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BR96 Conjugates of Highly Potent Anthracyclines

H. Dalton King,[a,*] Andrew J. Staab,[a] Kahnie Pham-Kaplita,[a] Derek Yurgaitis,[a] Raymond A. Firestone,[a,†] Shirley J. Lasch[b,‡] and Pamela A. Trail[b,‡]

[a]*Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 5100, Wallingford, CT 06492-7660, USA*
[b]*Bristol-Myers Squibb Pharmaceutical Research Institute, PO Box 4000, Princeton, NJ 08543-4000, USA*

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Abstract—The 6-maleimidocaproylhydrazone derivatives of highly potent antitumor agents 5-Diacetoxypentyldoxorubicin and Morpholinodoxorubicin were synthesized and conjugated to monoclonal antibody BR96 and control IgG. Immunoconjugate molar ratios were generally 7.5–8.5, and dimer aggregate levels were low. The linkers released parent drug at lysosomal pH 5, while they remained stable at neutral pH. BR96 conjugates were highly potent and antigen specific in vitro. The BR96–DAPDOX conjugate demonstrated an IC_{50} of 0.03μm and was at least 300-fold more potent than a non-binding IgG–DAPDOX control conjugate.

Doxorubicin (DOX) **1a** is a cytotoxic drug which is particularly well-suited for tumor delivery via conjugation to monoclonal antibodies.[1] Our group has synthesized several immunoconjugates that link an internalizing MAb and DOX via disulfide[2–4] or thioether[4–6] bonds to the MAb and hydrazone bonds to the DOX C-13 ketone. Immunoconjugates that utilize the C-13 hydrazone linkage have proven to be of particular utility in that they are stable at physiological pH 7–7.4, but release unmodified DOX following exposure to the acidic pH of endosomes/lysosomes. One such immunoconjugate, BR96–**1b** (BR96–DOX), demonstrated impressive antigen-specific activity in vitro and in vivo.[5] MAb BR96 has been shown to preferentially bind cells expressing the Lewis^y antigen, and then to be internalized into the lysosomal compartment of those cells.[7–9]

The chemistry utilized in the construction of this immunoconjugate is generally applicable to other anthracyclines which possess the α,α′-dihydroxyketone side chain. Within this group are anthracycline analogues that have modes of action different from that of DOX. Several of these are highly potent agents that have not been clinically useful because of their high systemic toxicity and poor therapeutic index. 5-Diacetoxypentyldoxorubicin (DAPDOX) **2a**[10,11] and Morpholinodoxorubicin (MorphDOX) **3a**[12,13] are two such analogues that are 2 to 250 times more potent against DOX-sensitive cell lines in vitro.[11,13] DAPDOX alkylates DNA and produces interstrand crosslinking.[10] MorphDOX causes DNA single strand breakage but is also metabolized in vivo to a DNA-alkylating species.[14] These drugs are also attractive as they are not substrates for *p*-glycoprotein and do not display the multidrug-resistant phenotype.[15]

MAb-directed delivery provides a mechanism to retain potent antitumor activity while reducing the systemic toxicity of extremely potent drugs. For example, immunoconjugates of calicheamicin, an extremely potent member of the enediyne family of antibiotics have demonstrated impressive antigen-specific activity and an acceptable therapeutic index.[16–18] Similarly, conjugates of MorphDOX **3a** with MAb LM609 have demonstrated antigen-specific cytotoxicity in vitro.[19,20] In the best cases, **3a** was linked via C-13 hydrazones, while the MAb linkage was made via amide formation with random lysine amino groups on LM609 to give molar ratios (MR) of 1.0–2.3 drugs per MAb.

In this study we describe the use of our thioether/hydrazone-based coupling chemistry to deliver DAPDOX **2a** and MorphDOX **3a**. Construction of BR96 immunoconjugates in this manner allows for the site-

*Corresponding author. Tel.: +1-203-677-6685; fax: +1-203-677-7702; e-mail: dalton.king@bms.com
†Current address: Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Rd., PO Box 368, Ridgefield, CT 06877-0368, USA.
‡Current address: Bayer Research Center, 400 Morgan Lane, West Haven, CT 06516-4175, USA.

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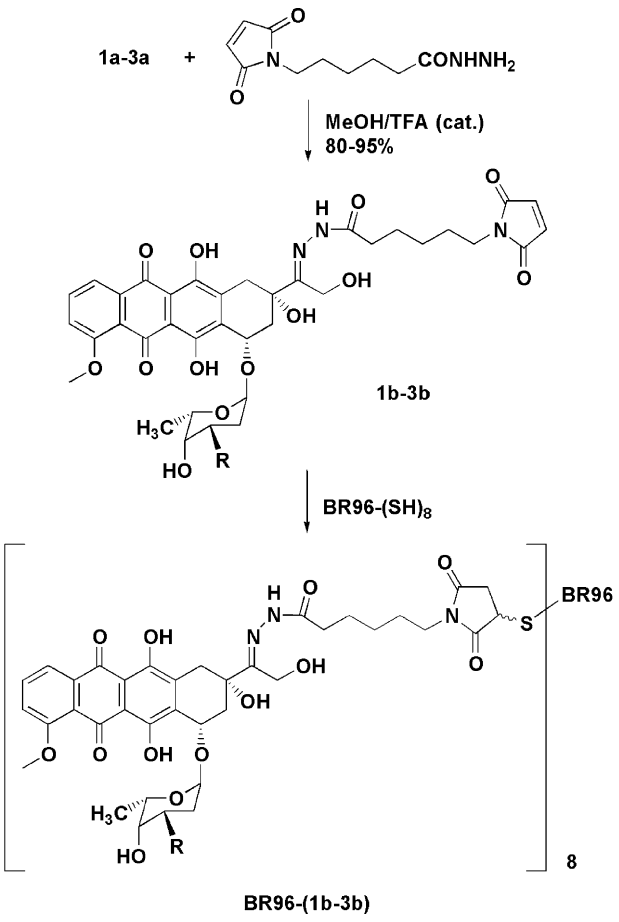

Scheme 1. Synthesis of linkers **1b–3b** and conjugates **BR96–(1b–3b)**. R is defined in Figure 1.

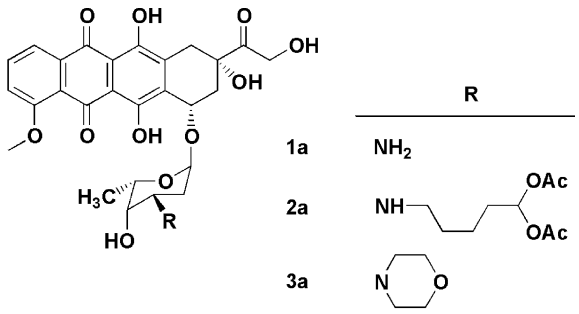

Figure 1. Structures of doxorubicin **1a** and highly potent analogues 5-diacetoxypentyldoxorubicin **2a** and morpholinodoxorubicin **3a**.

specific attachment of up to eight drugs per BR96 molecule without reducing the binding affinity of the mAb.[5,6] Thus, new linkers **2b** and **3b** have been synthesized and used to produce the DAPDOX conjugate BR96–**2b** and the MorphDOX conjugate BR96–**3b**. The linkers have been evaluated for their stability at pH 5 and 7. The new immunoconjugates were also evaluated for antigen specific activity in vitro.

Synthesis

The linkers **2b** and **3b**[21] and immunoconjugates BR96–**2b** and BR96–**3b** used in this study were synthesized as in Scheme 1, which is an identical approach to that used for our original linker **1b** and immunoconjugate BR96–**1b**.[6] As expected, formation of the acylhydrazone linkers **2b** and **3b** required similar reaction times and resulted in similar high yields compared to **1b**. The new linkers **2b** and **3b** were conjugated to BR96 and non-binding control IgG by our previous procedure.[6] Results are shown in Table 1. As with BR96–**1b**, the conjugation step was efficient, yielding immunoconjugates BR96–**2b** and BR96–**3b** with MR's almost equivalent to the SH titer generated. Dimer aggregate levels in the BR96 conjugates were generally low. The one exception (BR96–**2b**, larger batch), while acceptable, was likely a single batch abnormality. Final products contained virtually no unconjugated linker nor free drug. Linkers **2b** and **3b** therefore form BR96 conjugates analogous to **1b**, demonstrating the general applicability of our thioether/hydrazone-based coupling chemistry to this class of molecules.

Hydrolytic Stability

In order to evaluate the hydrolytic stability of new linkers **2b** and **3b**, thioether analogues **2c** and **3c** were generated by reaction with 2-mercaptoethanol as a model for the thioether linkage to BR96. This modification had the additional advantage of increasing their aqueous solubility. The hydrolysis of **2c** and **3c** to the parent drugs was followed at pH 5 and 7 by RP-HPLC and compared to previously studied **1c**.[6] $T_{1/2}$ values were calculated for disappearance of starting material and appearance of parent drug. Results are summarized in Table 2. At pH 7, all three model compounds are very stable with $T_{1/2}$ values greater than 4–5 days.[22] At pH 5, hydrolysis of the model compounds is relatively rapid,

Table 1. Immunoconjugates of **2b** and **3b**

Conjugate	SH titer	MR	Scale (g)	Yield (%)	Free drug (%)	Free linker (%)	Aggregate (%)
BR96–**2b**	8.6	7.6	0.05	80	2.4	BLD	1.0
BR96–**2b**	7.9	7.3	1.26	87	BLD[a]	0.6	10.7
IgG–**2b**	9.2	8.1	0.05	56	BLD	BLD	
IgG–**2b**	8.7	8.6	1.27	99	BLD	0.4	
BR96–**3b**	8.6	7.4	0.05	80	8.4	BLD	6.6
BR96–**3b**	7.6	8.2	1.09	100	0.3	0.8	BLD
IgG–**3b**	9.2	7.7	0.05	86	BLD	BLD	
IgG–**3b**	7.9	7.5	1.09	95	1.3	BLD	

[a]Below limits of detection.

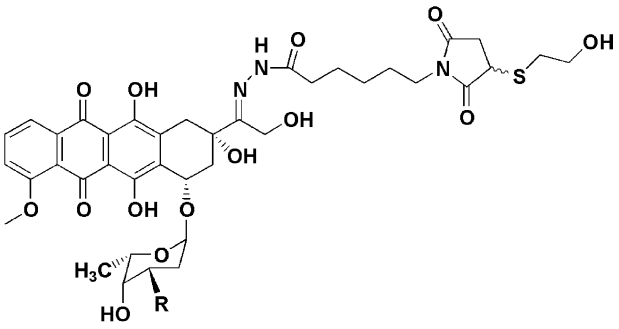

Figure 2. Thioether analogues **1c**–**3c**. R is defined in Figure 1.

yielding the free parent drugs with similar rates. Thus, we conclude that linkers **2b** and **3b** possess the desirable characteristics of high stability at physiological pH and rapid hydrolysis at lysosomal pH. Prior experience has shown that the BR96–**1b** is more stable than the unconjugated linker at both pH 5 and 7. Similar trends are likely to be observed for conjugates of **2b** and **3b** (Fig. 2).

In Vitro Cytotoxicity

The in vitro potency of immunoconjugates BR96–**2b** and BR96–**3b** is shown in Table 3. In all cases, the immunoconjugates were evaluated against the L2987 lung carcinoma cell line, a human lung carcinoma line that expresses the BR96 cell surface antigen. Cells were exposed to conjugates or free drugs for 2 h as described previously.[3] The BR96–**2b** conjugates produced potent antigen-specific cytotoxicity. In fact, the BR96–**2b** was 300 times more potent than a non-binding control conjugate IgG–**2b**. This high level of immunospecificity indicates in vitro stability and suggests that targeted delivery of DAPDOX **2a** via internalizing MAbs should increase the therapeutic index of this anthracycline analogue. The DAPDOX conjugates were substantially more potent than equivalent conjugates produced with DOX. BR96–**2b** was at least 30-fold more potent than the DOX conjugate BR96-**1b** and was similar in potency to unconjugated DAPDOX. The MorphDOX conjugate BR96–**3b** also demonstrated antigen-specific activity in vitro. BR96–**3b** was 10-fold more potent than the control conjugate IgG–**3b**. Unconjugated DAPDOX **2a** and MorphDOX **3a** were of similar potency. However, when delivered via the BR96 MAb, DAPDOX conjugate BR96–**2b** was >6-fold more potent than the MorphDOX conjugate BR96–**3b**. The molar ratios and binding activity of the BR96 conjugates were similar. It is not clear at present why the in vitro potency of the MorphDOX conjugate BR96–**3b** was substantially less than that of the DAPDOX conjugate BR96–**2b**.

Table 2. Hydrolytic stability of thioether analogues **1c**–**3c**

	$T_{1/2}$, (h), 37 °C	
Linker	pH 5.0[a]	pH 7.0[b]
1c	7.0	>120
2c	6.5	>100
3c	4.5	>95

[a]0.1 M NaOAc.
[b]0.1 M NaH_2PO_4.

Table 3. Cytotoxicity of immunoconjugates

Drug or conjugate	MR	IC_{50} (μM drug)[a]	Relative potency versus		Selectivity ratio[b]
			1a	BR96-**1b**	
DapDOX **2a**		0.03	10		
BR96–**2b**	7.6	0.03		30	300
IgG–**2b**	8.1	10			
MorphDOX **3a**		0.04	8		
BR96–**3b**	7.4	0.2		5	10
IgG–**3b**	7.7	2			
DOX **1a**		0.3	1		
BR96–**1b**	8	1.0		1	8

[a]L2987 cell line.
[b]Defined as IC_{50} IgG–drug/IC_{50} BR96–drug at 2 h exposure time.

In this work, we have demonstrated the general utility of the maleimidocaproylhydrazone approach for anthracyclines possessing the α,α′-dihydroxyketone side chain. The hydrazone linkage is stable in buffers at pH 7, but hydrolyzes rapidly at pH 5. These properties are highly desirable for immunoconjugates that are designed to be stable in systemic circulation and to liberate unmodified drug following antigen-specific internalization in tumor cells. The highly potent anthracyclines **2a** and **3a** have been conjugated to BR96 and non-binding control IgG achieving fully loaded conjugates with MR's of about 8. Both BR96 conjugates were highly active and selective in vitro when compared to the corresponding non-binding IgG conjugates, unconjugated parent drug and unconjugated DOX **1a**. Furthermore, DAPDOX conjugate BR96–**2b** is superior in vitro by a large margin to DOX conjugate BR96–**1b**.

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21. Analytical data for **2b**: ^{1}H NMR ($CDCl_3/CD_3OD$) selected peaks: δ 1.25 (d, 3H), 1.3 (m, 4H), 1.4–1.8 (m, 8H), 1.97 (s, 6H), 2.80 (t, 2H), 3.4 (m, 4H), 4.0 (s, 3H), 4.1 (m, 1H), 4.6 (m, 2H), 5.08 (m, 1H), 5.49 (m, 1H), 6.65 (m, 3H), 7.38 (d, 1H), 7.75 (t, 1H), 7.94 (t, 1H). MS (ESI): 937.4 $(M+H)^+$. Anal.: $C_{46}H_{56}N_4O_{17}\cdot 1.0TFA\cdot 1.0H_2O$: Theoretical C, 53.93; H, 5.56; N, 5.24. Found C, 53.79; H, 5.60; N, 5.59. FTIR: 3414, 2939, 1760, 1706, 1677, 1639, 1618, 1580, 1445, 1412, 1380, 1206, 1120, 990, 830 cm^{-1}. Analytical data for **3b**: ^{1}H NMR ($CDCl_3/CD_3OD$) selected peaks: δ 1.15 (t, 2H), 1.25 (d, 3H), 1.4–1.8 (m, 4H), 1.9–2.6 (m, 6H), 3.4 (m, 5H), 4.0 (s, 3H), 4.1 (m, 1H), 4.6 (m, 2H), 5.07 (m, 1H), 5.54 (m, 1H), 6.68 (m, 3H), 7.40 (d, 1H), 7.77 (t, 1H), 7.94 (t, 1H). MS (ESI): 821.4 $(M+H)^+$. Anal.: $C_{41}H_{48}N_4O_{14}\cdot 1.0TFA\cdot 1.5H_2O$: Theoretical C, 53.69; H, 5.45; N, 5.82. Found C, 53.62; H, 5.39; N, 5.84. FTIR: 3414, 2940, 1706, 1677, 1617, 1580, 1446, 1412, 1286, 1204, 1124, 1024, 994, 830 cm^{-1}.
22. Hydrolysis rates at pH 7 are generally reported as approximate ranges since the point at which 50% consumption of starting materials was not reached.