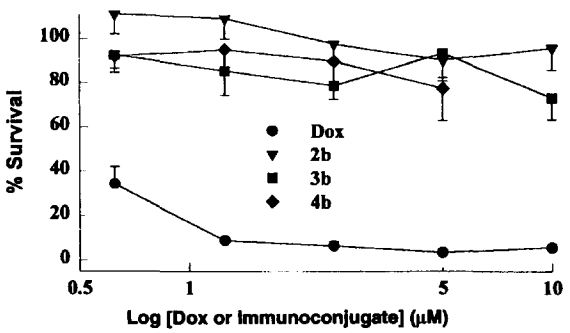


Figure 4. Dose–response curves for Dox and Dox 3'-*N*-amide immunoconjugates on LS174T cells (mean ± SD).

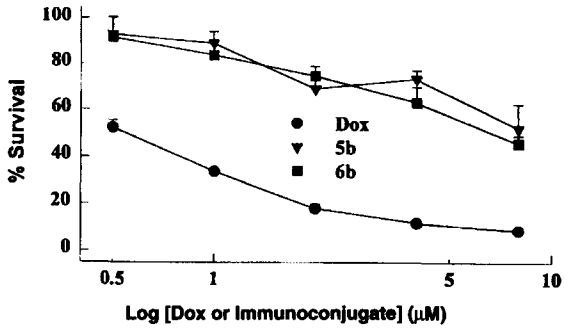


Figure 5. Dose–response curves for Dox and Dox C-13 hydrazone immunoconjugates on LS174T cells (mean ± SD).

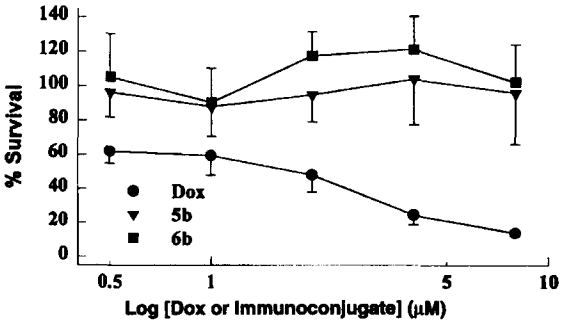


Figure 6. Dose–response curves for Dox and Dox C-13 hydrazone immunoconjugates on COLO320DM cells (mean ± SD).

The Dox(hydrazone-linked) immunoconjugates were more potent than Dox 3'-*N*-amide immunoconjugates, but not as potent as their starting material, Dox C-13 hydrazone derivatives. A gradual reduction in the percentage of surviving cells as the immunoconjugate concentration increased was observed when the high CEA-expressing cell line, LS174T, was evaluated. Selectivity was confirmed by the absence of conjugate effect on the low CEA-expressing cell line, COLO-320DM, with the same concentration range. The Dox(hydrazone-linked) immunoconjugates showed efficacy in *in vitro* testing; they were sufficiently cytotoxic to achieve 50% inhibition of LS174T cell growth at ~8 μM. The efficacy of immunoconjugates depends on the density of antigen expressed on the target cells and the quantity of drug delivered per antibody molecule. Thus, immunoconjugates prepared with a higher drug substitution should be more potent than low drug substitution, because more drug is delivered per antibody molecule bound to the target cell. Higher Dox:MAb ratio can be simply achieved by increasing the thiol groups on the MAb and/or Dox derivative for the conjugation reaction. However, we observed that significant losses in protein yield occurred following conjugation. Thus, around seven thiol groups per MAb and a 10-fold molar excess of Dox derivative to MAb were adopted for the final conjugation reaction. Dox:MAb ratios of 1.75–2.63 obtained in our immunoconjugates are probably too low for good antitumor activity.

The present report describes the synthesis of two new linkers, **13** and **14**. The preliminary testing results show

that Dox 3'-*N*-amide immunoconjugates **2b, 3b** and **4b** are not active *in vitro* while Dox(hydrazone-linked) immunoconjugates **5b** and **6b** are active *in vitro* and appear to provide a more promising approach. Further investigation of the correct spacer groups for effective release of the drug appears to be essential for development of doxorubicin immunoconjugates. The improvement of conjugation conditions is also essential to prevent protein aggregation and precipitation. These will hopefully contribute to the development of better conjugates for antibody mediated targeting.

Experimental

Monoclonal antibody

MAb (11-285-14) was purified from ascitic fluid on a protein-A affinity column according to the procedure of Ford *et al.*[22]

Cell lines

LS174T and COLO320DM (both are human colon adenocarcinoma cell lines) were obtained from the American Type Culture Collection (Maryland). LS174T cells were propagated in RPMI-1640 containing 8.8% fetal calf serum, glutamine and penicillin–streptomycin. COLO320DM were maintained in minimum essential medium with 8.8% fetal calf serum, glutamine, non-essential amino acids and penicillin–streptomycin. Cells were grown at 37 °C in a humid atmosphere with 5% CO_2.

Materials

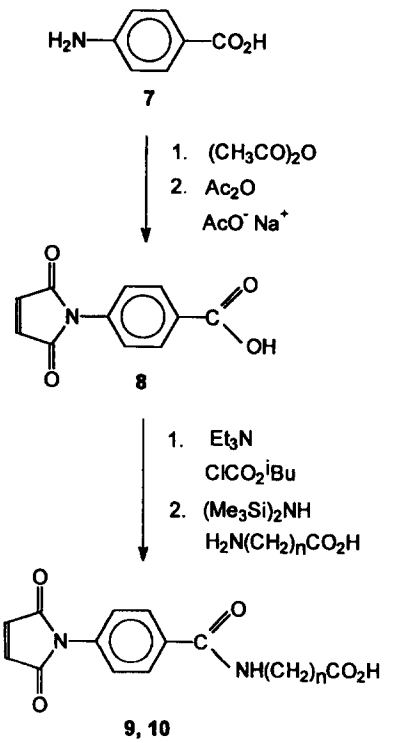
Anhydrous reactions were performed under an inert atmosphere of nitrogen. Unless otherwise noted, starting material, reactant and solvents were obtained commercially from Aldrich and were used as such or purified and/or dried by standard means.[23] Dox–HCl was a gift from Adria Laboratories Inc. (Ohio). Organic solvents were dried over $MgSO_4$, evaporated on a rotatory evaporator and under reduced pressure. All reactions were monitored by TLC. The plates were visualized by UV fluorescence. Commercial TLC plates were Sigma T6145 (polyester silica gel 60 Å 0.25 mm). Flash chromatography was performed according to the method of Still *et al.* on Merck grade 60 silica gel, 230–400 mesh.[24] Melting points were recorded on an Electrothermal 9100 apparatus and are uncorrected. The IR spectra were taken on a Nicolet model 205 FT-IR spectrophotometer. MS assays, (m/z) were obtained using a VG Micromass 7070 HS instrument with an ionization energy of 70 eV. Elemental analysis was conducted by Microanalysis Laboratories Limited, Markham, Ontario. NMR spectra were obtained in deuterated Me_2SO_4, MeOH and $CHCl_3$ on a General Electric GE 300-NB (300 MHz) instrument: chemical shifts were measured relative to internal standards: tetramethylsilane (TMS, δ 0.0 ppm) for ^1H and ^{13}C NMR spectra.

Thiolation of MAbs

Thiolation of MAbs with 2-iminothiolane (2-IT) was carried out as described before.[25] MAbs (1.48 or 1.70 mg mL^{-1} in 0.1 M phosphate buffer, 1 mM EDTA, pH 8.0) were treated with 0.1 M 2-IT to make the molar ratio of MAb to 2-IT 1:100. The reaction mixture was incubated for 2 h at rt and thiolated MAbs were separated from excess 2-IT on a Sephadex G-25 column equilibrated with PBS (0.9% NaCl:1 mM EDTA, pH 7.0). The thiolated MAbs were concentrated down using an Amicon ultrafiltration cell fitted with an Amicon PM10 ultrafilter (molecular weight cutoff 10,000). The average number of thiol groups per protein molecule was determined by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).[26]

Synthesis of heterobifunctional linkers and Dox derivatives

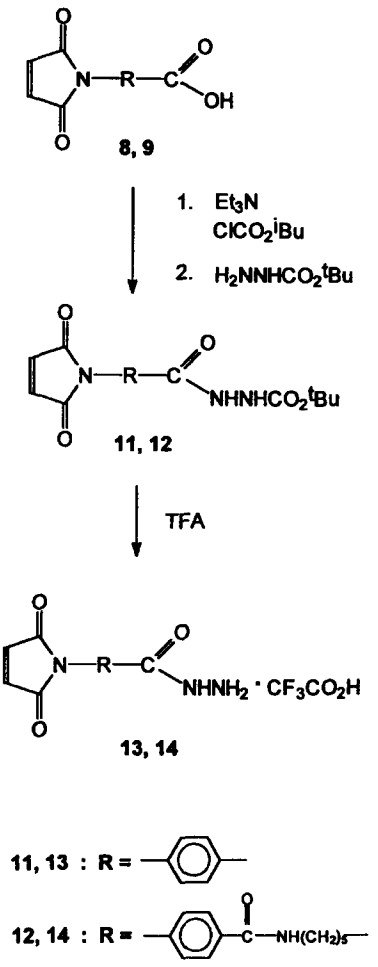
The synthesis and characterization of the linkers, 4-maleimido-benzoic acid (**8**), *N*-(4-maleimidobenzoyl)-6-aminocaproic acid (**9**) and *N*-(4-maleimidobenzoyl)-11-aminoundecanoic acid (**10**) (Scheme 1), and their Dox derivatives (**2a–4a**) were reported in the preceding paper.[25]



Scheme 1.

Derivatives **13** and **14** were prepared directly from acids **8** and **9**, respectively by converting the carboxylic acid group to protected hydrazide followed by treatment with

trifluoroacetatic acid (TFA) (Scheme 2), using a procedure modified from Willner *et al.*[8] Compound **8** (2.68 g, 1.3 mmol) was suspended in 60 mL CH_2Cl_2 and cooled to 0 °C before the addition of triethylamine (1.89 mL, 13.6 mmol) and isobutyl chloroformate (1.76 mL, 13.6 mmol). The mixture was stirred for 1 h at 0 °C and then *tert*-butyl carbazate (1.62 g, 12.3 mmol) in 10 mL CH_2Cl_2 was added dropwise. Stirring was continued for 2 h at rt. The reaction mixture was diluted with EtOAc (150 mL) and washed with saturated $NaHCO_3$ (2 × 50 mL), 0.1 N HCl (2 × 50 mL), saturated NaCl (2 × 50 mL), H_2O (50 mL), then dried ($MgSO_4$) and evaporated to give crude protected hydrazide **11**. The product was purified by flash chromatography, using two mixtures of solvent: initially 1:2 and then 2:3 Me_2CO:hexane, to yield 2.44 g (60%) of **11**: mp 182–184 °C; IR (KBr) 3545, 3329, 3093, 2991 and 1710 cm^{-1}; ^1H NMR (Me_2CO-d_6): δ 9.60 (*s*, 1H), 8.02 and 7.53 (two *d*, 4H, *J* = 8.6 Hz), 7.07 (*s*, 2H), 2.99 (*s*, 1H), 1.45 (*s*, 9H); ^{13}C NMR (Me_2CO-d_6): δ 169.06 (2), 165.63, 155.47, 134.79, 134.34 (2), 131.38, 127.68 (2), 125.59 (2), 79.50, 27.30 (3); Anal. Calcd for $C_{16}H_{17}N_3O_5$: C, 57.97; H, 5.17; N, 12.69. Found: C, 58.06; H, 5.14; N, 12.73.



Scheme 2.

Compound **11** (500 mg, 1.51 mmol) was dissolved in ice-cold TFA (5 mL) and stirred for 30 min in an ice-bath. The TFA was removed under vacuum at rt to give compound **13** quantitively: mp 142–144 °C; IR (KBr) 3500–2500, 3277, 3111, 2901, 2720 and 1710 cm^{-1}, ^1H NMR (DMSO-d_6): δ 11.62 (*bs*, 1H), 8.63 (*bs*, 3H), 8.01 and 7.56 (two *d*, 4H, *J* = 8.5 Hz), 7.24 (*s*, 2H); ^{13}C NMR (DMSO-d_6): δ 169.60 (2), 165.43, 135.47, 134.97 (2), 129.38, 128.38 (2), 126.46 (2); Anal. Calcd for $C_{13}H_{18}N_3O_5F_3$: C, 44.17; H, 5.14; N, 11.89. Found: C, 44.16; H, 5.06; N, 11.93.

Compound **9** (1.05 g, 3.18 mmol) was treated with the same procedure as above for the preparation of **11** to yield **12** (1.01 g, 72%): mp 137–139 °C; IR (KBr) 3310, 3082, 2978, 2934 and 1707 cm^{-1}; ^1H NMR (Me_2CO-d_6): δ 8.80 (*s*, 1H), 8.00 and 7.48 (*d*, 2H, *J* = 8.7 Hz), 7.73 (*t*, 1H, *J* = 4.7 Hz), 7.48 (*d*, 2H, *J* = 8.7 Hz), 7.07 (*s*, 2H), 3.41 (*q* apparent, 2H, *J* = 6.9 Hz), 2.88 (*s*, 1H), 2.21 (*t*, 2H, *J* = 7.3 Hz), 1.64 (*m*, 4H), 1.44 (*m*, 2H), 1.41 (*s*, 9H); ^{13}C NMR (Me_2CO-d_6): δ 172.14, 170.22 (2), 166.47, 156.45, 135.39 (2), 135.19, 134.94, 128.45 (2), 126.65 (2), 80.17, 40.24, 34.15, 29.91 (superimposed on acetone peak), 28.36 (3), 27.08, 25.64; Anal. Calcd for $C_{22}H_{28}N_4O_6$: C, 59.43; H, 6.35; N, 12.61. Found: C, 59.48; H, 6.31; N, 12.58.

Compound **12** (500 mg, 1.12 mmol) was deprotected with the same procedure as above for the preparation of **13** to yield **14** quantitatively as an oily yellowish gum: IR (KBr) 3500–2500, 3107, 2944 and 1717 cm^{-1}; ^1H NMR (DMSO-d_6): δ 10.91 (*bs*, 1H), 9.40 (*bs*, 3H), 8.57 (*t*, 1H, *J* = 5.5 Hz), 7.95 and 7.45 (two *d*, 4H, *J* = 8.5 Hz), 7.22 (*s*, 2H), 3.28 (*q* apparent, 2H, *J* = 6.6 Hz), 2.25 (*t*, 2H, *J* = 7.3 Hz), 1.57 (*m*, 4H), 1.34 (*m*, 2H); ^{13}C NMR (DMSO-d_6): δ 171.79, 169.75 (2), 165.51, 134.86 (2), 133.95, 133.78, 127.82 (2), 126.22 (2), 40.22 (superimposed on DMSO peak), 32.73, 28.84, 26.01, 24.49. Anal. Calcd for $C_{19}H_{21}N_4O_6F_3$: C, 49.77; H, 4.62; N, 12.23. Found: C, 49.80; H, 4.57; N, 12.27.

Synthesis of derivative ***5a***. Dox–HCl (20.58 mg, 0.036 mmol) and **13** (41.28 mg, 0.120 mmol) were dissolved in 5 mL MeOH with the addition of 3 μL TFA. The mixture, protected from light, was stirred at rt for 1 day. The methanolic solution was concentrated to 1 mL under nitrogen at rt. Acetonitrile (4 mL) was added for precipitation and the suspension was allowed to stand at 4 °C for 1 day. The red precipitate was isolated by centrifugation and then dried under vacuum to give compound **5a** (20.2 mg, 72%). The product can be reprecipitated from the remaining solution and further purified by TLC. **5a**: ^1H NMR (DMSO-d_6): δ 14.07 (*s*, 1H), 13.35 (*bs*, 1H), 11.86 (*s*, 1H), 7.93–7.64 (*m*, 9H), 7.50 (*d*, 2H, *J* = 8.4 Hz), 7.22 (*s*, 2H), 5.49 (*d*, 1H, *J* = 5.6 Hz), 5.31 (*bs*, 1H), 5.12 (*bs*, 1H), 4.63 (*s*, 2H), 4.56 (*d*, 1H, *J* = 5.7 Hz), 3.98 (*s*, 3H), 3.55 (*bs*, 1H), 1.18 (*d*, 3H).

Synthesis of derivative ***6a***. Dox–HCl (20.34 mg, 0.035 mmol) and **14** (53.65 mg, 0.117 mmol, assuming 100% yield obtained from 52 mg of **12**) were dissolved in 5 mL MeOH with the addition of 3 μL TFA. The reaction condition and the precipitation procedure were similar to the preparation of **5a**. The red precipitate was dried

under vacuum to give 28.2 mg (90%) of **6a**; ^{1}H NMR (DMSO-d_6): δ 14.10 (*s*, 1H), 13.32 (*s*, 1H), 10.34 (*s*, 1H), 8.46 (*t*, 1H, *J* = 5.3 Hz), 7.90 (*m*, 7H), 7.60 (*dd*, 1H, *J* = 4.3 and 2.6 Hz), 7.42 (*d*, 2H, *J* = 7.4 Hz), 7.21 (*s*, 2H), 5.49 (*bs*, 1H), 5.31 (*bs*, 1H), 4.95 (*t*, 1H, *J* = 6.4 Hz), 4.42 (*s*, 2H), 4.04 (*q*, 1H), 3.95 (*s*, 3H), 1.18 (*d*, 3H).

Conjugation of thiolated MAbs with Dox derivatives

Derivative **2a, 3a, 4a, 5a** or **6a** was dissolved in DMF and added to 2-IT thiolated MAbs in PBS (0.9% NaCl:1 mM EDTA, pH 7.0). In a typical experiment, 10 equivalents of **5a** were added to MAbs containing 6.55 thiol groups. Conjugation reaction was incubated at rt for 1.5 h. Then, the immunoconjugates was separated from unreacted **5a** by passage through a Sephadex G-25 column equilibrated with PBS (pH 7.2). The conjugate solution was concentrated down by ultrafiltration and then subjected to dialysis (2 × 8 h in PBS, pH 7.2). The absorbances of conjugates were determined at 280 nm and 495 nm to estimate protein and drug concentrations respectively, using the molar extinction coefficients summarized in Table 1. To correct for the overlap of Dox absorbance at 280 nm, the amount of MAb was determined according to the following formula

$$\text{MAb } (\mu\text{M}) = \frac{A_{280} - (\varepsilon_{280} \times A_{495}/\varepsilon_{495})}{214{,}600}$$

where *A* is the observed absorbance at noted wavelength and ε is the molar extinction coefficient of the Dox derivative at noted wavelength. The Dox:MAb molar ratios ot the conjugates are presented in Table 2.

Microcytostasis assay

Chemosensitivity of cell lines to Dox, Dox derivatives and the immunoconjugates were determined using the MTT assay.[27] The cells (1 × 10^4 cells $well^{-1}$) were grown in sterile 96-well culture plates at 37 °C in a humidified incubator with 5% CO_2 for 24 h, then incubated for 24 h with Dox, Dox derivatives or Dox immunoconjugates diluted in culture medium. After incubation, the cells were washed with PBS and incubated for 24 h, and then treated with MTT for 4 h. The precipitate was solubilized in DMSO and the plate was read spectrophotometrically at 570 and 630 nm. Absorbance was recorded and percentage cell survival was determined by comparing drug-, drug derivative-, or immunoconjugate-treated cells with the untreated control.

Acknowledgements

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Table 1. Molar extinction coefficient (ε) of Dox derivatives **2a–6a**

Compound	λ_{max} (nm)	ε	ε_{280}	ε_{495}	Solvent
2a	267	16853	12508	12380	DMF
3a	267	16248	11413	13236	DMF
4a	267	16017	11754	13061	DMF
5a	268	26320	18510	12531	DMF
6a	268	16028	12315	13051	DMF
IgG	280	214600			PBS

Dox 3'-*N*-amine derivatives (**2a–4a**).
Dox C-13 hydrazone derivatives (**5a** and **6a**).

Table 2. Spectrophotometric evaluation of the Dox-11-285-14 conjugates

C	C A 280nm	C A 495nm	Dox A 280nm	MAb A 280nm	Dox (μM)	MAb (μM)	Dox/ MAb
2b	1.82	0.24	0.24	1.58	19.38	7.36	2.63
3b	2.10	0.24	0.21	1.89	18.13	8.81	2.06
4b	1.13	0.11	0.10	1.03	8.42	4.80	1.75
5b	1.41	0.15	0.23	1.18	11.97	5.50	2.18
6b	1.16	0.14	0.13	1.03	10.72	4.80	2.23

C = Conjugate, A = absorbance.

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