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Minor groove binder antibody conjugates employing a water soluble β-glucuronide linker

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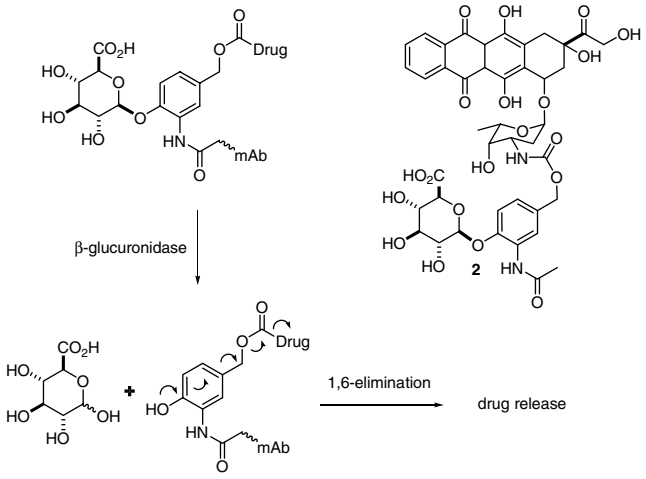
Abstract—The minor groove binder β-glucuronide drug-linker **3** was constructed from amino CBI **1** and determined to be a substrate for *Escherichia coli* β-glucuronidase (EC 3.2.1.31), resulting in facile drug release. Compound **3** was conjugated to mAbs cAC10 (anti-CD30) and h1F6 (anti-CD70) to give antibody-drug conjugates (ADCs) with potencies comparable to that of free drug **1**. The ADCs were largely monomeric at intermediate loading levels (4–5 drug/mAb), in contrast to highly aggregated *p*-aminobenzylcarbamate dipeptide-based ADCs of **1** previously reported. Significant levels of immunologic specificity were observed with cAC10-**3** by comparing antigen positive versus negative cell lines and binding versus non-binding control ADCs. The water soluble β-glucuronide linker is stable in plasma and effectively delivers drugs to target cells leading to potent cytotoxic activities.

Recently, we reported the development of a novel antibody-drug conjugate (ADC) linker system in which the enzyme β-glucuronidase releases free drug through the cleavage of a β-glucuronide glycosidic bond (Scheme 1).[1] The linker was an extension of the doxorubicin antibody dependent enzyme prodrug therapy (ADEPT) agent **2** reported by Desbène and coworkers, which efficiently released doxorubicin upon activation by a β-glucuronidase fusion protein.[2] This prodrug construct was modified to allow for attaching drugs to monoclonal antibodies (mAbs). ADCs of 2-pyrrolinodoxorubicin, and monomethyl auristatin E and F were constructed and found to be highly potent and immunologically specific both in vitro and in vivo.[1] The β-glucuronide linker provided for facile drug release and was highly stable in rat plasma. Importantly, the resulting ADCs were non-aggregated and monomeric even when heavily loaded (8 drugs/mAb) with hydrophobic anticancer drugs.



Scheme 1.

The cyclopropyl indole minor groove binders (MGB) alkylate double-stranded DNA in a sequence selective manner resulting in single-strand breaks and subsequent cell death.[3–5] These compounds, including their seco halide derivatives, have been viewed as attractive molecules for mAb targeted delivery based on their synthetic tractability, mechanism of action, and potent cytotoxic activity. Our work in this area has focused on the amino cyclopropylbenz[e]indole (amino CBI) seco-chloride **1** in which dipeptide-based drug linkers were designed to stabilize the drug while attached to

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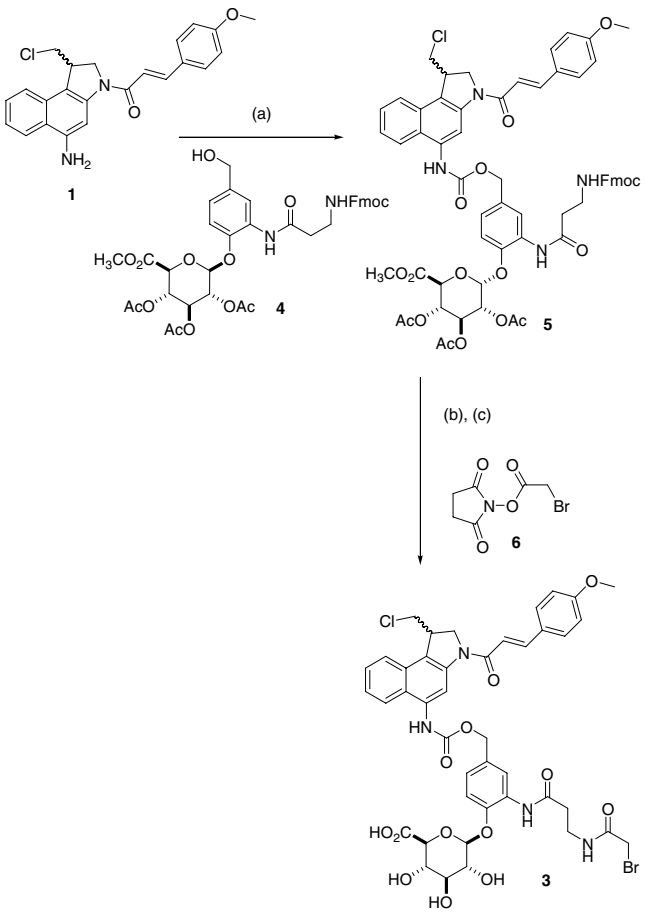
the mAb and liberate drug under the action of a proteolytic enzyme such as cathepsin B once delivered inside the cancer cell.[6,7] Others have published in this area using both peptide and hydrazone-based linker strategies.[8,9]

Two key challenges in working with this class of molecules are their propensities to cause ADC aggregation, due presumably to their planar hydrophobic structures and their inherent instability. It has been found that functionalizing the activating heteroatom (N or O) as a carbamate, amide or ether renders the molecules inactive while attached to the mAb. Several linker strategies were developed to address aggregation, all of which employed a polyethylene glycol unit as part of the solution. Here, we describe a follow up investigation using the β-glucuronide linker, specifically the preparation of ADCs of the hydrophobic MGB **1**, with a goal of developing active ADCs and understanding the impact a soluble β-glucuronide linker might have on ADC aggregation.

The β-glucuronide drug-linker **3** was prepared in three steps from **1**[6,10] and the protected β-glucuronide intermediate **4** (Scheme 2).[1] Reaction of **4** with diphosgene and capture of the resulting chloroformate with **1** proceeded to give a modest yield of the carbamate **5**. Treatment of **5** with lithium hydroxide removed the acetates and Fmoc protecting group, and saponified the methyl ester.[2] Finally, the free amine was capped by **6** to afford the bromoacetamide drug-linker **3**. The β-glucuronide linker is an effective solubilizing element for **1**; drug-linker **3** was soluble at 1 mM in phosphate buffered saline, pH 7.4, whereas the free drug **1** had a solubility of only 5 μM in phosphate buffered saline, pH 7.4, containing 10% DMSO.



Scheme 2. Reagents and conditions: (a) diphosgene (2.0 equiv), pyridine, CH_2Cl_2, **1**, 37%; (b) LiOH–H_2O, CH_3OH/H_2O (1:1); (c) **6**, DIPEA, DMF, 21% two steps.

Compound **3** was determined to be a substrate for *Escherichia coli* β-glucuronidase (EC 3.2.1.31) using an HPLC/MS based assay[1]. Compound **1** was the sole released drug. No deglucuronidated intermediate could be detected, suggesting that once the β-glucuronide group is cleaved, drug release via 1,6-elimination is fast. In the absence of β-glucuronidase, incubation of **3** for 24 h in buffer gave no measurable drug release.

ADCs of **3** were prepared with chimeric AC10 (IgG1 against CD30 antigen) and humanized 1F6 (IgG1 against the CD70 antigen). The CD30 antigen resides on activated B cells and is a marker for both Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL).[11] The CD70 antigen is overexpressed on renal cell carcinoma (RCC) lines as well as some hematologic malignancies.[12–16] mAb interchain disulfides were partially reduced using stoichiometric quantities of tris(2-carboxyethyl) phosphine to give on average approximately 4 free thiols/mAb as previously described.[17,18] The reduced mAbs were purified via gel filtration and conjugated to **3** at pH 8. An excess of drug (1.4 drug/thiol) was required to fully consume all free thiols and the resulting ADCs were purified by cation exchange chromatography.

Analysis of the conjugates by size exclusion chromatography revealed that they were largely monomeric. The cAC10-**3** ADC contained 4.8 drugs/mAb and was 79% monomeric while the h1F6-**3** contained 4.4 drugs/mAb and was 85% monomeric (Table 1). Increasing mAb loading to 8 drugs resulted in high levels of aggregation (70% for h1F6-**3**). The results with the glucuronides compare favorably to our previous results[7] with the dipeptide-based (valine–citrulline) *p*-aminobenzyl carbamates (PABC) MGB-linker in preventing ADC aggregation. The valine–citrulline PABC of **1**, when conjugated to reduced cAC10 with 4.5 available thiols/mAb, was only 32% monomeric. Finally, we note that the conjugation conditions have not yet been fully optimized. Such studies, which would require analyses of several parameters such as reaction time, temperature and pH, as well as the effects of concentration and stoichiometry, could lead to even lower levels of aggregate formation than those reported here.

In vitro cytotoxicity studies demonstrated that the ADCs were potent and selective for antigen positive cells (Table 1). The cAC10-**3** ADC was highly potent against the CD30 positive lines Karpas 299, L428, and L540cy with IC_{50} values comparable with those of free drug **1**. The cytotoxic activity of the h1F6-**3** against the CD70 positive cell line Caki-1 also was equivalent to **1**. These

Table 1. Characterization and in vitro cytotoxicity of conjugates and free drug

Cytotoxic agent	Antigen	Drug loading	Percent monomer	Karpas 299[a] (CD30+)	L428[a] (CD30+, CD70−)	L540 Cy[a] (CD30+)	WSU-NHL[a] (CD30−)	Caki-1[a] (CD70+)
cAC10-**3**	CD30	4.8	79	0.4	0.4	0.9	>32[b]	—
1F6-**3**	CD70	4.4	85	—	>29[b]	—	—	0.6
1		—	—	1.2	0.1	0.5	—	0.7

[a] 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 ADC.
[b] No activity at highest concentration tested.

data suggest that the drug is efficiently delivered to the cell. The cAC10-**3** ADC gave good selectivity for antigen positive cells versus the antigen negative line WSU-NHL, and the non-binding control h1F6-**3** was significantly less active against the CD30 positive line L428. The in vitro profile of the β-glucuronide ADCs of **1** with respect to both cytotoxic activity and specificity is comparable to that of the dipeptide reagents previously disclosed.[7]

Investigations by our group and others have demonstrated the importance of linker composition for developing clinically viable ADCs.[18–20] Linker stability and drug release kinetics are parameters inherent to the linker construct and profoundly impact ADC safety and efficacy. In addition, the linker can have a marked influence on aggregation, particularly when hydrophobic drugs are employed. The data presented here and previously suggest that the β-glucuronide linker, with its high aqueous solubility, long plasma half-life, and facile drug release, is a complementary alternative to PABC dipeptide, disulfide, and hydrazone-based linkers.[21–24] The β-glucuronide linker, when used to tether the hydrophobic amino CBI **1**, significantly reduced aggregation relative to PABC dipeptide-based ADCs. ADCs of **3** were immunologically specific and displayed high cytotoxic activity against Hodgkin's lymphoma lines L428 and L540cy, the ALCL line Karpas 299, and RCC line Caki-1. These results provide strong justification for further developing this promising agent by investigating its efficacy in preclinical in vivo models for human cancer.

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