Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1529–1532

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Doxorubicin Immunoconjugates Containing Bivalent, Lysosomally-Cleavable Dipeptide Linkages

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Received 13 November 2001; accepted 15 February 2002

Abstract—Bivalent doxorubicin (DOX)-dipeptides (**16a**–**c**) were prepared and conjugated to the monoclonal antibody BR96. The dipeptides are cleaved by lysosomal proteases following internalization of the resulting immunoconjugates. Conjugate **18b** demonstrated antigen-specific in vitro tumor cell killing activity ($IC_{50} = 0.2\,\mu M$) that was equipotent to DOX with a near doubling of drug molecules/MAb. Size exclusion chromatography showed **18b** to be a noncovalent dimer that was formed immediately upon conjugation.

The success of antigen-dependent drug delivery to target tumor cell populations can be limited by the cell surface density of expressed antigen by saturation of the receptors with immunoconjugate that is endocytosed at a given rate.[1] In this regard, the more drug molecules an immunoconjugate is able to carry without compromising tumor localization, drug release, and aqueous solubility the better. One way of increasing drug loading without coating the surface of a monoclonal antibody (MAb) with (usually lipophilic) drug moieties is to use polyvalent linkers in conjunction with carefully controlled conjugation methods. Herein we report the synthesis of branched drug-dipeptide linker compounds that can be conjugated to free thiol groups generated on MAbs by treatment with limiting amounts of dithiothreitol (DTT).[2] The dipeptides are cleaved by lysosomal proteases following internalization of the resulting immunoconjugates.[3] The liberation of active drug by these dipetide conjugates requires the presence of a self-immolative *p*-aminobenzyloxycarbonyl (PABC) spacer,[4] presumably because of steric constraints in the enzyme active site.[3]

Design and Synthesis

To make the linkers as hydrophilic as possible, we wanted to minimize their size while ensuring that the site of desired proteolytic cleavage was unencumbered so that liberation of free drug could occur as rapidly as with single-chain dipeptide linkers. Previous hydrazone-containing branched linkers used glutamic acid as the branching device.[5] However, to generate symmetrical linkers and to avoid the issue of chirality we chose to use iminodiacetyl (IDA) and iminodipropionyl (IDP) units in order to vary the distance between the branch point and the dipeptide. The maleimidoethyl-containing intermediates **7a** and **7b** were prepared as shown in Scheme 1. Mono-protected ethylenediamine **3** was prepared in 55% overall yield by treatment with 2 equiv of benzyloxycarbonyl chloride followed by partial acidolysis in concd HCl/acetic acid.[6] Alkylation using either *t*-butyl bromoacetate or t-butyl acrylate, followed by hydrogenolysis in the presence of acetic acid gave the sensitive amino diesters **5a** and **5b**. These were immediately treated with maleic anhydride followed by TMSCl-mediated cyclization to give the maleimidoesters **6a** and **6b** in ca. 60% yield. Significant decomposition occurred when deprotection was attempted with TFA or HCl in dioxane. However, overnight stirring with 3 equiv of *p*-toluenesulfonic acid (TsOH) in CH_2Cl_2 provided the diacid ammonium salts **7a** and **7b**. In each case, a solid precipitate was formed

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0960-894X/02/$ - see front matter
PII: S0960-894X(02)00194-4

containing 1.2–1.4 equiv of TsOH that did not interfere in the next reaction.

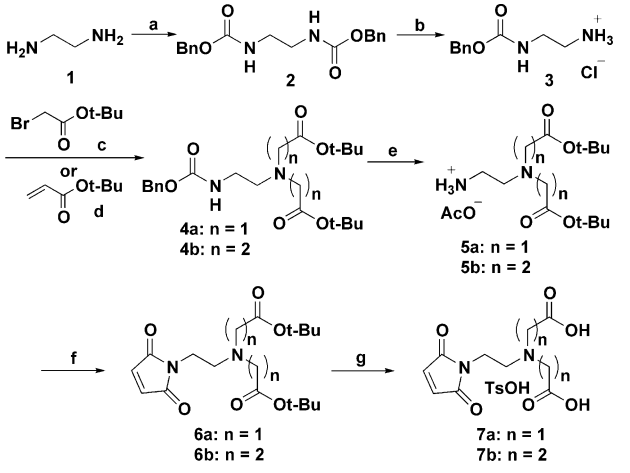
N-Maleimidopropionyl-iminodiacetic acid **11** was prepared as shown in Scheme 2 in a straightforward manner from commercially available maleimido-propionic acid **8** and di-*t*-butyliminodiacetate.

Coupling of the diacids (**7a**, **7b**, or **11**) with two equiv of the dipeptide-benzyl alcohol, Phe-Lys(MMT)-PABOH **12**,[7] was accomplished under standard conditions by in situ formation of the NHS active esters using DCC (Scheme 3), giving the branched benzylic alcohols **13** in very good yields. Formation of the activated *p*-nitrophenyl carbonates **14** and coupling with DOX were carried out essentially as described for the single-chain peptide linkers.[7] The two *p*-monomethoxytrityl (MMT) protecting groups were cleanly removed by treatment of **15** with excess dichloroacetic acid (20 equiv)/anisole (200 equiv) in CH_2Cl_2. Interestingly, in the case of the IDP compound **16b** the branching nitrogen atom was able to remain protonated giving, after precipitation with ethyl acetate, the tris–Cl_2CHCO_2H salt. On the other hand, ^{1}H NMR of **16a** showed the less basic IDA amine to be unprotonated following isolation.[8]

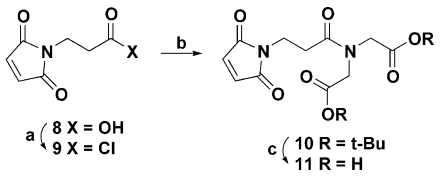
This clear pK_a difference between the two amines led to unusual side products. When a sample of **16a** was left in DMF at room temperature, HPLC showed its gradual disappearance over several hours coinciding with the appearance of two new components in a 1:1 ratio having molecular weights identical to **16a**. This decomposition could be inhibited to a varying extent by the presence of 2–10% acetic acid and by 'capping' the maleimide with mercaptoethanol, giving the corresponding thioether **18a**. In addition, **16b** and **16c** were completely stable under the same conditions. To us, this evidence suggests the formation of the diastereoisomeric 18-membered cyclic amides **17** perhaps initiated by transient proton transfer from Lys to the weakly-basic IDA nitrogen (Scheme 4). ^{1}H NMR of the newly-formed products was consistent with the structural assignment **17** in that there appeared additional complexity in an already complex spectrum–as might be expected upon cyclization–and the usually prominent maleimide CH signal ($\delta = 7.0$ ppm) was no longer visible.

Cathepsin B-mediated Drug Release and Plasma Stability

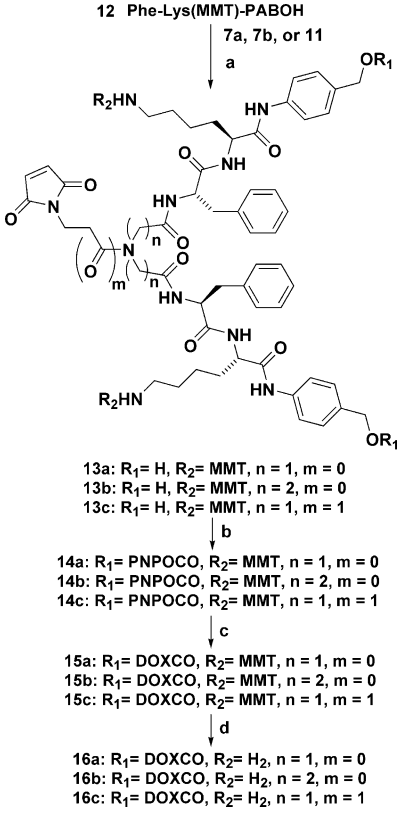
Thioether **18a**, a model compound representing the most sterically-demanding bivalent linker, was tested for cathepsin B-mediated liberation of DOX under conditions used for single-chain linkers.[3] In this case, complete drug release occurred nearly as rapidly ($t_{1/2} = 20$ min) as measured for the model compound Z-Phe-Lys-PABC-DOX ($t_{1/2} = 8$ min) suggesting that the presence of the second drug-peptide arm should present no liability. In addition, **18a** showed no detectable instability in freshly-drawn human plasma at 37 °C over 6 h.



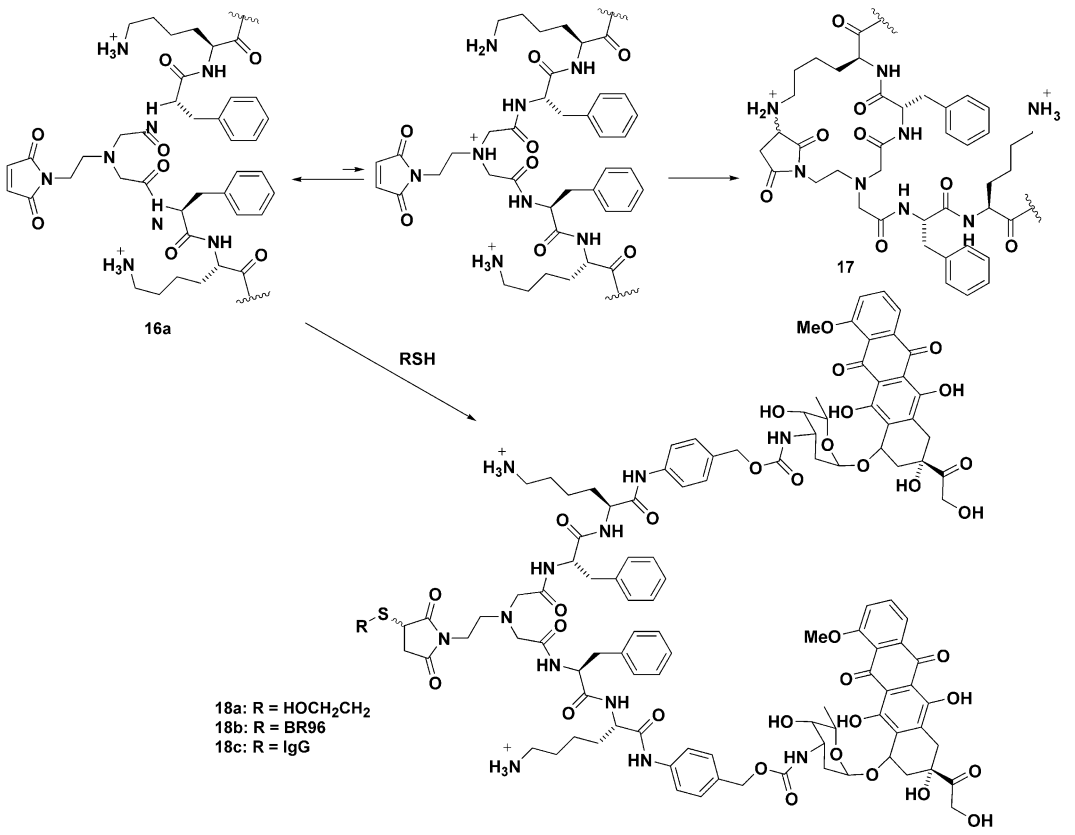
Scheme 1. (a) CBZ-Cl, KOH, quant; (b) concd HCl, AcOH, Δ, 55%; (c) $KHCO_3$, DMF, 73%; (d) Et_3N, CH_3OH/CH_2Cl_2, 96%; (e) H_2, Pd-C, AcOH, quant; (f) (1) maleic anhydride, CH_2Cl_2; (2) TMSCl, Et_3N, MeCN, Δ, 55–63%; (g) TsOH (3 equiv), CH_2Cl_2, quant.



Scheme 2. (a) oxalyl chloride, cat. DMF, CH_2Cl_2; (b) $HN(CH_2CO_2t\text{-}Bu)_2$, DIEA, quant; (c) TFA, CH_2Cl_2, 90%.



Scheme 3. (a) DCC, HOSu, DME, 76–90%; (b) PNP_2CO, DIEA, CH_2Cl_2, 68–94%; (c) DOX-HCl, DIEA, NMP, 60–93%; (d) Cl_2CHCO_2H, anisole, CH_2Cl_2, 90–100%.



Scheme 4.

Conjugation and In Vitro Cytotoxicity

Bivalent maleimide **16a** was conjugated to chimeric BR96 and a control IgG that had been thiolated as reported previously,[9] using a protocol modified for less soluble dipeptide-drug-linker compounds.[10] Immunoconjugate **18b** had a drug/MAb mole ratio (MR) of 14.1, close to the theoretical limit of 16 that would result from functionalization of all eight free thiol residues generated by controlled DTT reduction (protein yield 64%), while the MR for the nonbinding IgG conjugate **18c** was 11.4.

Against the antigen-positive L2987 lung carcinoma cell line (2 h exposure),[5] **18b** had an IC_{50} of 0.2 μM (equivalent DOX), while **18c** was much less active (IC_{50} = 5.0 μM). Thus, **18b** demonstrated antigen-specific cell killing activity that was equipotent to DOX with a near doubling of drug molecules/MAb. However, size exclusion chromatography (SEC) showed **18b** and **18c** to exist almost exclusively as non-covalent dimers.[11] Manipulation of conjugation conditions failed to prevent this aggregation which was found to occur almost immediately upon addition of **16a** to the thiolated MAb. Although the dimeric nature of **18b** does not appear to impair its in vitro activity, protein aggregates, even noncovalent ones, are unlikely to be effective therapeutics in vivo as they are liable to be recognized as foreign bodies and cleared by the reticuloendothelial system.[12] Without exception, we find that non-covalently aggregated immunoconjugates are susceptible to cathepsin B-mediated drug release and show in vitro potency that one would expect for an 'unaggregated' conjugate. Whether this means that the 'site of aggregation' is far from the site of drug linkage or whether disaggregation occurs prior to approach of the protease-perhaps as a result of the lower pH of the lysosome-is unknown. In subsequent reports, we will show how this problem was successfully circumvented.

References and Notes

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8. All new compounds gave satisfactory analytical data. For **16a**: ^{1}H NMR ($CDCl_3/CH_3OD$) δ 1.19 (d, 6H), 1.33 (m, 4H), 1.52 (m, 6H), 1.74 (m, 6H), 2.14 (ABq, 4H), 2.49 (brt, 2H), 2.93 (m, 12H), 3.25 (brs, 4H), 3.51 (m, 2H), 3.77 (m, 2H), 3.90 (s, 6H), 4.04 (m, 2H), 4.32 (m, 2H), 4.65 (s, 4H), 4.82 (m, 4H), 5.05 (brs, 2H), 5.33 (brs, 2H), 5.82 (brs, 2H), 6.63 (s, 2H), 7.01 (m, 14H), 7.32 (m, 6H), 7.63 (t, 2H), 7.79 (d, 2H). MS (ESI) 1078.8 $(MH_2)^{2+}$, 2156.8 $(MH)^+$.

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11. When subjected to gel electrophoresis under denaturing conditions, **18b** showed no covalently-crosslinked polypeptides, only DOX-substituted heavy and light chains. Under normal conditions, no monomeric species were detected for **18b** and **18c**. In comparison with BR96, the control IgG is much more heterogeneous, making it difficult to directly compare the degree of aggregation of IgG- and BR96-conjugates.
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