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Enabling ScFvs as Multi-Drug Carriers: A Dendritic Approach

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Abstract—To enable scFvs as multi-drug carriers, we designed and synthesized dendritic linker molecules bearing up to nine chlorambucil residues at the branch ends. A maleimide group was used at the focal point of the dendron for easy linkage to the scFv. Originally designed molecules showed poor water solubility. To address this problem, a lysine residue with an unprotected carboxylic acid group was inserted into the dendron branches. The new molecules showed excellent water solubility and are now suitable for conjugation. Such dendritic molecules will allow studies to understand the relationship between the drug/antibody ratio and the potency of the immunoconjugates. The dendritic approach could also be applied to drugs other than chlorambucil and carriers other than scFvs to greatly increase the drug/carrier ratio.

Introduction

The successes of antibody-based therapeutics have opened a new era for the treatment of cancer. Some monoclonal antibody (mAb)-based drugs such as Rituxan®, Herceptin®, and Mylotarg® have been approved by the FDA and more are in clinical trials. However, current generation mAb-based drugs can be limited by the physical properties of the mAbs which are intact immunoglobulin G (IgG) molecules with a molecular weight of 150 kDa. In order to address the limitations of large IgG molecules, smaller engineered single-chain Fv (scFv) fragments were developed.[1,2] These scFvs are composed of a variable region of the light chain (V_L) and a variable region of the heavy chain (V_H) linked by a peptide spacer and are expected to have improved tumor penetration and pharmacokinetic properties.[3,4]

Recently, we successfully developed high-affinity human scFvs to tumor-associated carbohydrate antigens sialyl Lewis^x, Lewis^x and G_{M3} using phage-display technology.[5,6] Prospective applications of these scFvs are significant and therefore we have examined the possibility of attaching anti-cancer drugs to form potent immunoconjugates. The use of mAb–drug conjugates targeted at tumor-associated antigens have improved efficacy and reduced systemic toxicity due to the high specificity of the antibody.[7,8] To improve the potency of such immunoconjugate drugs, efforts have been expended to select better antibodies as well as designing more efficient drug-conjugation and releasing strategies.[7–12] With our novel human scFvs, we are investigating new and more efficient methods to conjugate anti-cancer drugs.

It has been observed that the potency of immunoconjugates can be improved by increasing the quantity of drug delivered per mAb molecule.[13,14] Therefore, one critical aspect of investigation is to improve the loading of a conjugated drug with respect to the protein carrier. Typically, mAbs have multiple sites at which a drug molecule could be covalently attached without affecting the antigen binding of the mAb. In contrast, the small size of scFvs (25 kDa) limits the possible number of sites for drug conjugation. To address this problem, a linker unit is necessary that will enable multiple drug molecules to be attached onto one residue of the scFv. Reports have shown the utility of doubly branched linkers to carry two drug molecules onto one site of a mAb.[14] Hence, this increased the molar ratio (MR) of the drug/antibody by 2-fold. Since scFvs have far fewer sites for the attachment of the drugs, an alternative and robust way of increasing the MR is needed. We hypothesized that dendritic molecules could be excellent candidates.

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Dendrimers are hyper-branched macromolecules characterized by defined cascade structures and branch-end functionalities.[15,16] The unique properties of dendrimers enable their applications as both model compounds for fundamental research as well as applications in biomedical sciences.[17] In particular, a properly designed dendrimer with a well defined internal cavity can be used to host various small guest molecules and by adjusting the steric density of the dendrimer periphery allows the controlled release rate of the guest molecule.[18–20] In this respect, dendrimers have been extensively studied and applied in the drug delivery field.[21–24] However, for our purposes, we can not use the 'host–guest' or encapsulation concept because of the large dendrimeric size which would be required. A dendrimer designed for drug release would require a molecular weight of 10–20 kDa or even more and attachment of such a large molecule to an scFv could impair its specificity, tumor penetration and other pharmacokinetic advantages. Another way in which a dendrimer could be used for drug delivery would be the attachment of drug molecules covalently onto the dendrimer periphery. The sizes of a dendrimer required for this purpose would vary depending on the loading desired and the mechanism of tumor-cell targeting. Frechét and others have reported a drug-attached dendrimer, used for both drug delivery and tumor targeting, that preferentially accumulated in the solid tumor tissue;[25,26] the dendrimer used was 23.5 kDa. Our goal is to apply our newly developed scFvs as the specificity device while the dendritic linker functions only as a drug carrier, thus we require only small dendritic molecules with multiple surface groups. In addition, we specifically require a dendritic unit or dendron, which will have two functionalizable termini denoted as the focal point/core and the surface groups.

Our dendritic approach to enable scFvs as multi-drug carriers is depicted in Figure 1. At first, we designed and synthesized two model compounds containing small dendritic linkers with three and nine chlorambucil (CBL) moieties on the surface and a maleimide group at the focal terminus for attachment to an scFv. Synthetically we have disclosed this approach.[27] However, when attempting to conjugate these molecules to an scFv, we discovered that the solubility in a buffer medium was insufficient to allow reaction. Hence, we now report our newly designed and synthesized water-soluble version of these molecules as well as the experimental details for all compounds.

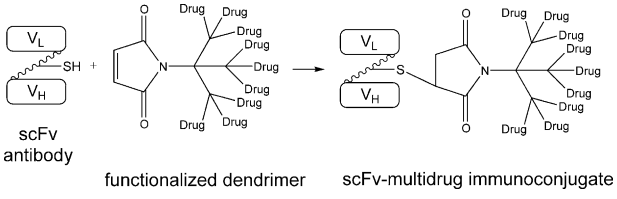


Figure 1. Cartoon depicting the preparation of an antibody–multidrug immunoconjugate. Here an scFv is equipped with a cysteine residue and a dendrimer carrying multiple drug residues is functionalized for conjugation at the core with a maleimide group.

Results and Discussion

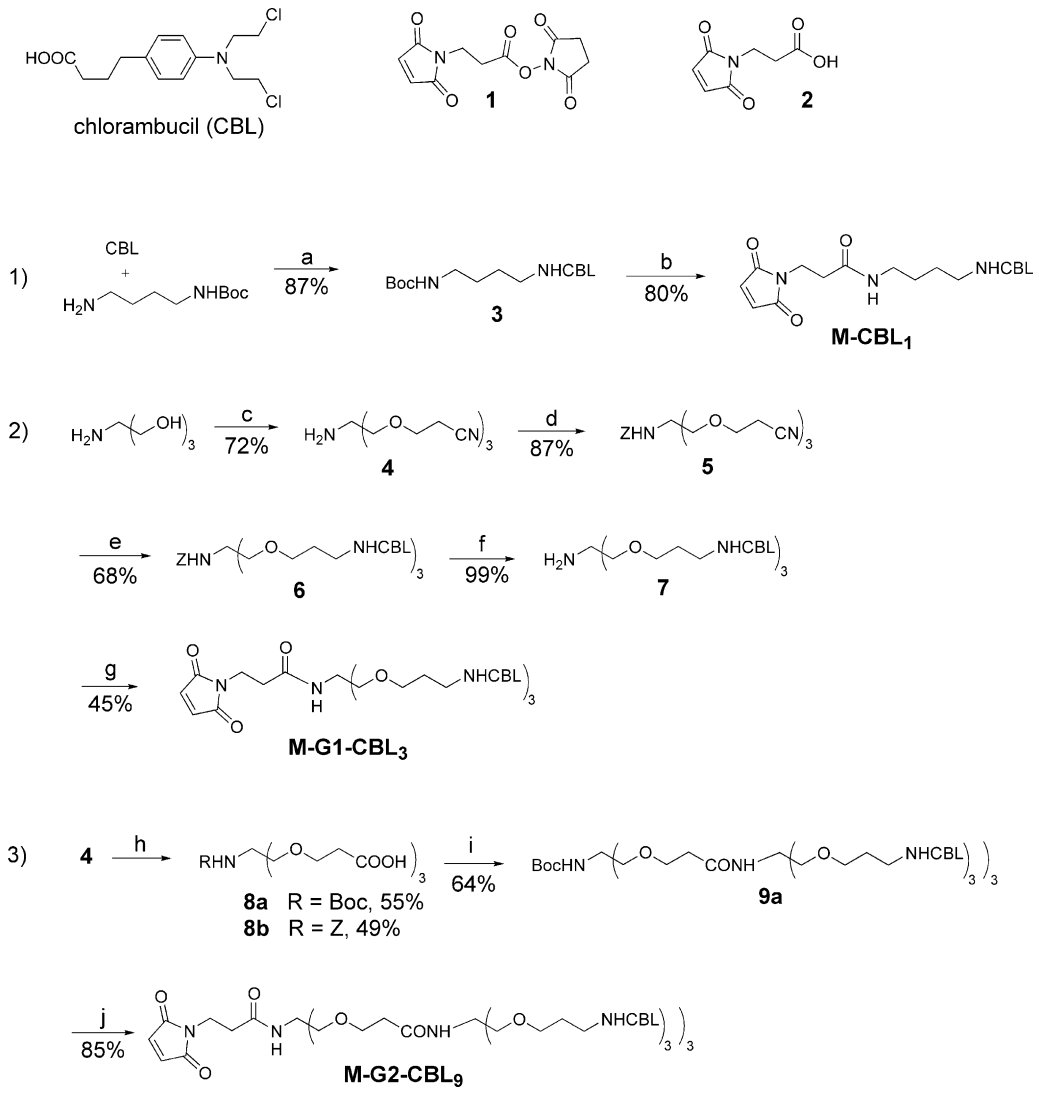
Original design and synthesis

Dendritic molecules with a polyamidoether backbone were selected as our desired multivalent drug carriers. The syntheses of these three molecules have been previously discussed,[27] and thus are just outlined in Scheme 1. As shown, **M-CBL$_1$** carries one, **M-G1-CBL$_3$** three and **M-G2-CBL$_9$** nine CBL moieties. The **M-G2-CBL$_9$** is the primary compound that we hoped to study while the other two were prepared for comparison purposes. These molecules were designed to have CBL at one end and a maleimide group at the other for covalent linkage with a cysteine residue site-specifically engineered into the scFv.[28] Notably, the dendritic nonamer is only 5.5 kDa, which is about 20% of the scFv weight. Unfortunately, the water solubility of these compounds were quite disappointing. None of the three molecules had a solubility greater than 200 μM in phosphate buffer with 10% DMSO as cosolvent. Therefore, water-soluble versions of these three molecules were designed and synthesized.

Water-soluble dendritic molecules

There are a number of routes to make a water-soluble dendrimer. One example would be to install hydrophilic peripheral groups like hydroxyl, amino or carboxyl. Since our compounds will have CBL on the peripheral termini and we would like to keep the already established convergent synthetic methodology to create homogenous drug carriers, the only feasible way is to insert a water-soluble unit into the branches. Polyethylene glycol (PEG) units are well known for their hydrophilic property.[29] However, insertion of a hexaethylene PEG into the dendrimers failed to give soluble molecules. Inserting longer PEG chains might be beneficial, but this would also increase the molecular weight of the dendrons. Therefore, we decided to insert amino acid residues with a hydrophilic side chain. Both lysine and glutamic acid were good candidates and have been incorporated in dendrimer scaffolds.[30–33] We chose lysine for synthetic ease. Two points were taken into consideration for the arrangement of the three functional groups on lysine. First, it is known that amide bond linkage to CBL does not impair its alkylating activity while ester conjugates of CBL may be not as active as the free drug. Hence one of the amino groups must be linked to the carboxylic group on CBL. Second, since CBL itself is in the acid form, it would be better if the final compound also were in the acid form. Consequently, the carboxylic group must be protected and the other lysine amino group connected with the dendron. At the final stage, the carboxylic acid would be deprotected to afford a water soluble molecule. According to these considerations, a set of three new compounds **CBL$_1$-Lys(M)-OH**, **CBL$_3$-Lys(M-G$_1$)-OH**, and **CBL$_9$-Lys(M-G$_2$)-OH** were designed as shown in Figure 2. The compound **CBL$_9$-Lys(M-G$_2$)-OH** is composed of four building blocks, namely the maleimide focal terminus, a dendritic linker, a lysine residue and a CBL moiety.

Syntheses of the compounds were based on our previous routes with modifications. Thus, di-protected lysine **10**



Scheme 1. (a) HBTU, NMM, DMF; (b) (1) 50% TFA, CH_2Cl_2; (2) **1**, NMM, CH_2Cl_2; (c) acrylonitrile, KOH, 1,4-dioxane; (d) CbzCl, aq $NaHCO_3$, 1,4-dioxane; (e) (1) $NaBH_4$, $CoCl_2{\cdot}6H_2O$; (2) CBL, HBTU, NMM, DMF; (f) H_2, 10% Pd/C, MeOH; (g) **1**, NMM, CH_2Cl_2; (h) (1) H_2SO_4, EtOH, reflux; (2) Boc_2O, TEA, or CbzCl, aq $NaHCO_3$, 1,4-dioxane; (3) 3 N aq NaOH; (i) **7**, HBTU, NMM, DMF; (j) (1) 50% TFA, CH_2Cl_2; (2) **2**, HBTU, NMM, DMF.

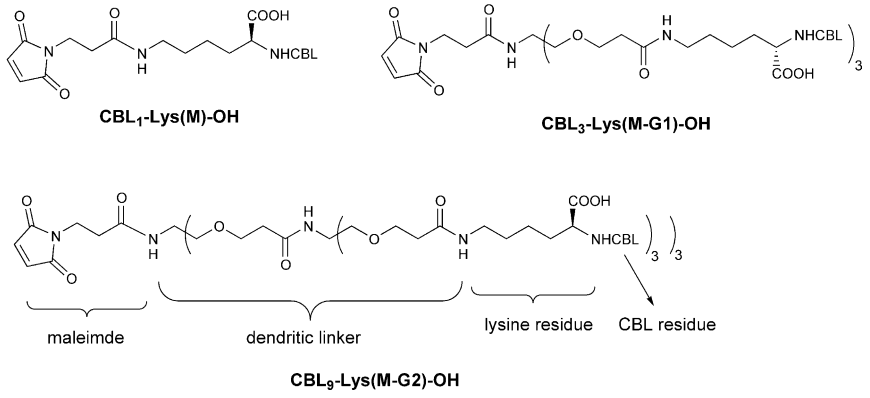
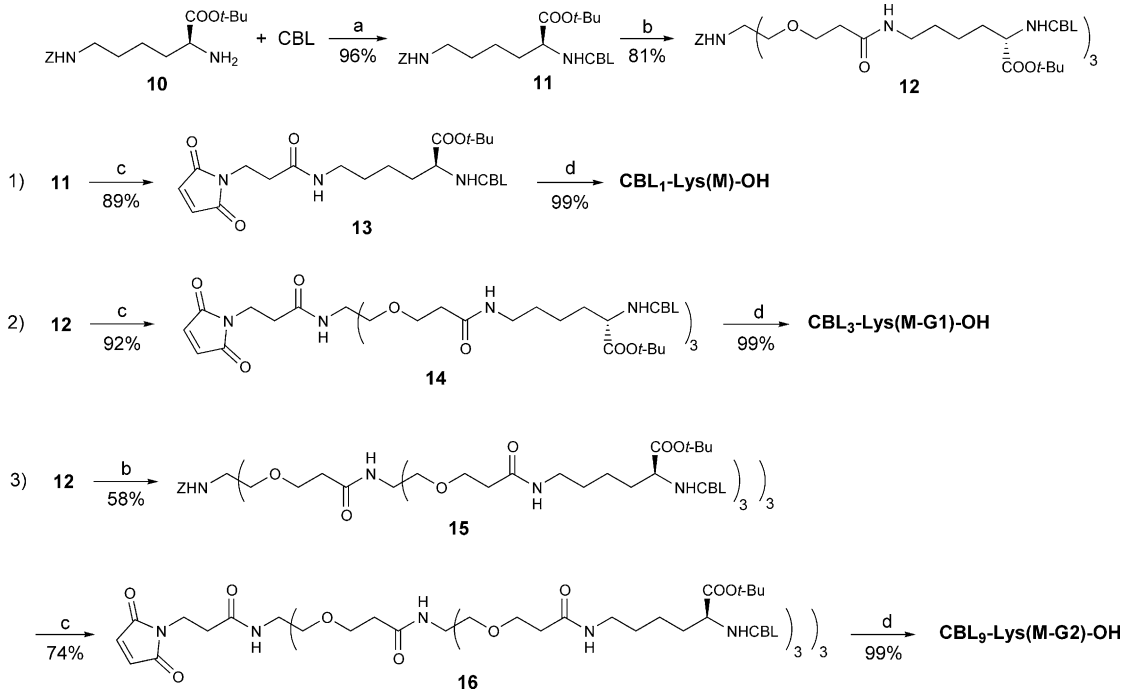


Figure 2. Dendritic drug compounds with improved water solubility as a result of lysine insertion.

was coupled first with CBL to generate **11** in 96% yield. Deprotection of the ε-Z group by Pd/C hydrogenation, HBTU coupling with maleimido-propionic acid **2** followed by TFA deprotection of Boc gave **CBL_1-Lys(M)-OH** in 88% yield. The ε-deprotected amine from **11** was coupled with tri-acid **8b** to give the trimer **12** in 81% yield. Repeating the above procedures of hydrogenation, HBTU coupling and TFA deprotection gave the final trimer **CBL_3-Lys(M-G_1)-OH** in 91% yield. The Z group on **12** was then removed by hydrogenation and



Scheme 2. (a) HBTU, NMM, DMF; (b) (1) H_2, 10% Pd/C, MeOH; (2) **8b**, HBTU, NMM, DMF; (c) (1) H_2, 10% Pd/C, MeOH; (2) **2**, HBTU, NMM, DMF; (d) 50% TFA, CH_2Cl_2.

the free amine was coupled to **4b** to provide the nonamer **15** in a convergent fashion. The nonamer **15** was again hydrogenated, coupled with **2** and treated with TFA to reveal the final nonameric CBL dendron **CBL$_9$-Lys(M-G$_2$)-OH**. It is worth noting two synthetic aspects in the preparation of these compounds. First, it was previously observed that the introduction of the maleimide moiety by coupling with the active ester **1** suffered from lower yields as the compounds increased in size. Therefore, the HBTU coupling method of the free acid was adopted throughout and provided consistently good yields. Second, the convergent methodology gave us control of the homogeneity of the final nonamer. Since this method worked well, we did not invoke the divergent method as described for the original compounds. We then examined the solubility of the new dendrons and found all to be soluble to at least 2 mM in phosphate buffer (pH 7.4)/10% DMSO, or >10-fold more soluble than the original compounds (Scheme 2).

Conclusion

Recently, we reported the design and synthesis of three compounds containing one, three and nine CBL drug molecules at the branching termini and a maleimide moiety at the core terminus of a dendron. With these compounds we attempted to link them to our scFvs to form drug/scFv immunoconjugates for use in cancer therapy. Such conjugates would enable for the first time the loading of an scFv with nine drug molecules. However, solubility problems with the dendrons precluded conjugate formation. To solve this problem, we designed and synthesized three new compounds with lysine residues added at interior sites in the dendron branches. The free carboxylic acid functionality on lysine greatly increased the water solubility of the compounds.[34] The method of using a dendritic linker molecule to connect multiple drug molecules with scFvs has not been previously reported. Yet, the strategy is not limited to scFvs, since any antibody, including Fab fragments and whole IgG, or other protein carriers could be utilized which would effectively increase the drug loading and the drug/carrier ratio. Furthermore, we chose to attach chlorambucil since this drug is commercially available, inexpensive and robust and allowed facile demonstration of the concept as part of our anticancer investigations. However, other drugs could be used. Future studies will incorporate more potent anticancer drugs, such as novel enediynes, and will be reported in due course.

Experimental

General methods

^{1}H and ^{13}C NMR spectra were recorded on a Brucker AMX-500 spectrometer at 500 MHz and 125 MHz, or Bruker AMX-600 at 600 and 150 MHz, respectively. Chemical shifts (ppm) were reported on the δ scale relative to internal $CDCl_3$ (^{1}H, 7.26 ppm and ^{13}C, 77.0 ppm) and acetone-d_6 (^{1}H, 2.04 ppm and ^{13}C, 29.9 ppm). MS spectra were recorded using electrospray ionization (ESI) or MALDI techniques. Glassware and solvents were dried by standard methods. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh) and thin-layer chromatography on glass plates coated with a 0.25 mm (analytical) or 0.50 mm (preparative) layer of silica gel 60 F_{254}. Chlorambucil and other chemical reagents and solvents were from Aldrich Chem. Co. or Sigma, unless otherwise noted,

and used without further purification. Compounds **4**,[35] **5**,[36] **8a**,[37] **8b**[38] were synthesized according to literature procedures. Reactions were carried out at room temperatures unless otherwise noted.

Preparation of compound 3. To a cooled (0 °C) solution of chlorambucil (0.61 g, 2.0 mmol) and 4-methylmorpholine (0.44 mL, 4.0 mmol) in 5 mL DMF was added HBTU (0.80 g, 2.1 mmol). After 10 min, *N*-Boc-1,4-diaminobutane (0.38 g, 2.0 mmol) was added and the mixture was stirred for 3 h. The solution was diluted with 20 mL of EtOAc and washed with water. The organic layer was then dried over $MgSO_4$ and evaporated. After chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3–96/4), the product was obtained in a yield of 0.82 g (87%) as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (d, 2H, J = 8.8 Hz), 6.60 (d, 2H, J = 8.8 Hz), 5.78 (s, 1H, NH), 4.67 (s, 1H, NH), 3.65 (m, 8H), 3.24 (dd, 2H, J = 6.4, 12.3 Hz), 3.11 (m, 2H), 2.54 (t, 2H, J = 7.5 Hz), 2.16 (t, 2H, J = 7.5 Hz), 1.90 (tt, 2H, J = 7.5, 7.5 Hz), 1.49 (m, 4H), 1.42 (s, 9H); ^{13}C NMR (125 MHz, CDCl3) δ 172.8, 156.1, 144.2, 130.6, 129.6, 112.1, 79.1, 53.5, 40.5, 40.0, 39.0, 35.9, 34.0, 28.3, 27.6, 27.4, 26.7; HRMS (MALDI) calcd for $C_{23}H_{37}N_3O_3Cl_2$ $[M+Na]^+$ 496.2104, found 496.2116.

Preparation of M-CBL$_1$

A solution of **3** (0.10 g, 0.21 mmol) in CH_2Cl_2 (2.0 mL) was treated with TFA (2 mL) at 0 °C. After completion, the mixture was evaporated with toluene (three times). The residue was dissolved in 5 mL CH_2Cl_2 and 4-methylmorpholine (55 μL, 0.50 mmol) was added followed by 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (compound **1**, 80 mg, 0.30 mmol). After 2 h stirring, the reaction mixture was evaporated and the residue was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3 to 95/5), the product was obtained in a yield of 88 mg (80%) as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (d, 2H, J = 8.8 Hz), 6.68 (s, 2H), 6.61 (d, 2H, J = 8.8 Hz), 6.16 (s, 1H, NH), 5.80 (s, 1H, NH), 3.82 (t, 2H, J = 7.0 Hz), 3.65 (m, 8H), 3.24 (m, 4H), 2.52 (m, 4H), 2.17 (t, 2H, J = 7.5 Hz), 1.90 (tt, 2H, J = 7.5, 7.5 Hz), 1.50 (m, 4H); ^{13}C NMR (133 MHz, CDCl3) δ 173.15, 170.50, 169.81, 144.29, 134.19, 130.61, 129.64, 112.12, 53.54, 40.51, 39.04, 38.87, 35.96, 34.70, 34.31, 34.05, 27.41, 26.98, 26.50; HRMS (MALDI) calcd for $C_{25}H_{34}N_4O_4Cl_2$ $[M+Na]^+$ 547.1849, found 547.1869.

Preparation of compound 6. To a stirred methanol solution of the tri-nitrile compound 5 (0.95 g, 2.3 mmol) and cobalt (II) chloride hexahydrate (3.28 g, 13.8 mmol) was added $NaBH_4$ (5.2 g, 137 mmol) in small portions (violent gas evolution). The mixture was stirred for 2 h and then acidified with concentrated hydrochloric acid (15 mL). The solvents were removed under vacuum and the resulting deep blue residue was taken up in concentrated ammonia solution (20 mL) and $CHCl_3$ (20 mL). The insoluble solid was filtered off and the aqueous phase was extracted with $CHCl_3$ (3 × 20 mL). The combined organic layer was dried over $MgSO_4$, evaporated and then co-evaporated with toluene. The resulting tri-amine (0.92 g, 94% yield) is a thick yellow liquid, which was used without further purifications. To a cooled (0 °C) solution of chlorambucil (0.304 g, 1.0 mmol) and 4-methylmorpholine (0.22 mL, 2.0 mmol) in 5 mL DMF was added HBTU (0.40 g, 1.05 mmol). After 10 min, the above crude tri-amine (0.128 g, 0.3 mmol) was added and the mixture was stirred overnight. The solution was diluted with 10 mL of EtOAc and washed with water. The organic layer was then dried over $MgSO_4$ and evaporated. After chromatography over a silica gel column eluted with $CHCl_3$/MeOH/NH_4OH (96/4/0.3), the product was obtained in a yield of 0.28 g (72%) as a slight yellow powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34–7.29 (m, 5H), 7.04 (d, 6H, J = 8.3 Hz), 6.60 (d, 6H, J = 8.3 Hz), 6.09 (t, 3H, NH, J = 5.3 Hz), 5.00 (s, 2H), 3.69–3.59 (m, 30H), 3.45 (t, 6H, J = 5.7 Hz), 3.28 (td, 6H, J = 6.2, 5.3 Hz), 2.52 (t, 6H, J = 7.5 Hz), 2.15 (t, 6H, J = 7.5 Hz), 1.88 (tt, 6H, J = 7.5, 7.5 Hz), 1.71 (tt, 6H, J = 5.7, 6.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.87, 155.25, 144.22, 136.37, 130.76, 129.60, 128.50, 128.15, 127.96, 112.16, 70.02, 69.26, 66.32, 58.63, 53.56, 40.49, 36.92, 35.94, 34.13, 29.25, 27.46; ESI-MS (positive) calcd for $C_{63}H_{89}N_7O_8Cl_6$ $[M+H]^+$ 1282.5, found 1282.3.

Preparation of compound 7. Compound **6** (0.25 g, 0.195 mmol) in MeOH (10 mL) was hydrogenated with 10% Pd/C (98 mg) using a hydrogen balloon. After 45 min, the reaction mixture was filtered through Celite and the filter cake was washed with MeOH. The filtrate was evaporated and the product (224 mg, 99% yield) was used without further purifications. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.48 (m, NH, 3H), 7.04 (d, 6H, J = 8.3 Hz), 6.89 (s, NH, 2H), 6.59 (d, 6H, 8.3 Hz), 3.71–3.39 (m, 42H), 2.52 (m, 6H), 2.24 (m, 6H), 1.87 (m, 6H), 1.71 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.70, 144.27, 130.85, 129.63, 112.19, 69.48, 68.22, 53.59, 40.61, 36.08, 35.70, 34.25, 29.47, 27.73; ESI-MS (positive) calcd for $C_{55}H_{83}N_7O_6Cl_6$ $[M+H]^+$ 1148.5, found 1148.7.

Preparation of compound M-G1-CBL$_3$. This compound was prepared from **7** (60 mg, 52 μmol) and **1** (42 mg, 156 μmol) following analogous procedures as in the synthesis of M-CBL$_1$. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3–95/5) as a white powder in a yield of 30 mg (45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (d, 6H, J = 8.8 Hz), 6.65 (s, 2H), 6.61 (d, 6H, J = 8.8 Hz), 6.03 (t, 3H, NH, J = 5.9 Hz), 5.30 (s, 1H), 3.78 (t, 2H, J = 7.3 Hz), 3.71–3.59 (m, 30H), 3.44 (t, 6H, J = 5.6 Hz), 3.31 (td, 6H, J = 6.0, 6.0 Hz), 2.57–2.52 (m, 8H), 2.18 (t, 6H, J = 7.5 Hz), 1.90 (tt, 6H, J = 7.5, 7.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.00, 170.43 (2), 144.30, 134.12, 130.73, 129.64, 112.16, 69.89, 68.72, 59.72, 53.58, 40.55, 36.57, 35.98, 35.08, 34.40, 34.14, 29.42, 27.50; HRMS (MALDI) calcd for $C_{62}H_{88}N_8O_9Cl_6$ $[M+Na]^+$ 1321.4697, found 1321.4714.

Preparation of compound 9a. To a cooled (0 °C) solution of **8a** (34 mg, 78 μmol) and 4-methylmorpholine (51 μL, 0.47 mmol) in 1.5 mL DMF was added HBTU (99 mg, 0.26 mmol). After 30 min, **7** (0.30 g, 0.26 mmol) was

added and the mixture was stirred for 16 h. The solution was diluted with 10 mL of EtOAc and washed with water. The organic layer was then dried over $MgSO_4$ and evaporated. After chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3 to 96/4), the crude product was purified again on P-TLC developed with $CHCl_3$/MeOH (94/6). The product was obtained in a yield of 0.19 g (64%) as a slightly yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.04 (d, 18H, J = 8.5 Hz), 6.59 (d, 18H, J = 8.5 Hz), 3.67–3.58 (m, 102H), 3.44 (br-t, 18H, J = 5.3 Hz), 3.28 (m, 18H), 2.52 (br-t, 18H, J = 7.5 Hz), 2.40 (br-t, 6H), 2.18 (t, 18H, J = 7.4 Hz), 1.89 (m, 18H), 1.71 (m, 18H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.09, 172.94, 171.32, 154.89, 144.26, 130.70, 129.59, 112.14, 70.01, 69.71, 68.89, 67.57, 59.79, 58.62, 53.54, 40.53, 37.43, 36.64, 35.96, 34.20, 29.41, 28.44, 27.58; ESI-MS (positive) calcd for $C_{173}H_{258}N_{22}O_{22}Cl_{18}$ M_{ave} 3836.4, found 3835.7.

Preparation of compound M-G2-CBL$_9$. A solution of **9a** (15 mg, 3.9 μmol) in CH_2Cl_2 (0.5 mL) was treated with TFA (0.5 mL) at 0 °C. After completion, the mixture was evaporated with toluene (three times). The residue was dissolved in 0.5 mL DMF and added to a DMF solution containing **2** (1 mg, 5.9 μmol), NMM (1 μL, 10 μmol) and HBTU (2.2 mg, 5.9 μmol). The mixture was stirred for 3 h, diluted with 10 mL of EtOAc and washed with water. The organic layer was then dried over $MgSO_4$ and evaporated. After P-TLC developed with $CHCl_3$/MeOH (94/6), the product was obtained in a yield of 13 mg (85%) as a slightly yellow powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.04 (d, 18H, J = 8.3 Hz), 6.66 (s, 2H), 6.61 (d, 18H, J = 8.3 Hz), 6.45 (s, 9H, NH), 3.75 (t, 2H, J = 7.5 Hz), 3.69–3.59 (m, 102H), 3.43 (t, 18H, J = 5.7 Hz), 3.28 (td, 18H, J = 6.1, 5.7 Hz), 2.52 (m, 20H), 2.40 (br-t, 6H), 2.18 (t, 18H, J = 7.5 Hz), 1.89 (tt, 18H, J = 7.5, 7.5 Hz), 1.71 (m, 18H); ESI-MS (positive) calcd for $C_{185}H_{271}N_{23}O_{27}Cl_{18}$ M_{ave} 3887.4, found 3887.3.

Preparation of compound 11. This compound was prepared from CBL (1.0 g, 3.3 mmol) and H-Lys(Z)-O*t*-Bu hydrochloride (1.1 g, 3.3 mmol) following analogous procedures as in the synthesis of **3**. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (98/2) as a white powder in a yield of 1.95 g (96%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99 (s, 1H, NH), 7.34–7.27 (m, 5H), 7.04 (d, 2H, J = 8.3 Hz), 6.59 (d, 2H, J = 8.3 Hz), 6.10 (d, 1H, J = 7.9 Hz, NH), 5.04 (s, 2H), 4.46 (m, 1H), 3.68–3.58 (m, 8H), 3.16 (td, 2H, J = 6.6, 6.6 Hz), 2.52 (t, 2H, J = 7.5 Hz), 2.19 (t, 2H, J = 7.5 Hz), 1.89 (tt, 2H, J = 7.5, 7.5 Hz), 1.79 (m, 1H), 1.60 (m, 1H), 1.51 (m, 2H), 1.44 (s, 9H), 1.35 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.47, 171.69, 162.43, 156.41, 144.16, 136.52, 130.54, 129.58, 128.39, 127.95, 127.94, 112.02, 81.97, 66.41, 53.47, 52.12, 40.49, 40.43, 38.49, 36.37, 35.66, 33.92, 32.18, 31.31, 29.28, 27.89, 27.86, 27.22, 22.12; HRMS (MALDI) calcd for $C_{32}H_{45}N_3O_5Cl_2$ $[M+Na]^+$ 644.2628, found 644.2637.

Preparation of compound 12. Compound **11** (0.50 g, 0.80 mmol) in MeOH (15 mL) was hydrogenated with 10% Pd/C (155 mg) using a hydrogen balloon. After 2 h, the reaction mixture was filtered through Celite and the filter cake was washed with MeOH. The filtrate was evaporated and the product was dissolved in 2 mL DMF and added to a DMF solution containing **8b** (0.10 g, 0.21 mmol), NMM (97 μL, 0.88 mmol) and HBTU (0.33 g, 0.88 mmol). The mixture was stirred for 2 h, diluted with 50 mL of EtOAc and washed with water. The organic layer was then dried over $MgSO_4$ and evaporated. After chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3–96/4), the product was obtained in a yield of 0.32 g (81%, two steps) as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35–7.29 (m, 5H), 7.05 (d, 6H, J = 8.8 Hz), 6.64 (t, 3H, J = 5.7 Hz, NH), 6.61 (d, 6H, J = 8.8 Hz), 6.25 (d, 3H, J = 7.5 Hz), 5.40 (s, 1H, NH), 5.01 (s, 2H), 4.42 (m, 3H), 3.70–3.60 (m, 36H), 3.19 (m, 6H), 2.54 (t, 6H, J = 7.5 Hz), 2.35 (t, 6H, J = 5.9 Hz), 2.21 (t, 6H, J = 7.6 Hz), 1.89 (m, 6H), 1.75 (m, 3H), 1.63 (m, 3H), 1.51 (m, 6H), 1.45 (s, 27H), 1.32 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.78, 171.71, 171.33, 163.11, 144.26, 130.55, 129.64, 128.49, 128.13, 127.96, 112.10, 82.01, 69.36, 67.35, 58.69, 53.54, 52.30, 40.51, 38.89, 36.63, 35.72, 34.04, 32.20, 28.94, 27.98, 27.38, 22.45; HRMS (MALDI) calcd for $C_{93}H_{140}N_{10}O_{17}Cl_6$ $[M+Na]^+$ 1901.8421, found 1901.8363.

Preparation of compound 13. This compound was prepared from **11** (0.25 g, 0.40 mmol) and **2** (74 mg, 0.44 mmol) following analogous procedures as in the synthesis of **12**. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (98/2) as a white powder in a yield of 227 mg (89%, two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (d, 2H, J = 8.4 Hz), 6.68 (s, 2H), 6.61 (d, 2H, J = 8.4 Hz), 6.11 (d, 1H, J = 8.1 Hz, NH), 5.98 (br-t, 1H, J = 5.9 Hz, NH), 4.45 (m, 1H), 3.80 (t, 2H, J = 7.0 Hz), 3.71–3.60 (m, 8H), 3.19 (m, 2H), 2.55 (t, 2H, J = 7.5 Hz), 2.49 (t, 2H, J = 7.0 Hz), 2.22 (t, 2H, J = 7.5 Hz), 1.91 (tt, 2H, J = 7.5, 7.5 Hz), 1.88 (m, 1H), 1.61 (m, 1H), 1.51 (m, 2H), 1.45 (s, 9H), 1.33 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.73, 171.72, 170.46, 169.67, 144.24, 134.15, 130.54, 129.63, 112.09, 82.16, 77.25, 77.19, 76.99, 76.74, 53.53, 52.00, 40.48, 39.06, 35.79, 34.58, 34.25, 33.97, 32.51, 28.52, 27.96, 27.32, 22.33; HRMS (MALDI) calcd for $C_{31}H_{44}N_4O_6Cl_2$ $[M+Na]^+$ 661.2533, found 661.2539.

Preparation of CBL$_1$-Lys(M)-OH. A solution of **13** (50 mg, 78 μmol) in CH_2Cl_2 (2.0 mL) was treated with TFA (2 mL) at 0 °C. After completion, the mixture was evaporated with toluene (three times). The product is a slightly yellow powder, which is used without further purifications. ^{1}H NMR (500 MHz, acetone-d_6) δ 7.32–7.28 (m, 2H, NH), 7.07 (d, 2H, J = 8.8 Hz), 6.83 (s, 2H), 6.70 (d, 2H, J = 8.8 Hz), 4.41 (m, 1H), 3.78–3.70 (m, 10H), 3.15 (m, 2H), 2.53 (t, 2H, J = 7.5 Hz), 2.43 (t, 2H, J = 7.3 Hz), 2.25 (t, 2H, J = 7.5 Hz), 1.89–1.80 (m, 3H), 1.69 (m, 1H), 1.51–1.36 (m, 4H); ^{13}C NMR (125 MHz, acetone-d_6) δ 173.84, 173.53, 171.35, 170.49, 145.43, 135.17, 131.59, 130.35, 113.04, 53.88, 52.73, 41.57, 39.30, 35.75, 35.19, 35.08, 34.72, 31.92, 23.64; HRMS (MALDI) calcd for $C_{27}H_{36}N_4O_6Cl_2$ $[M+Na]^+$ 605.1904, found 605.1908.

Preparation of compound 14. This compound was prepared from **12** (0.14 g, 74 μmol) and **2** (19 mg, 0.11 mmol) following analogous procedures as in the synthesis of **12**. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3 to 96/4) as a white powder in a yield of 0.13 g (92%, two steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.05 (d, 6H, J = 7.9 Hz), 6.68 (s, 2H), 6.64 (t, 3H, J = 5.7 Hz, NH), 6.61 (d, 6H, J = 7.9 Hz), 6.52 (s, 1H, NH), 6.30 (d, 3H, J = 7.9 Hz, NH), 4.42 (m, 3H), 3.76 (t, 2H, J = 7.0 Hz), 3.70–3.60 (m, 36H), 3.21 (m, 6H), 2.55–2.50 (m, 8H), 2.36 (t, 6H, J = 5.7 Hz), 2.22 (t, 6H, J = 7.5 Hz), 1.90 (m, 6H), 1.79 (m, 3H), 1.64 (m, 3H), 1.52 (m, 6H), 1.45 (s, 27H), 1.36 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.79, 171.72, 171.46, 170.50 170.25, 144.27, 134.18, 130.54, 129.64, 112.09, 82.00, 69.35, 67.36, 59.63, 53.53, 52.33, 40.52, 38.97, 36.49, 35.74, 35.03, 34.33, 34.04, 32.21, 28.98, 27.98, 27.38, 22.49; HRMS (MALDI) calcd for $C_{92}H_{139}N_{11}O_{18}Cl_6$ $[M+Na]^+$ 1918.8322, found 1918.8321.

Preparation of CBL$_3$-Lys(M-G1)-OH. This compound was prepared from **14** (50 mg, 26 μmol) following analogous TFA deprotection procedures as in the synthesis of **CBL$_1$-Lys(M)-OH**. The product is a slightly yellow powder, which is used without further purifications. ^{1}H NMR (500 MHz, acetone-d_6) δ 11.45 (br-s, 13H, COOH), 7.81–7.73 (m, 6H, NH), 7.07 (d, 6H, J = 8.6 Hz), 6.82 (s, 2H), 6.72 (d, 6H, J = 8.6 Hz), 4.46 (m, 3H), 3.77–3.66 (m, 38H), 3.26 (m, 6H), 2.55–2.46 (m, 14H), 2.33 (t, 6H, J = 7.5 Hz), 1.91–1.87 (m, 9H), 1.74 (m, 3H), 1.59–1.42 (m, 12H); ^{13}C NMR (125 MHz, acetone-d_6) δ 175.07, 173.73, 173.60, 171.81, 171.43, 159.18, 158.86, 158.54, 158.22, 145.25, 135.19, 131.67, 130.38, 119.38, 117.10, 114.83, 113.26, 112.55, 69.93, 68.16, 64.68, 60.98, 54.01, 53.07, 41.51, 39.92, 36.94, 35.84, 35.75, 35.07, 34.77, 31.86, 30.54, 28.67, 23.67; MS (MALDI-TOF) calcd for $C_{23}H_{37}N_3O_3Cl_2$ $[M+H]^+$ 1729, found 1729.

Preparation of compound 15. This compound was prepared from **12** (0.88 g, 0.47 mmol) and **8b** (61 mg, 0.13 mmol) following analogous procedures as in the synthesis of **12**. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (97/3 to 95/5) as a white powder in a yield of 0.43 g (58%, two steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32–7.30 (m, 5H), 7.04 (d, 18H, J = 8.5 Hz), 6.60 (d, 18H, J = 8.5 Hz), 6.48 (d, 6H, J = 7.9 Hz), 5.01 (s, 2H), 4.40 (m, 9H), 3.69–3.59 (m, 120H), 3.18 (m, 18H), 2.52 (t, 18H, J = 7.5 Hz), 2.35 (m, 24H), 2.22 (t, 18H, J = 7.5 Hz), 1.88 (m, 18H), 1.76 (m, 9H), 1.64 (m, 9H), 1.51 (m, 18H), 1.44 (s, 81H), 1.32 (m, 18H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.76, 171.62, 171.27, 162.38, 144.15, 130.48, 129.50, 128.35, 127.69, 111.99, 81.71, 68.97, 67.41, 67.27, 59.62, 58.82, 53.40, 52.41, 40.44, 38.82, 36.34, 35.55, 33.96, 31.91, 31.26, 29.00, 27.87, 27.32, 22.51; ESI-MS (positive) calcd for $C_{276}H_{425}N_{31}O_{53}Cl_{18}$ M_{ave} 5663.7, found 5664.7.

Preparation of compound 16. This compound was prepared from **15** (0.16 g, 28 μmol) and **2** (7.3 mg, 43 μmol) following analogous procedures as in the synthesis of **12**. The product was purified by chromatography over a silica gel column eluted with $CHCl_3$/MeOH (95/5) as a slightly yellow powder in a yield of 122 mg (74%, two steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (s, 6H, NH), 7.04 (d, 18H, J = 8.3 Hz), 6.68 (s, 2H), 6.60 (d, 18H, J = 8.3 Hz), 6.50 (m, 9H, NH), 4.39 (m, 9H), 3.77 (t, 2H, J = 7.0 Hz), 3.69–3.59 (m, 120H), 3.18 (m, 18H), 2.54–2.51 (m, 20H), 2.36 (m, 24H), 2.22 (t, 18H, J = 7.5 Hz), 1.88 (m, 18H), 1.76 (m, 9H), 1.64 (m, 9H), 1.51 (m, 18H), 1.44 (s, 81H), 1.32 (m, 18H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.89, 171.75, 171.50, 171.45, 170.52, 170.48, 144.26, 134.24, 130.56, 129.63, 112.10, 81.93, 69.05, 67.51, 67.39, 59.94, 59.77, 53.52, 52.51, 40.53, 38.93, 36.50, 35.70, 35.02, 34.30, 34.07, 32.08, 29.09, 27.98,27.42, 22.60; ESI-MS (positive) calcd for $C_{275}H_{424}N_{32}O_{54}Cl_{18}$ M_{ave} 5680.6, found 5681.9.

Preparation of CBL$_9$-Lys(M-G2)-OH. This compound was prepared from **16** (50 mg, 8.8 μmol) following analogous TFA deprotection procedures as in the synthesis of **CBL$_1$-Lys(M)-OH**. The product is a slightly yellow powder, which is used without further purifications. ^{1}H NMR (500 MHz, acetone-d_6) δ 10.34 (br-s, 33H, COOH), 7.06 (d, 18H, J = 8.8 Hz), 6.85 (s, 2H), 6.69 (d, 18H, J = 8.8 Hz), 4.46 (m, 9H), 3.74–3.69 (m, 122H), 3.24 (m, 18H), 2.54–2.47 (m, 44H), 2.31 (t, 18H, J = 7.5 Hz), 1.89–1.86 (m, 27H), 1.76 (m, 9H), 1.58–1.45 (m, 36H); ^{13}C NMR (125 MHz, acetone-d_6) δ 174.51, 174.08, 173.05, 172.98, 172.91,171.59, 158.99, 158.66, 158.34, 158.02, 145.42, 135.32, 131.55, 130.41, 119.31, 117.04, 114.77, 113.12, 112.50, 69.84, 68.54, 68.39, 61.11, 61.01, 53.94, 52.99, 41.64, 39.74, 37.78, 37.17, 35.90, 34.84, 31.98, 31.96, 28.66, 27.54, 23.80; HRMS (MALDI) calcd for $C_{239}H_{352}N_{32}O_{54}Cl_{18}$ M_{ave} 5175.7, found 5175.2.

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