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Dipeptide-based highly potent doxorubicin antibody conjugates

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Abstract—Highly potent and novel derivatives of doxorubicin were linked to monoclonal antibodies (mAbs) for site-specific drug delivery. Drug linker **5** consisted of a dipeptide linker attached directly to the daunosamine nitrogen of the *n*-butyldiacetate doxorubicin derivative **2a**. Upon hydrolysis of the peptide linker and acetate groups, the free daunosamine nitrogen is able to form the highly potent 2-pyrrolinodoxorubicin (**3a**). The second approach involved the use of an oxazolidine carbamate (**13**) to mask an activating aldehyde group until proteolytic hydrolysis releases **3a**. Both drug linkers were shown to be substrates for the lysosomal enzyme cathepsin B. Each molecule was conjugated to the mAbs c1F6 (anti-CD70) and cAC10 (anti-CD30) to give potent drug conjugates against renal cell carcinoma and anaplastic large cell lymphoma cell lines, respectively. The activities were immunologically selective, since antigen negative cell lines were much less sensitive to treatment with the drug conjugates. The approaches described here for attaching highly potent doxorubicin derivatives to mAbs are novel and allow for control of drug stability while covalently bound to the delivery agent.

A great deal of attention has surrounded the use of monoclonal antibodies (mAbs) for the delivery of anticancer drugs to tumor cells. Several mAb–drug conjugates have displayed pronounced activities in preclinical cancer models, and there are now three approved antibody–cytotoxin conjugates for cancer therapy: Mylotarg™ (gemtuzumab ozogamicin), Zevalin™ (ibritumomab tiuxetan), and Bexxar™ (tositumomab). Research surrounding the critical parameters for therapeutic success has suggested that highly potent drugs are required for mAb-based delivery strategies, and the linker used to attach the drug to the mAb should be highly stable in circulation. A significant body of literature describes efforts in this field using an assortment of cytotoxic payloads including doxorubicin,[1,2] taxanes,[3] maytansinoids,[4] minor groove binders,[5–8] and others. We have recently reported ADC technologies based on the antimitotic agent monomethyl auristatin E[9–11] and minor groove binders[8] released through proteolytic cleavage of dipeptide linkers. This communication describes our work with highly potent derivatives of doxorubicin which employ a similar proteolytic release strategy. In one example, we employed a novel masking strategy for tethering to the mAb through a latent aldehyde group to result in highly potent cytotoxic activity.

Cyclic derivatives of doxorubicin involving the daunosamine sugar nitrogen were first reported by Acton et al.[12] The morpholino cyanide compound **1** was reported to be intensely potent, displaying cytotoxic activities several log units higher than doxorubicin. Structure–activity relationship studies suggested that the potential for iminium formation through displacement of the cyanide group was key to increased potency of **1** relative to doxorubicin. More recently, Farquhar[13] reported *n*-butyl and *n*-pentyl diacetate derivatives of doxorubicin (**2a**,**b**) which displayed a similar potency profile to **1**. The basis for activity likely involves esterase-mediated hydrolysis of the acyclic diacetates **2a**,**b**. The resulting aldehydes cyclize to form derivatives **3a**,**b**[14] (Scheme 1). These compounds alkylate double-stranded DNA, through the corresponding cyclic iminium, resulting in interstrand crosslinking and apoptosis.[15] In addition, they are capable of overcoming P-glycoprotein-mediated multidrug resistance.[13] A recent report demonstrated the use of the pentyl diacetate **2b** tethered through a hydrazone linkage to the mAb BR96 which displayed improved potency relative to a doxorubicin hydrazone linker.[16] We wanted to explore

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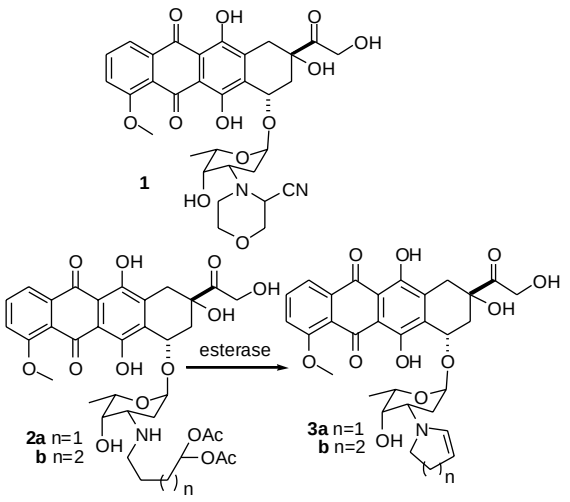


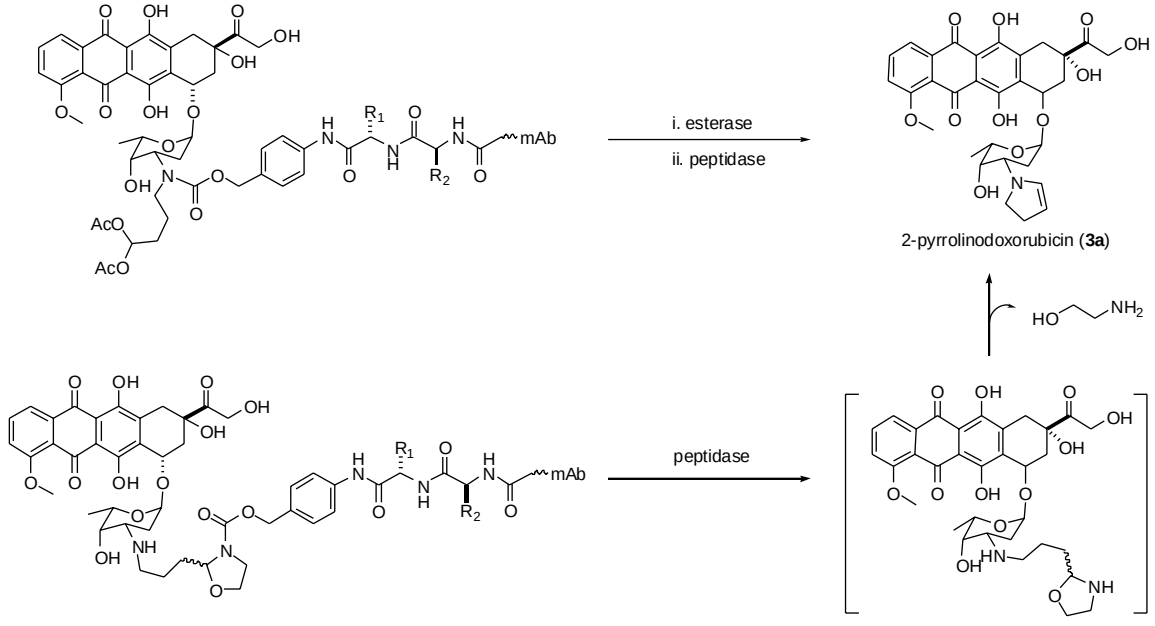
Scheme 1.

conjugates based on these highly potent doxorubicin derivatives employing a more stable dipeptide linkage for mAb-targeted delivery. We have already shown that these molecules can be stably masked and activated by a targeted mAb-enzyme conjugate.[17]

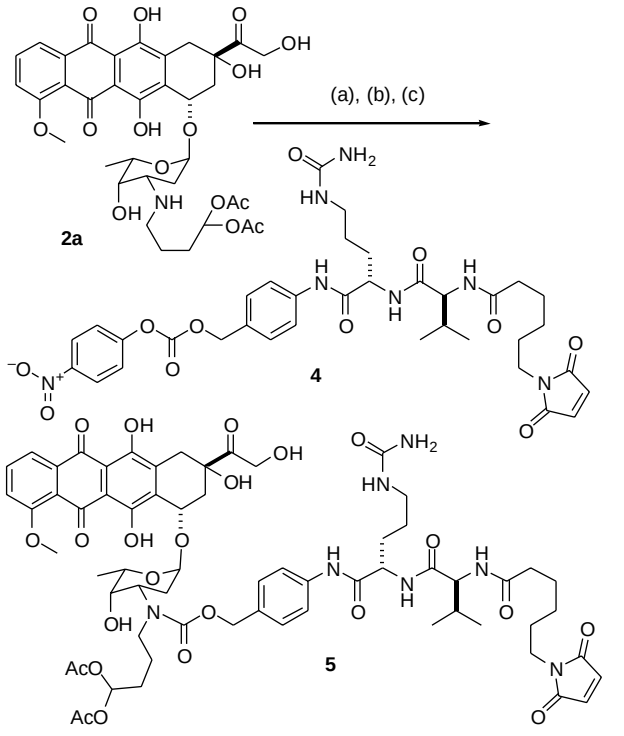
Two approaches were conceived for targeted delivery and protease-mediated release of **3a** (Scheme 2). The key feature of both methods was that the formation of a reactive pyrroline ring would not occur until the drug was released from the mAb by proteolytic hydrolysis of the peptide linker. This is in contrast to an approach previously taken with this class of molecules in which the drug could undergo activation while bound to the mAb carrier.[16] The first approach illustrated in Scheme 2 involved attaching the dipeptide linker, through a *p*-aminobenzyl carbamate spacer (PABC), to the daunosamine nitrogen of **2a**. Hydrolysis of both the geminal diacetate group and the dipeptide (resulting in 1,6-elimination of drug through the PABC spacer), and loss of a water molecule would generate **3a**. The second approach involved tethering the molecule through an oxazolidine carbamate a group which would stably mask the activating aldehyde group until proteolytic cleavage from the mAb. By tethering through a masked aldehyde, we hoped to prevent potentially undesired chemistry involving a free aldehyde that might compromise the drug while attached to the mAb. Cleavage of the dipeptide would result in 1,6-elimination to liberate an intermediate that should rapidly cyclize to **3a**. The peptide sequence val-ala was chosen over val-cit to reduce hydrophobicity and the potential for aggregation.

To construct the molecule described as Approach I (Scheme 2), compound **2a** was reacted with the *p*-nitrophenyl (PNP) carbonate of the valine-citrulline (val-cit) dipeptide linker **4**[18,19] (Scheme 3). This chemistry was problematic, due to the hindered nature of daunosamine nitrogen of **2a**. Nevertheless, low yields of the desired agent could be obtained by first protecting **2a** as its mono-TBS ether. Coupling with **4** and deprotection afforded the drug-linker **5**.

To prepare the oxazolidine carbamate described as Approach II (Scheme 2), the benzyl alcohol **6** was activated using diphosgene and reacted with the oxazolidine **7** formed through treatment of aldehyde **8** (prepared from 1,4-butanediol in two steps) with ethanolamine in the presence of 4 Å molecular sieve powder (Scheme 4). The resulting product was N-deprotected with piperidine to give **9**. Coupling with Fmoc-Val-OSu and deprotection of both the TBDPS and Fmoc groups with TBAF afforded **10**. This was followed by coupling of the free amine to maleimidocaproyl *N*-hydroxysuccinamide ester (MC-OSu) and oxidation of the alcohol



Scheme 2. Approach I: *n*-butyl diacetate carbamate. Approach II: oxazolidine carbamate.

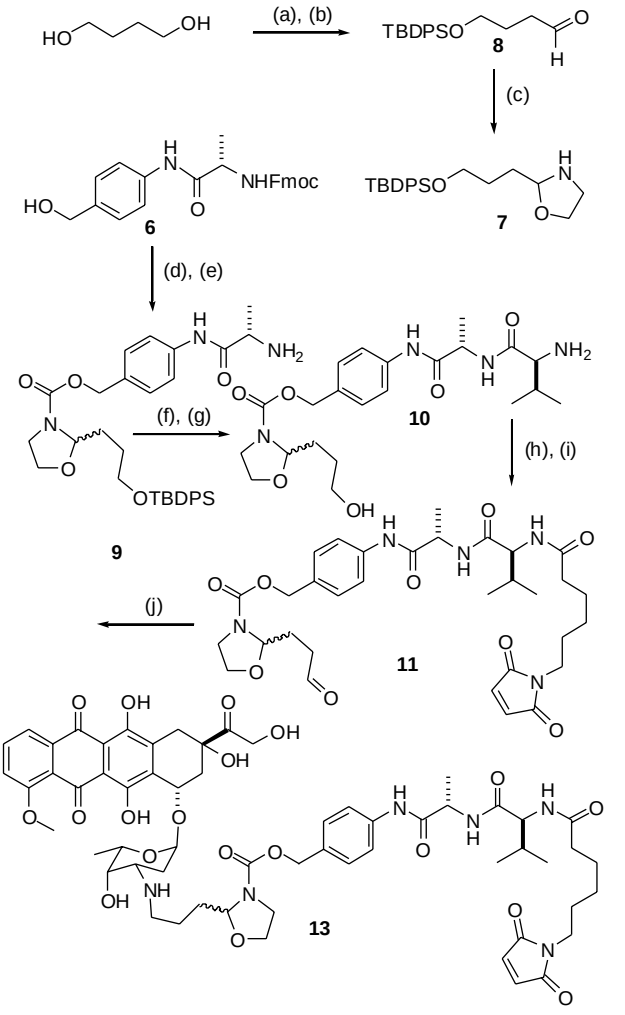


Scheme 3. Reagents and conditions: (a) TBSOTf, 2,6-DTBP, CH_2Cl_2, 81%; (b) **4**, pyridine, DMF, rt, 48 h, 36%; (c) HF–pyridine complex, CH_3CN, 47%.

group to the corresponding aldehyde **11**. Finally, reductive alkylation with doxorubicin hydrochloride (**12**) using sodium cyanoborohydride afforded the desired drug linker **13** with the aldehyde stably masked as the oxazolidine carbamate.

To determine if the drug-linkers **5** and **13** were likely to release free drug once transported by the mAb to the lysosomes of target cells, the reactive maleimide groups were quenched with cysteine (cys) and the resulting compounds were exposed to the human lysosomal enzyme cathepsin B.[19] Both drug-linkers were substrates for cathepsin B, with the val-cit compound cys-**5** being cleaved at approximately twice the rate of the val-ala compound cys-**13** (Table 1). The linker cys-**5** cleanly released the butyl diacetate **2a**, while the linker cys-**13** released 2-pyrrolinodoxorubicin **3a** directly as determined by LC–MS analysis of the sample digests. Both reactions went to completion. In the absence of cathepsin B, both cysteine-quenched drug-linkers were highly stable.

Compounds **5** and **13** were conjugated to the chimeric mAbs 1F6 and AC10. Chimeric 1F6 binds to CD70 (TNF receptor superfamily) which has a high relative expression profile on both hematologic and renal cell carcinoma lines compared to normal tissues.[20–23] cAC10 binds to the CD30 antigen found on malignant B cell lines.[24] For the preparation of conjugates, both mAbs were treated with dithiothreitol (DTT) to reductively cleave the heavy–heavy and heavy–light chain disulfide linkages. Full reduction provides a mAb with approximately eight free sulfhydryl groups. Exposure



Scheme 4. Reagents and conditions: (a) TBDPSCl, NaH, 56%; (b) Dess–Martin, CH_2Cl_2, 97%; (c) ethanolamine, benzene, 4 Å powdered sieves; (d) diphosgene (2 equiv), pyr (8 equiv), CH_2Cl_2, −78 °C, 2 h, then **7**, −78 °C to rt, 61%; (e) DMF, piperidine (4:1); (f) Fmoc-Val-OSu, DMF, 94% two steps; (g) TBAF, THF, 82%; (h) MC-OSu, DMF, 88%; (i) Dess–Martin, CH_2Cl_2, 91%; (j) doxorubicin–HCl (**12**), CH_3CN, H_2O (2:1), $NaCNBH_3$, 0 °C to rt, 26%.

to the drug linkers **5** and **13** resulted in conjugates that were largely monomeric (Table 2) as determined by size exclusion chromatography. Achieving a high level of loading with **5** (>4 drugs/mAb) was complicated by precipitation and aggregation of the resulting ADCs. In contrast, **13** could be loaded with 7.4 drugs/mAb without exceeding an acceptable level of aggregation (>10%). Drug loading was determined using spectrometric methods by comparing the absorbance of the

Table 1. Cathepsin B digest of cysteine-quenched **5** and **13**

Compound	Human cathepsin B hydrolysis ($t_{1/2}$ min)
Cys-**5**	72
Cys-**13**	142

Results from a single experiment. Substrate:enzyme ratio = 20,000. Final substrate concentration of 2.5 mM. Half-life was determined by LC measuring percent remaining starting material at 254 nm.

Table 2. Characterization and in vitro cytotoxicity of the conjugates[a]

Compound	Target antigen	Drug loading	% Aggregation	Caki-1 cells[b] (CD70+, CD30−)	786-O cells[b] (CD70+, CD30−)	Karpas 299 cells[b] (CD30+, CD70−)
2a	—	—	—	0.039	0.006	0.099
Doxorubicin (**12**)	—	—	—	112	64.5	29.2
c1F6-**5**	CD70	2.4	<1	1.6	0.4	—
cAC10-**5**	CD30	3.3	3	65	108	1.42
c1F6-**13**	CD70	4.9	<1	0.65	2.3	>50
cAC10-**13**	CD30	7.4	10	>50	>50	0.57

[a] The activities of the ADCs were compared to that of doxorubicin, and a highly potent doxorubicin derivative (**2a**) that leads to the same molecule released from the conjugates.

[b] Cells were treated with the test agents for 96 h and viability was determined by reduction of resazurin. The IC_{50} values indicated are the concentrations (nM) of the drug component of the conjutates.

doxorubicin chromophore (490 nm) to the protein absorbance (280 nm).

Compound **2a** showed exquisite cytotoxic activity on the three cell lines evaluated: the renal cell carcinoma (RCC) lines Caki-1 (CD70+) and 786-O (CD70+), and ALCL line Karpas 299 (CD30+) with potencies ranging from 6 to 99 pM (Table 2). Doxorubicin (**12**) was 2.5–4 orders of magnitude less active on the same cell lines.

When the active drug **3a** was conjugated to the mAbs c1F6 or cAC10 either via drug-linker **5** or **13**, significant levels of cytotoxic activity were obtained and the effects were immunologically specific. The cells ranged from 41- to 270-fold more sensitive to binding conjugates than to non-binding conjugates. In addition, the IC_{50} values of conjugates with these two drugs were similar, suggesting that both linker constructs delivered the active drug **3a** with equal effectiveness. However, the potency of the conjugates was significantly less than that of the free drugs **2a**. This might be due to the fact that passive cellular uptake of free drug may lead to higher intracellular concentrations compared to those obtained through mAb-mediated delivery.

We compared the potency of our ADCs (relative to doxorubicin) to that of the hydrazone-based BR96 conjugate of **2b** previously described by King et al.[16] For the hydrazone of **2b** on BR96, an increased potency of 10-fold was seen over the free drug doxorubicin. The increased potency of ADCs based on **5** and **13** ranged from 70- to 170-fold, relative to doxorubicin. We believe the increased relative potency of our ADCs stems from the potency of the released drug. In our hands, compound **2a** was approximately 10-fold more potent than **2b** on a series of cell lines (data not included).

In summary, the drug linkers **5** and **13**, based on the potent doxorubicin derivative **3a**, were designed, synthesized, and linked to mAbs that recognize tumor associated antigens on RCC and ALCL tumors. The cysteine-quenched adducts of **5** and **13** were substrates for the lysosomal enzyme cathepsin B and were cleaved at comparable rates. Conjugation to the mAbs c1F6 and cAC10 gave ADCs with single-digit nanomolar to sub-nanomolar cytotoxicity IC_{50}s, and >40-fold specificity when evaluated against an antigen negative cell line and non-binding control ADCs. Both drug-linkers gave conjugates that were >70-fold more potent than doxorubicin when evaluated on the basis of drug concentration. Results from the in vivo evaluation of ADCs based on **5** and **13** will be reported elsewhere.

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