Monoclonal Antibody Conjugates of Doxorubicin Prepared with Branched Peptide Linkers: Inhibition of Aggregation by Methoxytriethyleneglycol Chains

H. Dalton King,*[,†] Gene M. Dubowchik,[†] Harold Mastalerz,[†] David Willner,[†] Sandra J. Hofstead,[†] Raymond A. Firestone,[†,‡] Shirley J. Lasch,[§,||] and Pamela A. Trail[§,||]

Bristol Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492, and Princeton, New Jersey 08543

Received April 8, 2002

High mole ratio BR96 immunoconjugates were synthesized using branched peptide−doxorubicin linkers designed to liberate doxorubicin following antigen-specific internalization into lysosomes. However, these immunoconjugates are highly prone to noncovalent, dimeric aggregation. We hypothesize that this is due to (1) the hydrophobic nature of the peptides, (2) the loss of positive charge upon amide formation at the 3′-amino group of doxorubicin, and (3) the proximity of the peptide hydrophobic residues to form efficient intermolecular stacking interactions. By introducing a hydrophilic methoxytriethylene glycol chain onto the doxorubicin portion of the branched peptide linkers, aggregation has been eliminated or greatly reduced in the immunoconjugate products. The methoxytriethylene glycol chain was linked to the doxorubicin moiety of the linker via a hydrazone bond that is stable at pH 7 but hydrolyzes rapidly at pH 5 to release free drug. BR96 immunoconjugates synthesized from methoxytriethylene glycol-modified branched peptide−doxorubicin linkers are highly potent and immunospecific in vitro. The data suggest that the methoxytriethylene glycol chain hydrolyzes as designed upon antigen-specific internalization into tumor lysosomes in vitro, where enzymatic degradation of the peptide linker releases free doxorubicin.

BR96 is a chimeric (mouse/human IgG1) mAb which identifies a Le^y-related tumor-associated antigen expressed on carcinomas of the lung, colon, breast, and ovary.[1] We have demonstrated that it is well suited for targeting cytotoxic drugs in view of its ability to internalize into tumor cells following antigen-specific binding.[2] In an effort to reduce the amount of mAb required for potent activity in BR96-drug immunoconjugates, we set out to maximize the molar ratio (i.e., the number of the drugs carried per BR96 molecule) while retaining the binding affinity of the BR96 mAb. Immunoconjugates with higher molar ratios are more potent on a mAb basis, reducing the amount of immunoconjugate necessary to deliver a lethal dose of drug and produce the desired antitumor effect.[3] Our preferred method of conjugation uses a discrete set of eight thiol groups, generated by mild dithiothreitol (DTT) reduction of four interchain disulfides, as the linkage site on BR96 to attach the drug carrier.[2,4] Since this limits molar ratios to a maximum value of 8 mol drug/mol BR96 for single chain linkers, the use of a bivalent, or branched linker represents a feasible method to further increase molar ratio. We have demonstrated the advantages of this approach in the synthesis of BR96 conjugates of **1**[4] and **2**[3] (Scheme 1), obtaining molar ratios of up to 8 and 16 mol doxorubicin (DOX)/mol BR96, respectively. Both types of conjugates are monomeric entities with molecular weight 160 000 kD. BR96-**2** immunoconjugates were shown to be more potent in vitro than BR96-**1** on both a DOX and mAb basis.

BR96 immunoconjugates have also been constructed using the enzyme-cleavable single chain peptide linkers **3a**−**b**[5] and their branched congeners **4a**−**b**[6,7] (Scheme 2). Analogous to BR96-**2**, BR96-**4a** and -**4b**, both nearly double the molar ratio (13−14 mol DOX/mol mAb) and are more potent in vitro when compared to BR96-**3a** and -**3b** (8 mol DOX/mol mAb). Linkers **4a** and **4b** are problematic though, since their BR96 conjugates are obtained as completely aggregated dimers regardless of reaction conditions. Aggregated mAb conjugates are unsuitable for in vivo use, due to accelerated clearance, heterogeneity, and increased liver toxicity.[8] The nature of this aggregate was confirmed to be noncovalent since gel electrophoresis under denaturing conditions showed no covalently cross-linked chains. High drug loading alone does not explain the aggregation observed in BR96-**4a** or BR96-**4b**. For example, BR96-**2** is typically characterized by equal or higher mole ratios (14−16 mol DOX/mol mAb) but low aggregate levels (0.5−7%). A more likely explanation involves the hydrophobic nature of the peptide-PABC portion of **4a** and **4b**, in tandem with the DOX amide linkage which eliminates the positively charged 3′-amino group. The aromatic, hydrogen bond donor−acceptor characteristics of the PABC spacer may also play an important role in these interactions. We hypothesize, therefore, that in the distinctive compact environment created by **4a** and **4b**, multiple peptide−DOX chains from different conjugate molecules stack noncovalently with one another to form the dimer complexes (Figure 1).

* Corresponding author. Address: Bristol Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492. Telephone: (203) 677-6685. FAX: (203) 677-7702. E-mail dalton.king@bms.com.

† Wallingford, CT.

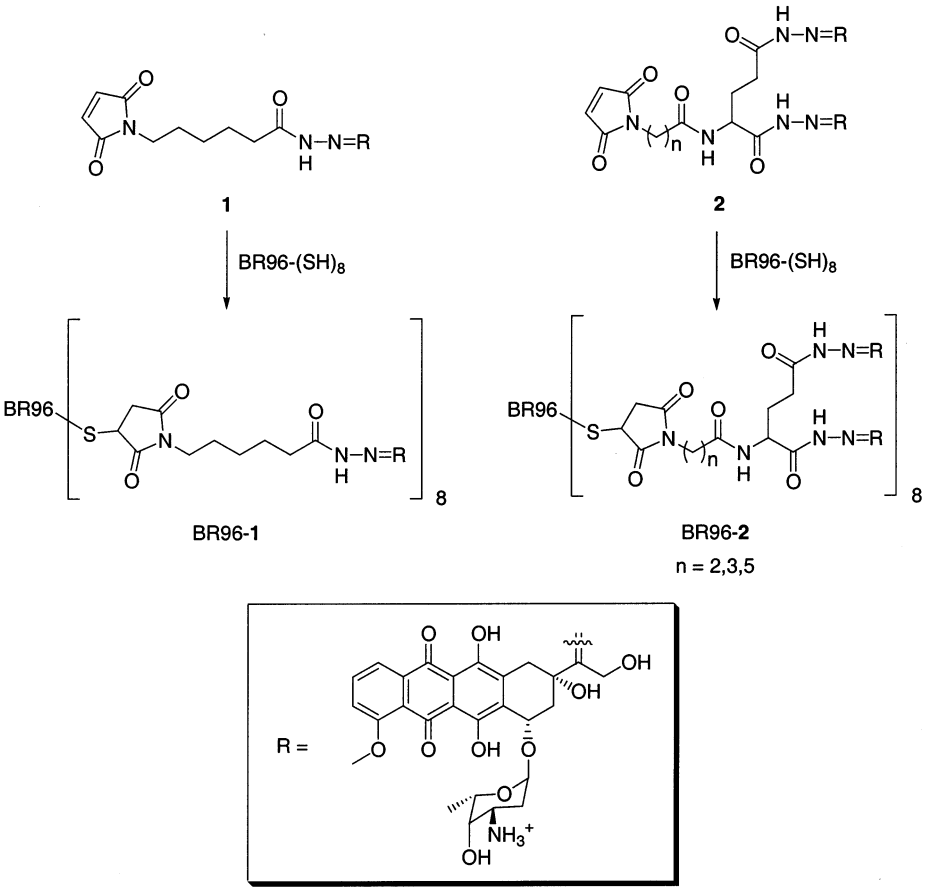
‡ Current address: Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT 06877.

§ Princeton, NJ.

|| Current address: Bayer Research Center, West Haven, CT 06516.

10.1021/jm020149g CCC: $22.00
Published on Web 08/14/2002

Scheme 1. DOX Hydrazone Linkers and BR96 Conjugates



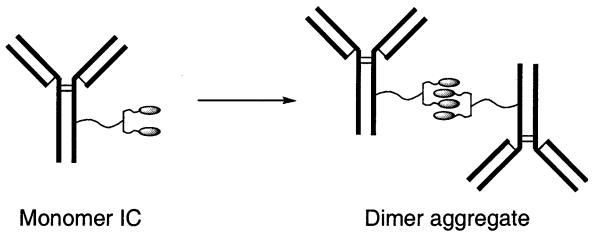


Figure 1. Dimerization process induced by the branched DOX peptide linker.

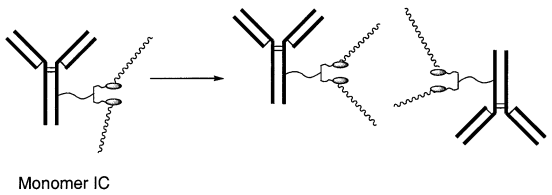
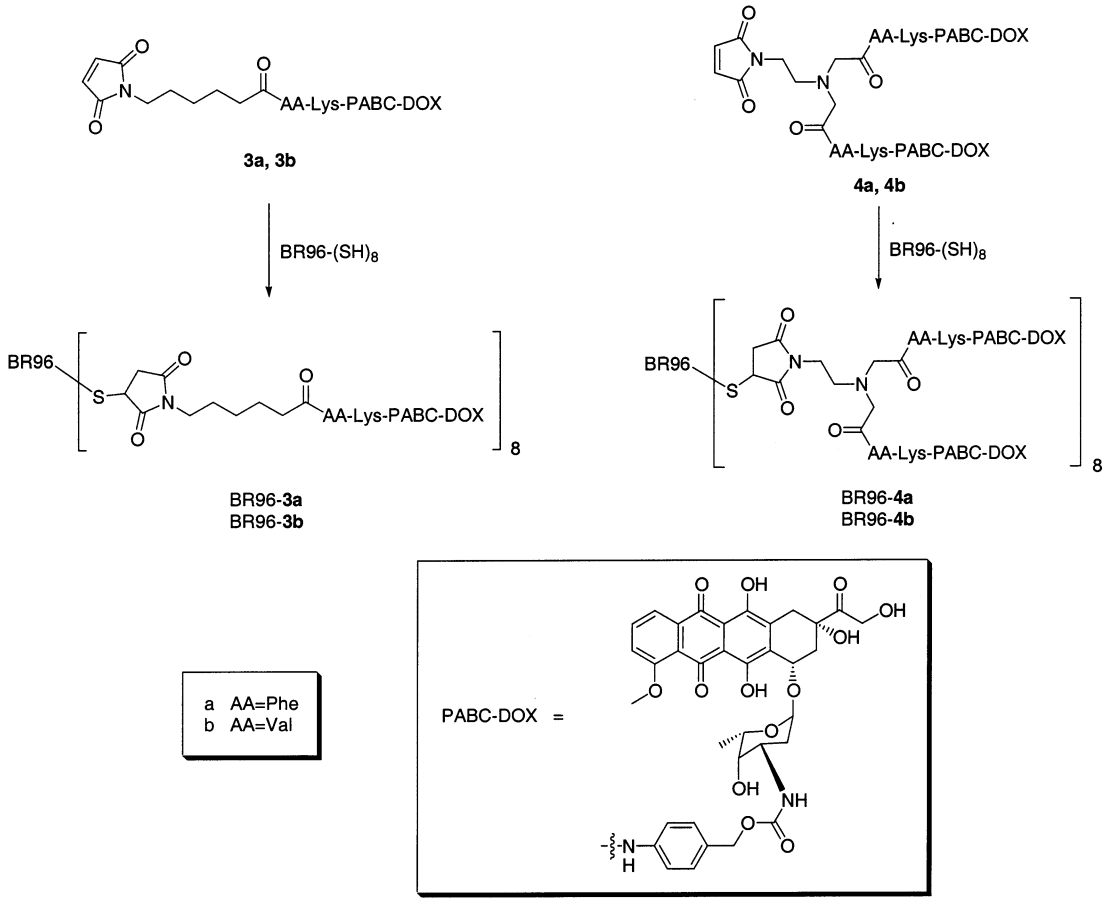


Figure 2. Dimerization process inhibited by hydrophilic chains.

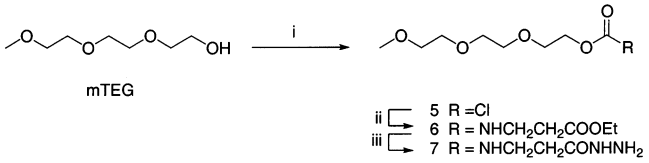
If such interactions could be inhibited, then aggregation levels might be reduced. One possible means by which to achieve this goal is through the synthesis of immunoconjugates with low molar ratios.[9] These constructs would present a lower density of drug and linker moieties on which to initiate the noncovalent stacking process which leads to aggregation. In fact, we have synthesized unaggregated, low mole ratio BR96 immunoconjugates using various drugs which otherwise tend to cause complete aggregation at higher loading.[10] However, low mole ratio immunoconjugates of drugs such as DOX lack the potency required to produce meaningful antitumor effects at acceptable doses of Ab. Another possible means by which to reduce aggregation is by introducing hydrophilic chains into the immunoconjugate. This might block intermolecular associations by sterically disrupting the formation of the potential aggregate and by introducing an additional shell of water molecules between the linkers (Figure 2). Ideally, this structure would (1) be added to the immunoconjugate in a controlled and reproducible fashion, (2) be tolerated by the chemical properties of the immunoconjugate, and (3) have no effect on the in vitro or in vivo activity of the immunoconjugate.

We were attracted to poly(ethylene glycol) (PEG), which has been used to alter the surface properties of various proteins and mAb's. PEG's unique hydrophilic properties and conformational flexibility in combination with its steric stabilization effect make it a good candidate for our purposes.[11,12] In this instance, though, we felt that random BR96 modification with diffuse polymeric preparations of PEG would yield an undesirable heterogeneous immunoconjugate product. As an alternative, we chose to modify the drug portion of our immunoconjugate with shorter PEG-like chains of defined composition. In contrast to PEG, no systematic investigations with homogeneous oligomeric ethylene glycols to alter aggregating properties of proteins are known. In the case of **4a** and **4b**, it is advantageous that the side chain ketone group of DOX remains available as a point of attachment for the chain. We reasoned that prior to conjugation, a modification of **4a**−**b** could be constructed with a hydrolyzable linkage similar to that

Scheme 2. DOX Peptide Linkers and BR96 Conjugates



Scheme 3. Synthesis of mTEG-Hydrazide **7**[a]



[a] Reagents: i, phosgene/toluene, 92%; ii, β-alanine ethyl ester hydrochloride, triethylamine, 99%; iii, anhydrous hydrazine, ethanol, 100%.

used in **1** and **2**.[3,4] Instead of PEG, oligomeric methoxytriethylene glycol (mTEG) was evaluated as a chain long enough to provide the necessary aggregate disruption. In contrast to PEG, commercially available mTEG is a homogeneous material. mTEG was derivatized to the hydrazide **7** (Scheme 3), which in turn could form a hydrazone at the DOX C-13 groups of the branched peptide linkers **4a**−**b** (Scheme 4). The final assemblies **8a** and **8b** were then attached to BR96 by our standard protocol[3] (Scheme 4). Immunoconjugates constructed with **8a** and **8b** were anticipated to have sufficient stability to localize to the tumor and undergo antigen-specific endocytosis in an intact state. Hydrazone hydrolysis and enzymatic degradation of the peptide would then follow in tumor lysosomes to liberate free DOX.

In this paper, we present the synthesis of novel BR96 immunoconjugates based on our new linkers **8a** and **8b** and demonstrate the ability of the mTEG hydrazone unit to disrupt immunoconjugate aggregation. We also present the results of our in vitro cytotoxicity studies with these immunoconjugates.

Experimental Procedures

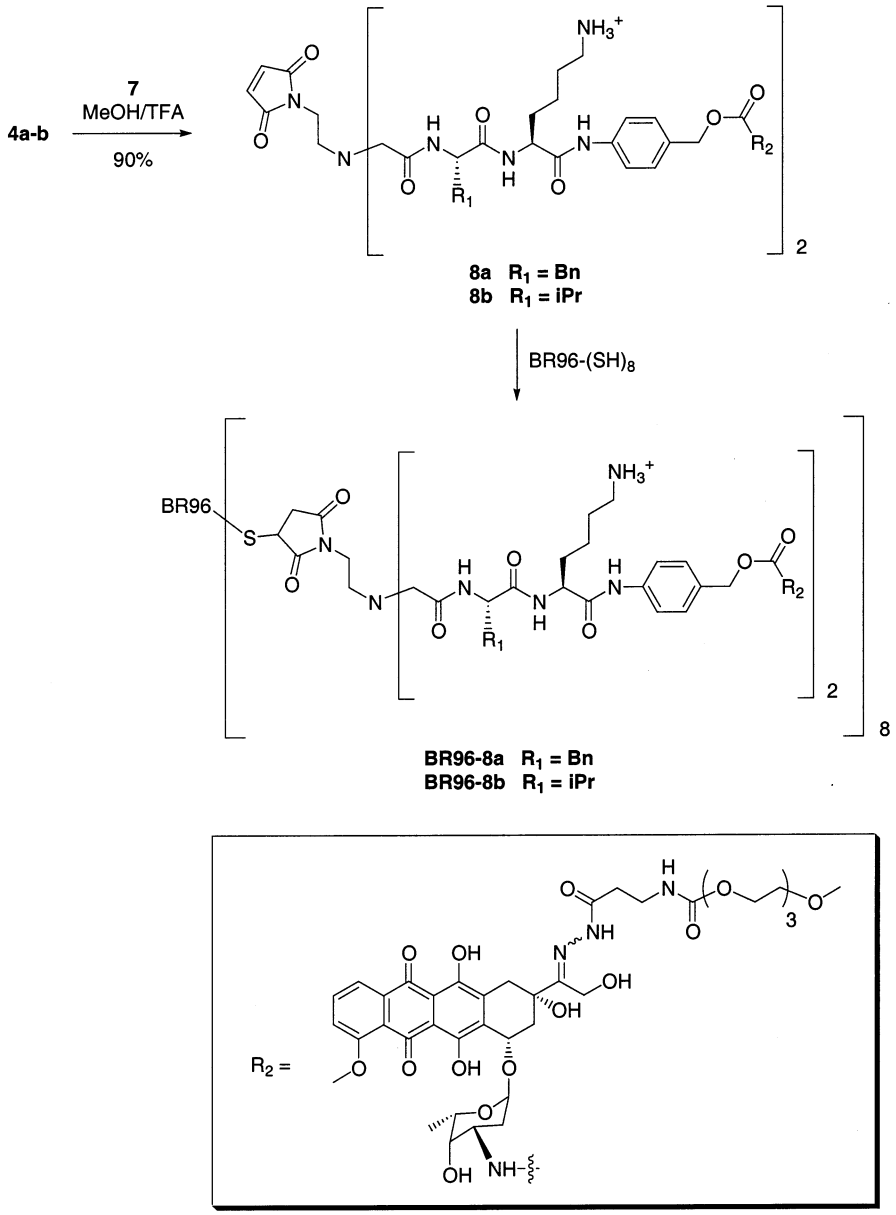
All reaction solvents were freshly opened Aldrich "Sure-Seal" quality. All other solvents and reagents were reagent-grade and used without further purification. Bio-Beads SM-2 chromatographic support was supplied by Bio-Rad Laboratories. ^{1}H NMR and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively, in the indicated solvents. Internal referencing was used, and chemical shifts are reported in parts per million. FTIR's of oils and liquids were obtained as films and of solids as KBr pellets. Elemental analyses were provided by Oneida Research Services. Routine reverse-phase HPLC of synthetic compounds was performed on a Perkin-Elmer 410 Pump and LC-235 Detector. Unless otherwise indicated, HPLC conditions were: isocratic 25% 50 mM triethylamine formate pH (2.8), 75% methanol at 1.5 mL/min; Phenomenex IB−Sil 5 C-18 column.

Synthesis

2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethylchloroformate 5. Under a N_2 atmosphere at room temperature, a 20% solution of phosgene in toluene (210 mL, 0.40 mol) was added over 45 min to a solution of mTEG (16.42 g, 0.10 mol) in 200 mL toluene. The reaction was stirred for 3.5 h. The solvent was evaporated and dried under vacuum. The residue was distilled under vacuum to yield **5** as a colorless liquid (20.80 g, 92%). ^{1}H NMR ($CDCl_3$): δ 3.30 (s, 3H), 3.49 (dd, 2H), 3.57 (m, 6H), 3.70 (m, 2H), 4.37 (dd, 2H). ^{13}C NMR ($CDCl_3$): δ 58.9, 68.2, 70.6, 70.7, 71.9, 150.7. Mass Spec.: DCI 227 ($M + H)^+$. Anal. $C_8H_{15}O_5Cl$: theoretical C, 42.39; H, 6.67; Cl, 15.64. Found: C, 42.47; H, 6.71; Cl, 15.71. FTIR: 2878, 1778, 1452, 1352, 1248, 1170, 1130, 844, 688 cm^{-1}.

3-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxycarbonylamino}-propionic Acid Ethyl Ester 6. Triethylamine (6.13 mL, 44 mmol) was added in portions to a suspension of **5** (4.53 g, 20 mmol) and β-alanine ethyl ester hydrochloride (3.38 g, 22

Scheme 4. Synthesis of mTEG-Modified Linkers and BR96 Conjugates



mmol) in 125 mL CH_2Cl_2 at 0 °C. The reaction was warmed to room temperature and stirred for 18 h. After filtration of the solid, the filtrate was extracted with 10% citric acid and water. The organic layer was dried over Na_2SO_4 and concentrated to an oil **6** (6.09 g, 99%).

^{1}H NMR ($CDCl_3$): δ 1.15 (t, 3H), 2.42 (t, 2H), 3.27 (s, 3H), 3.33 (q, 2H), 3.45 (m, 2H), 3.54 (m, 8H), 4.03 (q, 2H), 4.09 (t, 2H), 5.30 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.1, 34.4, 36.4, 58.9, 60.6, 63.9, 69.5, 70.4, 70.5, 71.8, 156.2, 172.1. Mass Spec.: DCI 308 $(M + H)^+$. Anal. $C_{13}H_{25}NO_7$: theoretical C, 50.80; H, 8.20; N, 4.56. Found: C, 50.64; H, 8.24; N, 4.49. FTIR: 3342, 2880, 1728, 1532, 1456, 1376, 1252, 1188, 1110, 1030, 854 cm^{-1}.

3-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxycarbonylamino}-propionyl Hydrazide 7. A solution of **6** (2.53 g, 8.23 mmol) in 15 mL EtOH was treated with anhydrous hydrazine (4 mL) at room temperature for 4 h. The reaction was concentrated by rotary evaporation and dried under high vacuum to yield a colorless oil **7** (2.4 g, 100%).

^{1}H NMR ($CDCl_3$): δ 2.39 (t, 2H), 3.35 (s, 3H), 3.45 (q, 2H), 3.55 (m, 2H), 3.64 (m, 8H), 4.20 (m, 2H). Mass Spec.: DCI 294 $(M + H)^+$. Anal. $C_{11}H_{23}N_3O_6$: theoretical C, 45.04; H, 7.90; N, 14.33. Found: C, 44.90; H, 7.79; N, 14.50.

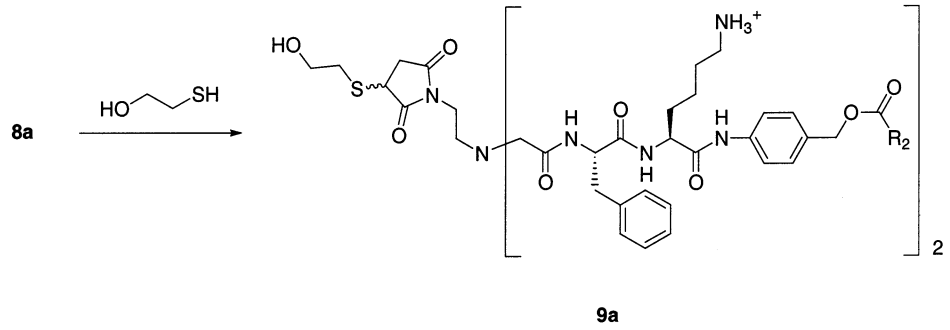
mTEG Hydrazone of Maleimidoethyliminodiacetyl-bis(Phe-Lys-PABC-DOX) 8a. **7** (254 mg, 866 μmol) was dissolved in 10 mL of methanol along with 50 μL TFA. This solution was added to a suspension of **4a** (276 mg, 108 μmol) in 40 mL anhydrous methanol. The reaction was stirred for 3.5 h at room temperature. The reaction mixture was rotary evaporated to the point of precipitation, then 1 mL CH_2Cl_2 was added to redissolve. This was added dropwise to 250 mL ether, precipitating a red solid. The solid was filtered, washed with ether, and dried under high vacuum to yield **8a** (300 mg, 90%).

^{1}H NMR (d^7-DMF): (selected peaks) δ 1.23 (d, 6H), 2.34 (t, 4H), 3.28 (s, 6H), 3.55 (s, 24H), 4.03 (s, 6H), 4.10 (t, 4H), 6.98 (s, 2H). Mass Spec.: FAB 1354.6 $(M+2H)^{2+}$. Anal. $C_{132}H_{164}N_{18}O_{44}\cdot 3.0TFA\cdot 4.0H_2O$: theoretical C, 53.11; H, 5.65; N, 8.08. Found: C, 52.99; H, 5.96; N, 8.64. FTIR: 3412, 2938, 1702, 1681, 1616, 1520, 1412, 832, 696 cm^{-1}.

mTEG Hydrazone of Maleimidoethyliminodiacetyl-bis(Val-Lys-PABC-DOX) 8b. Starting with **7** (761 mg, 2.6 mmol), **4b** (1.06 g, 0.43 mmol), and TFA (200 μL), **8b** (1.51 g, 90% yield) was obtained via the method described for **8a** above.

^{1}H NMR (d^7-DMF): (selected peaks) δ 0.91 (m, 12H), 1.24 (dd, 6H), 3.27 (s, 6H), 3.47 (s, 24H), 4.04−4.07 (2s, 6H), 6.86 (m, 2H). Mass Spec.: TOF 2610.2 $(M + H)^+$, 1305.6 $(M+2H)^{2+}$. Anal. $C_{124}H_{164}N_{18}O_{44}\cdot 3.0TFA\cdot 2.0H_2O$: theoretical C, 52.24; H, 5.77; N, 8.44. Found: C, 52.33; H, 5.95; N, 8.61. FTIR: 3429, 2938, 1699, 1684, 1617, 1578, 1520, 1446, 1413, 833 cm^{-1}.

Scheme 5. Synthesis of the Thioether **9a**



9a

R_2 defined in Scheme 4.

Synthesis of Thioether 9a. 8a (3 mg) was dissolved 1 mL methanol and treated with 10 μL 2-mercaptoethanol at room temperature for 10 min Complete conversion to the thioether **9a** was followed by HPLC (isocratic 25% 50 mM triethylamine formate [pH 2.8], 75% methanol at 1.5 mL/min). In this system, **9a** eluted at 7.0 min, **8a** at 8.1 min.

mTEG Hydrazone of DOX 10. 7 (293 mg, 1.0 μmol) and doxorubicin HCl (290 mg, 0.5 mmol) were dissolved in 25 mL methanol along with 50 μL TFA. The reaction was stirred for 6 h at room temperature. The reaction mixture was concentrated by rotary evaporation to 5 mL volume and added dropwise to 250 mL ether, precipitating a red solid. The solid was filtered, washed with ether, and dried under high vacuum to yield **10** (400 mg, 94%).

^{1}H NMR (d^4-MeOH): (selected peaks) δ 1.31 (t, 3H), 3.35 (s, 3H), 3.55 (t, 2H), 3.65 (m, 12H), 4.03 (s + t, 4H), 4.15 (m, 3H), 5.10 (t, 1H), 7.57 (d, 1H), 7.82 (t, 1H), 7.96 (d, 1H). Mass Spec.: FAB 819.6 $(M + H)^+$. Anal. $C_{38}H_{50}N_4O_{16}$·HCl·2.0H_2O: theoretical C, 51.21; H, 6.22; N, 6.29. Found: C, 51.48; H, 6.24; N, 6.59.

Conjugate Synthesis

Monoclonal Antibodies. BR96 is a chimeric (mouse/human IgG1) mAb which identifies a Le^y-related tumor associated antigen expressed on carcinomas of the lung, colon, breast, and ovary. The mAb is rapidly internalized following antigen-specific binding.[1,2,4] Immunoconjugates of **8a** and **8b** were prepared with both chimeric BR96 and a nonbinding control human IgG (Rockwell Inc., Gilbertsville, PA).

Conjugation. The following procedure, for the conjugation of BR96 and **8a**, is typical of that used for all cited preparations. To reduced BR96[3] (83 mL, 6.70 μmol mAb, 51.6 μmol thiol) was added dropwise at 0 °C under Ar a solution of **8a** (239 mg, 77.3 μmol) in 4.0 mL DMSO (final DMSO concentration 5%). After stirring for 30 min, the reaction was filtered through a 0.22 μ sterile filter. Conjugate was purified at 4 °C by percolation (approximately 2 mL/min) through a 1 in x 24 in Bio-Beads column (initially prepared by swelling and packing in methanol, then equilibrated in H_2O, and finally PBS, pH 7.4). The purified conjugate was filtered again through a 0.22 μ sterile filter to yield 95 mL of BR96-**8a** (BR96, 76.1 μM; DOX, 1.11 mM; molar ratio, 14.6 mol DOX/mol BR96; yield, 100%). Conjugate was frozen in liquid N_2 and stored at −80 °C.

Size Exclusion-HPLC of Conjugates. SEC−HPLC analysis of conjugates was carried out on a Toyo Soda 5μ TSK column (3000SW XL, 300 mm × 7.5 mm) equipped with a 5μ TSK (3000SW XL, 40 mm × 6.0 mm) guard column. Samples were eluted at a flow rate of 1.0 mL/min with isocratic 0.2 M KH_2PO_4, 0.9% NaCl, pH 6.8, and monitored at 280 and 495 nm.

***In Vitro* Cytotoxicity assays**. In vitro cytotoxicity assays were performed as described previously[3,13] using the BR96 expressing human lung carcinoma line L2987.[2] Briefly, monolayer cultures of L2987 cells were harvested and resuspended to 1 × 10^5/mL in RPMI-1640 containing 10% heat inactivated fetal calf serum (RPMI-10%FCS). Cells (0.1 mL/well) were added to each well of 96 well microtiter plates and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was removed from the plates and serial dilutions of DOX or mAb-DOX conjugates added to the wells. All dilutions were performed in quadruplicate. Cells were exposed to DOX or mAb-DOX conjugates for 2h at 37 °C in a humidified atmosphere of 5% CO_2. Plates were centrifuged (200*g*, 5 min), the drug or conjugate removed, and the cells washed three times with RPMI-10%FCS. The cells were cultured in RPMI-10% FCS (37 °C, 5% CO_2) for an additional 48 h. At this time the cells were pulsed for 2 h with 1.0 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA). Cells were harvested onto glass fiber mats (Skatron Instruments, Sterling, VA), dried, and filter bound [^{3}H]thymidine radioactivity determined (β-Plate scintillation counter, Pharmacia LKB Biotechnology, Piscataway, NJ). Inhibition of [^{3}H]thymidine uptake was determined by comparing the mean CPM for treated samples with that of the mean CPM of the untreated control.

Determination of Hydrolysis Kinetics of 10. The hydrolysis of **10** was examined according to a procedure used previously for the analysis of analogues of **1** and **2**.[3,4] In brief, a solution of **10** (30 mM) was incubated in either 100 mM NaOAc, pH 5.0, or 100 mM Na_2HPO_4, pH 7.0, at 37 °C. Both the formation of DOX and the disappearance of starting material were monitored by HPLC. Free DOX was quantified and represented as the percent of total DOX in solution versus time. Half-lives for the appearance of DOX were calculated from these curves and listed in Table 2.

Results and Discussion

Linker Synthesis. Since commercially available mTEG is homogeneous, synthesis and analysis of its derivatives are straightforward. Thus, mTEG was easily converted to the chloroformate **5**, which was reacted with β-alanine ethyl ester to give the carbamate **6**. Treatment with hydrazine yielded the hydrazide **7** in nearly quantitative overall yield (Scheme 3). The two DOX moieties of **4a** and **4b** were then condensed with excess **7** by our standard methods (ethanol, catalytic TFA).[3] **8a** and **8b** were isolated by precipitation in 90% yield and required no chromatographic purification (Scheme 4). The reactivity of **8a** with thiols was probed by treatment with 2-mercaptoethanol at room temperature in methanol, generating thioether **9a** quantitatively (Scheme 5).

Conjugate Synthesis. We have shown that mild treatment of BR96 with DTT reduced a maximum of four interchain disulfide bonds to yield eight thiol groups. Upon reaction with a bifunctional DOX linker such as **2**, immunoconjugates with molar ratios equal to approximately 16 were obtained.[3] These reduction and conjugation procedures have been found to be generally applicable and were used with minor modifications in this work to generate BR96 and IgG conjugates of **8a** and **8b**. Thus, mAb (either BR96 or control

Table 1. Representative Immunoconjugates

entry	immunoconjugate[a]	scale (mg)	DMSO (%)	SH titer	molar ratio	yield (%)	aggregate (%)
1	BR96-**4a**	14	18[b]	8.9	13.9	61	100
2	BR96-**4a**	600	3[b]	8.5	13.3	57	100
3	BR96-**4a**	250	10	8.7	14.1	64	100
4	BR96-**8a**	25	5	6.6	-	-	2
5	BR96-**8a**	25	9	6.6	-	-	9
6	BR96-**8a**	25	17	6.6	-	-	11
7	BR96-**8a**	75	5	6.6	12.6	100	1
9	BR96-**8a**	110	5	8.9	13.4	90	4
10	BR96-**8a**	1200	5	7.7	14.6	100	14
11	BR96-**8a**	1100	5	8.0	14.0	100	13
12	BR96-**4b**	100	17[b]	8.1	13.2	50	100
13	BR96-**8b**	25	5	9.3	16.1	70	6
14	BR96-**8b**	100	5	8.9	14.6	75	undetected
15	BR96-**8b**	1500	5	8.4	16.0	77	undetected
16	IgG-**4a**	450	19[b]	9.2	11.4	54	-
17	IgG-**8a**	1300	5	9.2	15.1	76	-
18	IgG-**8b**	1200	5	8.3	15.9	56	-

[a] Defined in Schemes 2 and 4. [b] DMF used as the cosolvent in place of DMSO.

IgG) was treated with an 8-fold excess of DTT with exclusion of O_2 at 37 °C for 3 h, generating SH titers of about 8 for BR96 and 10 for IgG. The conjugate was formed by addition of a 1.5 molar excess **8a** or **8b** (based on SH titer) as a DMSO solution for 30 min at 0 °C, followed by chromatography on Bio-Beads to remove unreacted linker and DMSO. Molar ratio values averaging 13−15 DOX/mAb were obtained for BR96-**8a** and 15−16 for BR96-**8b**, consistent with the SH titer generated. Protein recoveries were generally good with yields in the range of 70−100% (Table 1). The absence of unreacted Ab was verified by HIC chromatography and the immunoconjugate products were shown by RP-HPLC to contain <2−3% unconjugated DOX or DOX-linker.

SEC−HPLC was used to assay each preparation of BR96-**8** conjugate for aggregate formation. A typical set of chromatograms is shown in Figure 3, and results for all batches are displayed in Table 1. Our chromatographic method is capable of detecting monomer at 10.0 min and dimer at 8.5 min, as well as higher molecular weight components in the immunoconjugate.[14] Unconjugated BR96 elutes at 10.3 min. As shown in Table 1, it is readily apparent that aggregation as dimer is largely inhibited in all BR96 conjugates of **8a** and **8b** in contrast to **4a** and **4b**. We learned in our first few trial batches with **8a** (entries 4−7 in Table 1) that aggregate levels are directly related to the amount of DMSO cosolvent used to dissolve the linker. As final DMSO concentrations were increased from 5% to 17%, dimer levels increased from 2% to 11%. Since lowest aggregation was observed at 5% DMSO, this concentration was used for subsequent conjugations. Though some mole ratios ran slightly below the expected value of 2× SH titer, as we gained expertise working with these linkers, the mole ratios tended to approach the predicted values. BR96-**8a** conjugates with the highest mole ratios and/or those synthesized in largest scale exhibited the highest aggregate levels (up to 14%). The effects of scale may be related to extended reaction times resulting from adding large volumes of linker solutions in those batches. These results are still acceptable, with aggregate levels far below those of conjugates lacking the mTEG side chain. BR96-**8b** conjugates up to the maximum value of 16 were almost completely free of aggregate. This is posssibly due to a combination of factors which include (1) enhanced pi-stacking in the Phe-linker, (2) lower degree of hydrophobicity of the Val-linker, and (3) conformational/steric differences between the two linkers.

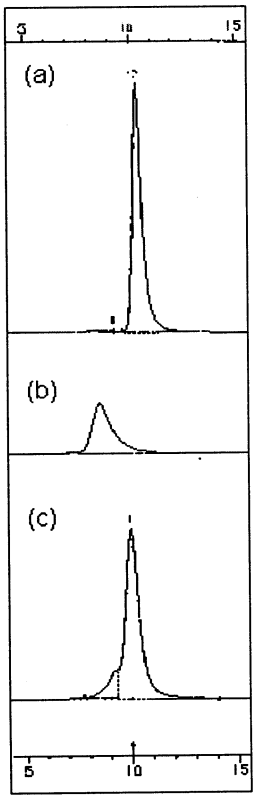
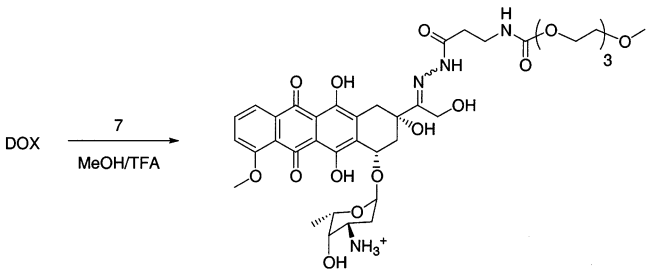


Figure 3. Representative SEC−HPLCs of (a) unconjugated BR96, (b) dimeric BR96-**4b** (MR = 13.2), and (c) monomeric BR96-**8b** (MR = 16.1, dimer 6%). In this system, SEC standards IgG (MW 158 000) and IgA (MW 300 000) run at 10.2 and 8.9 min, respectively.

Hydrolysis Kinetics. To maintain aggregate-free conjugate in circulation, the mTEG chain of BR96-**8a** and **-8b** should be stable under normal physiological conditions (pH 7). After the immunoconjugate undergoes endocytosis, the mTEG chain should be hydrolytically labile in the tumor lysosome (pH 5). In a formal sense, this process essentially converts BR96-**8a** and **-8b** to, respectively, BR96-**4a** and **-4b**, one of which (BR96-**4a**) has already been shown to degrade enzymatically in

Scheme 6. DOX Hydrazones



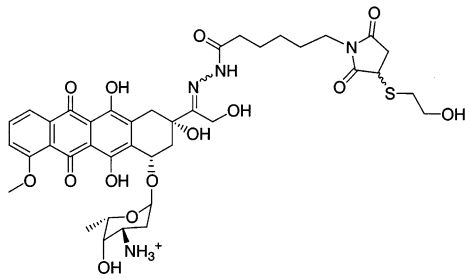


Table 2. Hydrolysis of DOX Hydrazones

compound	$T_{1/2}$ (h), pH 5, 37 °C[a]	$T_{1/2}$ (h), pH 7, 37 °C[b]
10	2.3	>96
11[c]	3.5	>120

[a] 100 mM NaOAc. [b] 100 mM Na_2HPO_4. [c] Ref 3.

Table 3. In Vitro Cytotoxicity of Linear and Branched Peptide DOX Conjugates

immunoconjugate	molar ratio	IC_{50} (μM DOX)	IC_{50} (μM mAb)	immunospecificity ratio[a]
BR96-**3a**	8.0	0.2	0.025	>50
BR96-**4a**	14.1	0.2	0.014	25
BR96-**8a**	12.6	0.2	0.016	>50
BR96-**3b**	8.0	0.4	0.050	>25
BR96-**8b**	15.4	0.1	0.007	>100
BR96-**8b**	16.1	0.2	0.012	>50
unconjugated DOX		0.3		

[a] Immunospecificity ratio defined as IC_{50} of control IgG-DOX/ IC_{50} BR96-DOX following a 2 h exposure time.

tumor lysosomes to release DOX.[6] Because of poor aqueous solubility, the hydrolysis of **8a** and **8b** could not be directly characterized. Instead, model compound **10** was synthesized (Scheme 6) and its conversion to free DOX monitored at pH 7 and 5, 37 °C, by RP-HPLC. Results are summarized in Table 2. Because of the residual positive charge, **10** is an imperfect model for **8a** or **8b**. Nonetheless, we observed almost identical kinetics when compared to the previously reported model DOX hydrazone **11**.[3] Hydrolysis of the mTEG chain from **10** is rapid at pH 5 (about 2−3 h), but at pH 7 the hydrazone linkage is stable.

In Vitro Cytotoxicity. The in vitro potency of immunoconjugates BR96-**8a** and BR96-**8b** is shown in Table 3. In all cases, the immunoconjugates were evaluated against the BR96-positive L2987 lung carcinoma line using a 2h drug or immunoconjugate exposure. Both BR96-**8a** and BR96-**8b** conjugates demonstrated potency on a DOX basis that was comparable to that of unconjugated DOX. BR96-**8a** and BR96-**8b** produced antigen-specific cytotoxicity and were at least 50−100 fold more potent than their respective IgG controls. This high immunospecificity is indicative of the excellent in vitro stability of the peptide linker. In comparing the TEGylated BR96-**8a** versus its unTEGylated parent BR96-**4a**,[6] it can be concluded that the mTEG chain has no deleterious effect on the antigen-specific toxicity of the conjugate.[15] The effects of branching the linker are apparent by examining BR96-**8a**, which is as potent as BR96-**3a** on a DOX basis, but almost twice as potent on a mAb basis. This phenomenon has been observed previously with conjugates represented by BR96-**2**.[3] In summary, BR96-**8a** and BR96-**8b** are highly potent and immunospecific in vitro. Importantly, on a mAb basis, the TEGylated branched peptide immunoconjugates are more potent than single chain peptide BR96 immunoconjugates and non-TEGylated branched peptide BR96 immunoconjugates.

Conclusion

We have prepared high mole ratio BR96 immunoconjugates using branched peptide-DOX linkers. Aggregation was prevented by the introduction of hydrophilic mTEG residues in a defined, homogeneous manner onto the DOX portion of the linkers via hydrazone links. These immunoconjugates are highly potent and immunospecific in vitro. The data indicate that the mTEG chains are stable at pH 7 but hydrolyze at lysosomal pH 5. The peptide linkers are also enzymatically hydrolyzed in lysosomes, releasing free DOX. The potential therapeutic utility of these immunoconjugates still requires further evaluation in preclinical models. Our utilization of short mTEG chains to inhibit nonspecific aggregation in these BR96 immunoconjugates represents a novel application of PEGylation technology. It is also applicable to the synthesis of immunoconjugates with other hydrophobic drugs and linkers. Those results will be reported in later publications.

References

(1) Hellstrom, I.; Garrigues, H. J.; Garrigues, U.; Hellstrom, K. E. Highly tumor-reactive, internalizing mouse monoclonal antibodies to Le^y-related cell surface antigen. *Cancer Res.* **1990**, *50*, 2183-2190.

(2) Trail, P. A.; Willner, D.; Lasch, S. J.; Henderson, A. J.; Hofstead, S. J.; Casazza, A. M.; Firestone, R. A.; Hellstrom, I.; Hellstrom, K. E. Cure of xenografted human carcinomas by BR96- doxorubicin immunoconjugates. *Science* **1993**, *261*, 212−215.

(3) King, H. D.; Yurgaitis, D.; Willner, D.; Firestone, R. A.; Yang, M. B.; Lasch, S. J.; Hellstrom, K. E.; Trail, P. A. Monoclonal antibody conjugates of doxorubicin prepared with branched linkers: A novel method for increasing the potency of doxorubicin immunoconjugates. *Bioconjugate Chem.* **1999**, *10*, 279−288.

(4) Willner, D.; Trail, P. A.; Hofstead, S. J.; King, H. D.; Lasch, S. J.; Braslawsky, G. R.; Greenfield, R. S.; Kaneko, T.; Firestone, R. A. (6-Maleimidocaproyl)hydrazone of doxorubicin - a new derivative for the preparation of immunoconjugates of doxorubicin. *Bioconjugate Chem.* **1993**, *4*, 521−527.

(5) Dubowchik, G. M.; Firestone, R. A.; Padilla, L.; Willner, D.; Hofstead, S. J.; Mosure, K.; Knipe, J. O.; Lasch, S. J.; Trail, P. A. Cathepsin B-labile dipeptide linkers for antigen-specific lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and *in vitro* anticancer activity. *Bioconjugate Chem.* **2002**, *13*, 855−869.

(6) Dubowchik, G. M.; Radia, S.; Mastalerz, H.; Leung, K.; Firestone, R. A.; King, H. D.; Hofstead, S. J.; Willner, D.; Lasch, S. J.; Trail, P. A. Doxorubicin immunoconjugates containing bivalent, lysosomally-cleavable dipeptide linkages. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1529−1532.

(7) The synthesis of **4a** and preliminary results for its conjugation to BR96 have been reported in ref 6. The synthesis of **4b** and its conjugation to BR96 closely follows that reported for **4a** in that reference.

(8) Finbloom, D. S.; Abeles, D.; Rifai, A.; Plotz, P. H. The specificity of uptake of model immune complexes and other protein aggregates by the murine reticuloendothelial system. *J. Immunol.* **1980**, *125*, 1060−1065.
(9) Patel, V. F.; Hardin, J. N.; Mastro, J. M.; Law, K. L.; Zimmermann, J. L.; Ehlhardt, W. J.; Woodland, J. M.; Starling, J. J. Novel acid labile COL1 trityl-linked difluoronucleoside immunoconjugates: synthesis, characterization, and biological activity. *Bioconjugate Chem.* **1996**, *7*, 497−510.
(10) King, D.; Dubowchik, G. M. Manuscript in preparation.
(11) Zalipsky, S.; Harris, J. M. Introduction to Chemistry and Biological Applications of Poly(ethylene glycol). In *Poly(ethylene glycol) Chemistry and Biological Applications*; J. M. Harris; S. Zalipsky, Eds.; American Chemical Society: Washington, DC, 1997; pp 1−13.
(12) Lasic, D. D. The Conformation of Polymers at Interfaces. In *Poly-(ethylene glycol) Chemistry and Biological Applications*; J. M. Harris; S. Zalipsky, Eds.; American Chemical Society: Washington, DC, 1997; pp 31−44.
(13) Trail, P. A.; Willner, D.; Lasch, S. J.; Henderson, A. J.; Greenfield, R. S.; King, D.; Zoeckler, M. E.; and Braslawsky, G. R. Antigen specific activity of carcinoma reactive BR64-Adriamycin conjugates evaluated *in vitro* and in human tumor xenograft models. *Cancer Res.* **1992**, *52*, 5693−5700.
(14) In some cases, the dimeric peak was not sufficiently resolved to yield an exact measure of dimer level. In these cases, the dimer peak was quantified by drawing a perpendicular line at the inflection point between dimer and monomer peaks (as in Figure 3, panel c). Dimer levels reported in this manner therefore represent a worst case estimate.
(15) BR96-**4b** was not tested; therefore, direct comparison to BR96-**8b** is not possible.

JM020149G