Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 2213–2215

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Syntheses of Dendritic Linkers Containing Chlorambucil Residues for the Preparation of Antibody–Multidrug Immunoconjugates

Chengzao Sun, Peter Wirsching* and Kim D. Janda*

Department of Chemistry, The Scripps Research Institute and the Skaggs Institute for Chemical Biology, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA

Received 11 March 2002; accepted 19 April 2002

Abstract—A novel dendritic molecule with nine chlorambucil (CBL) residues on the surface and a maleimide moiety at the core terminus was synthesized using a convergent synthetic methodology. This molecule is ready for attachment to single-chain Fv antibodies (scFvs) to form antibody–multidrug immunoconjugates in an effort to study the relevance of drug/antibody molar ratio and the potency of these drug–antibody immunoconjugates. A monomer and a trimer with a similar structural motif were also prepared for comparative purposes.

Monoclonal antibodies (mAbs) directed to tumor-associated antigens have been chemically conjugated with anti-cancer drugs to form immunoconjugates.[1] These immunoconjugates are expected to both improve efficacy and reduce systemic toxicity and therefore are actively involved in chemotherapeutic research programs. Efforts to design and refine immunoconjugates have focused on the selectivity of the mAbs as well as drug-linking/drug-releasing strategies.[1,2] To improve tumor penetration and the pharmacokinetic properties of these mAbs, smaller antibody fragments such as single-chain Fv antibodies (scFvs) have been constructed.[3] Recently, we have developed high-affinity human scFvs to tumor-associated carbohydrate antigens sialyl Lewis^x and Lewis^x using phage-display technology.[4] To utilize these antibodies to destroy cancer cells, we are moving forward in the preparation of immunoconjugates. Since the scFv unit is smaller than the whole IgG, there are fewer sites available for linkage of the drug(s) to the antibody. Consequently, we are exploring dendritic molecules with two functionalized termini to be used as linkers to connect multiple drug molecules to a scFv. Our plan to prepare this kind of scFv-multidrug immunoconjugate is shown in Figure 1.

A dendritic linker approach will increase the molar ratio of drug/antibody, which is related to the potency of the immunoconjugate.[5] The increased molar ratio can be controlled by varying the size or generation of the dendritic molecule, which will allow investigations into the relationship between the molar ratio of drug/antibody and the potency of the immunoconjugate.

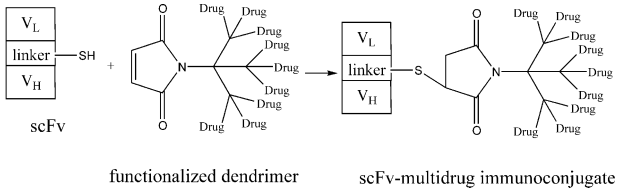

Figure 1. Cartoon depicting the preparation of an antibody–multidrug immunoconjugate. Here a scFv is equipped with a cysteine residue and a dendrimer carrying multiple drug residues is functionalized for conjugation at the core with a maleimide group.

Dendrimers are widely employed as macromolecular vectors for drug delivery purposes and other biomedical applications.[6] Indeed many of them have been used as encapsulating agents. In addition, drugs or other biologically active molecules can be attached to the surface of the dendrimer. For example, antibodies and synthetic porphyrins have been attached to the surface of a starburst dendrimer.[7] However, there are very few reports of using both termini, in another words, where the starting core and the surface branches of the dendrimer are functionalized in an orthogonal manner such that each end serves a different purpose. We report herein the design and synthesis of two dendritic compounds

*Corresponding author. Tel.: +1-858-784-2516; fax: +1-858-784-2595; e-mail: kdjanda@scripps.edu

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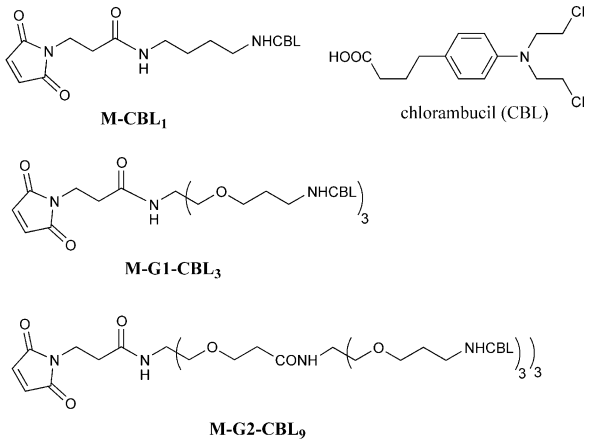


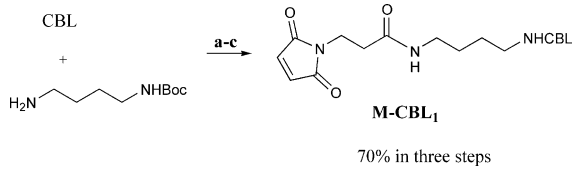
Figure 2. Drug molecules with one, three, nine CBL residues and a maleimide functionality.

with up to nine drug molecules covalently attached on the branches and the core functionalized for covalent linkage to an antibody. This provides a novel approach for using dendrimers for both multidrug carrying and drug targeting purposes.

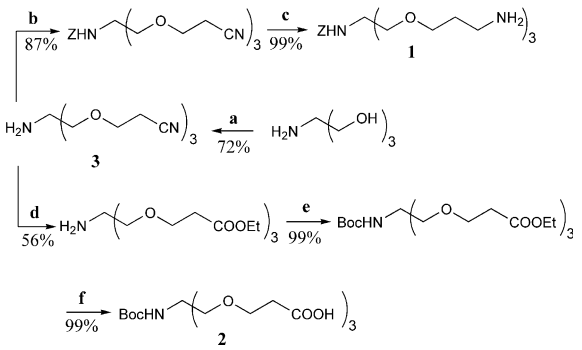
We designed and synthesized **M-CBL$_1$**, **M-G1-CBL$_3$** and **M-G2-CBL$_9$**, shown in Figure 2, as our model compounds. **M-CBL$_1$** was used to explore the general chemistry needed to access **M-G1-CBL$_3$** and **M-G2-CBL$_9$** containing chlorambucil (CBL). The latter two have dendritic linkers that carry three and nine CBL molecules, respectively. CBL is a nitrogen mustard alkylating agent used as an antineoplastic agent for chronic lymphocytic leukemia and Hodgkin's disease. It has been conjugated to carrier proteins such as antibodies[8] and more recently transferrin.[9] We chose this drug for our study because it is readily available and contains a carboxylic group for attachment to a dendrimer. It has been demonstrated that amide bond linkage to CBL does not impair its alkylating activity.[10] However, ester conjugates of CBL may be not as active as the free drug.[9] Consequently, the backbone of the dendrimer is designed as a polyamido-polyether linkage with amino functionality at the terminus to attach the CBL molecule. At the core of the dendron is an amino group, which allows introduction of a maleimide moiety for facile conjugation to a cysteine residue in an scFv.

M-CBL$_1$ was synthesized in three steps using 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (NHSMP), CBL and *N*-Boc-1,4-diaminobutane in 70% overall yield (Scheme 1).

Syntheses of **M-G1-CBL$_3$** and **M-G2-CBL$_9$** proceeded with the construction of building blocks **1** and **2** (Scheme 2). Tri-acid **2** was synthesized following literature precedent[11] while tri-amine **1** was synthesized using the same intermediate **3**. Thus, protection of the amino group in **3** with a Cbz group followed by reduction of the nitrile functionality to a primary amine using cobalt (II) ion-catalyzed $NaBH_4$ reduction in methanol gave **1**.[12] Coupling of 3.3 mol equivalents of CBL to **1** with HBTU gave **4** in 72% yield. Hydrogenation of trimer **4**



Scheme 1. (a) HBTU, NMM, DMF; (b) 50% TFA, CH_2Cl_2; (c) NHSMP, NMM, CH_2Cl_2. HBTU = *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyl-uronium hexafluoro-phosphate; NMM = 4-methylmorpholine; NHSMP = 3-maleimidopropionic acid *N*-hydroxysuccinimide ester.



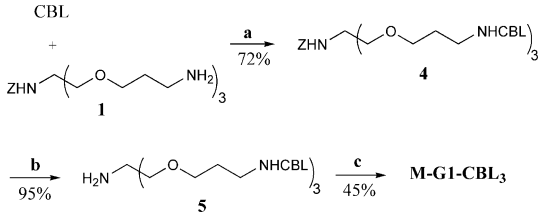
Scheme 2. (a) Acrylonitrile, KOH, 1,4-dioxane; (b) CbzCl, aq $NaHCO_3$, 1,4-dioxane; (c) $NaBH_4$, $CoCl_2{\cdot}6H_2O$; (d) H_2SO_4, EtOH, reflux; (e) Boc_2O, TEA; (f) 3 N aq NaOH.

with 10% Pd/C did not affect the chloroethyl moiety on CBL and yielded **5** almost quantitatively. The amine **5** was then treated with NHSMP to afford the desired trimeric **M-G1-CBL$_3$** (Scheme 3).

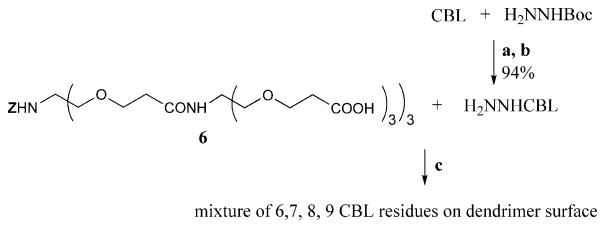
Our initial plan to synthesize **M-G2-CBL$_9$** was based on a seemingly straightforward and convenient divergent construction.[13] Thus, generation-2, a nona-acid dendrimer **6**[11b] would be coupled with the hydrazide derivative of CBL as shown in Scheme 4.

However, even with a large excess of $H_2NNHCBL$ we failed to obtain pure nonamer. In all attempts, the LC/MS results showed a mixture of hexamer through nonamer products. Therefore, we decided to change our approach to a convergent synthesis,[14] which hopefully would guarantee pure nonamer.

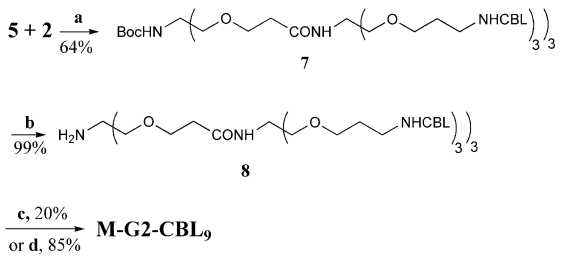
Thus, 3.3 mol equiv of **5** was coupled to the carboxylic terminus of **2** to furnish the generation-2 dendrimer-CBL compound **7** in 64% yield. Following a standard 50% TFA/CH_2Cl_2 deprotection of the Boc group to unmask the free amine **8**, attempts to add the maleimide group



Scheme 3. (a) HBTU, NMM, DMF; (b) 10% Pd/C, MeOH; (c) NHSMP, NMM, CH_2Cl_2.



Scheme 4. (a) Isobutylchloroformate, TEA, THF; (b) 50% TFA, CH_2Cl_2; (c) HBTU, NMM, DMF.



Scheme 5. (a) HBTU, NMM, DMF; (b) 50% TFA, CH_2Cl_2; (c) NHSMP, NMM, CH_2Cl_2; (d) MPA, HBTU, NMM, DMF.

using the active ester NHSMP were not successful. The reaction proceeded slowly and decomposition of the maleimide group was detected after 5 h. We surmized the increased steric hindrance around the amino group was the problem. Thus, we employed the more potent activating agent HBTU to couple **8** with 3-maleimidopropionic acid (MPA) and obtained the final fully functionalized compound **M-G2-CBL$_9$** in 85% yield (Scheme 5).

In conclusion, we have synthesized three molecules containing one, three and nine chlorambucil residues at one terminus and a maleimide moiety on the other. **M-G1-CBL$_3$** and **M-G2-CBL$_9$** take advantage of a dendritic display to carry multiple drug molecules. Site-specific attachment of these compounds to our recently prepared scFvs will allow studying the relationship between the molar ratio of drug/antibody and the potency of antibody–drug immunoconjugates and will be reported in due course.

Acknowledgements

This work was supported in part by grants from the NIH NIAID-AI47127, California Cancer Research Program (00–00757V-20012) and the Skaggs Institute for Chemical Biology.

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