Optimization of the N-Lost Drugs Melphalan and Bendamustine: Synthesis and Cytotoxicity of a New Set of Dendrimer−Drug Conjugates as Tumor Therapeutic Agents

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Bendamustine and melphalan are very promising alkylating drugs with applicability in the treatment of various tumoral diseases, e.g., chronic lymphocytic leukemia (CLL) or breast cancer. However, numerous adverse effects limited their use. Therefore, 1,3,5-tris(3-aminopropyl)benzene (**G0**) and its **G1** analogue 3,5-bis(3-aminopropyl)-*N*-(3-{3,5-bis[3-{3,5-bis(3-aminopropyl)benzoylamino}propyl]phenyl}propyl)benzamide were selected to design cytostatic drug−dendrimer conjugates to achieve tumor cell accumulation by endocytosis as already demonstrated for platinum complexes. The dendrimers act as carriers and an *N*-(2-hydroxyethyl)maleimide spacer between drug and carrier should guarantee a selective release of the cytostatics in the tumor cells. The resulting cytotoxicity was determined in vitro using the human MCF-7 and MDA-MB-231 breast cancer cell lines. It was demonstrated that melphalan caused cytotoxic effects depending on its free amino group (Boc protection strongly decreased the activity) but independent of a derivation of the carboxylic group (dendrimers and spacer binding). Esterification of bendamustine with the *N*-(2-hydroxyethyl)maleimide spacer strongly increased the hydrolytic stability of the N-lost moiety, so antiproliferative effects were yet observed in vitro.

INTRODUCTION

Designing miscellaneous dendritic structures containing both a drug and a targeting residue has played a very important role in drug development during the past years (*1*−*4*). The main objective has been to diminish the side effects induced through drug therapy. Accumulation of these conjugates in the tumor tissue is explained by the EPR (enhanced permeability and retention) effect, the property by which macromolecules build up in the tumor due to the special properties of the cells (e.g., poor lymphatic drainage) (*5, 6*). Current studies by Baker et al. showed remarkable results and proved the selective uptake of PAMAM dendrimer-based multifunctional conjugates in the tumor tissues (*7*−*9*).

Bendamustine is a drug with high potency in the treatment of several tumoral diseases. It was first synthesized by Ozegowski et al. and has been in clinical use since 1985 (*10*). The molecule consists of three structural elements: a mechlorethanamine group, a benzimidazole core, and a butyric acid chain. It was proposed that the latter (together with the protonated heterocycle) mediates water solubility and the benzimidazole moiety should lead to tumor cell accumulation by purine base transporters.

Bendamustine belongs to the class of nitrogen mustard agents like chlorambucil, melphalan, and cyclophosphamide and should induce cell death, possibly through apoptosis. Recent clinical trials have illustrated remarkable results in the treatment of chronic lymphocytic leukemia (CLL), and thus, in March 2008, bendamustine was also approved in the USA for the treatment of CLL (*11*). Additionally, bendamustine has a promising future in the treatment of breast cancer (*12*). The mechanism of action is thought to include both alkylating agent properties (comparable to melphalan) and purine antimetabolite characteristics.

Melphalan is a typical alkylating agent and contains two functional groups: the amino acid phenylalanine (drug targeting by using amino acid transporter of tumor cells) and the N-Lost moiety (cross-link of DNA strands). It is used in the treatment of cancer and other diseases, including ovarian cancer, malignant melanoma, multiple myeloma (bone-marrow cancer), breast cancer, and chronic myelogenous leukemia.

Both bendamustine and melphalan are very effective antitumor drugs, without having the proposed tumor selectivity. Therefore, we decided to use them in our approach to increase the selectivity for malignant cells following the concept of Kratz et al. They synthesized in 1998 a protein-conjugate with chlorambucil capable of releasing the drug in breast cancer (MCF-7) and leukemia (MOLT4) cells. Chlorambucil was attached through an ester or amide bond to a maleimide spacer, which is able to bind selectively to thiol groups of human serum albumin (HSA) (*13, 14*). HSA is a handy protein in pure form, is nontoxic and biodegradable, and is essentially assimilated by the tumor cells (*15, 16*). The resulting drug−HSA conjugates should accumulate in the tumors due to the EPR effect and then release the drug in the cells (drug targeting). The in vitro cell tests illustrated a relationship between the linker and the efficacy of the conjugates, and in vivo studies demonstrated the possibility of administering a conjugate with a lower IC_{50} value than chlorambucil in a higher dose to mice.

These positive results induced us to bind melphalan and bendamustine directly or through a cleavable maleimide spacer to the dendrimers **G0** and **G1** (see Figure 1) synthesized in a former SAR study (*17*). In vitro antitumor activity studies on MCF-7 and MDA-MB-231 cell lines showed for **G0** and **G1** no cytotoxic effects (see Supporting Information), making them suitable as carriers.

Initially, three different spacers were considered for the assembling of the conjugates (Figure 1). Due to the instability of the hydrazone linkers under the given reaction conditions,

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10.1021/bc900453f
Published on Web 09/17/2010

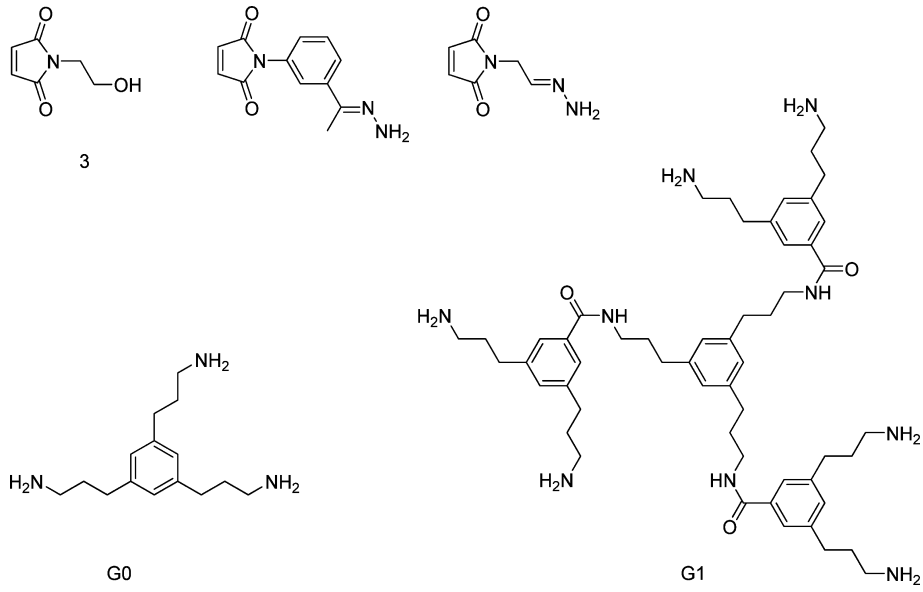


Figure 1. Structures of the considered spacers and dendrimers.

the synthesis was first limited to the use of *N*-(2-hydroxyethyl)maleimide **3**.

EXPERIMENTAL SECTION

General. ^{1}H and ^{13}C NMR spectra were taken on a Bruker Avance/DPX 400 MHz with TMS as internal standard. Melting points (uncorrected) were measured with a Büchi Melting Point B-545. All column chromatography purifications were done using silica gel 60 (0.063−0.1 mm, Merck). TLC was performed on silica gel 60 GF_{254} plates (Merck). Elemental analysis was carried out on a Vario EL (Hanau). EI spectra were recorded on a Thermo-Fisions VG Auto Spec (70 eV). The FAB spectra were measured on a CH-5, Varian MAT, Bremen. The ESI-TOF spectra were measured on an Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA. Solvent flow rate was adjusted to 4 μL/min, with spray voltage set to 4 kV. Drying gas flow rate was set to 15 psi (1 bar). All other parameters were adjusted for a maximum abundance of the relative $[M+H]^+$.

Synthesis. The following compounds were prepared according to literature procedures: **G0** (*17*), **G1** (*17*), *N-tert*-butoxycarbonyl-4-[bis(2-chloroethyl)amino]-L-phenylalanine (*2*) (*18*). Bendamustine hydrochloride was a gift from the company Bendalis. Melphalan hydrochloride was synthesized as described by Bergel (*19*). All other starting materials were purchased from commercial sources and used without purification. Solvents were dried under standard conditions.

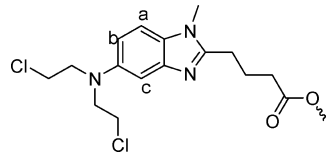
*O-{N-tert-Butoxycarbonyl-4-[bis(2-chloroethyl)amino]-L-phenylalanyl}-2-hydroxyethylmaleimide (***4***).* [1]MelBoc **2** (330 mg, 0.81 mmol), DMAP (tip of a spatula), and *N*-(2-hydroxyethyl)-maleimide **3** (441 mg, 3.1 mmol) were dissolved in 30 mL of dry dichloromethane at room temperature. DCC (176.5 mg, 0.85 mmol), dissolved in 25 mL of dry dichloromethane, was added dropwise to this solution within 1 h, and the solution stirred for additional 20 h. The solution was filtered and evaporated in vacuo. The red residue was chromatographed on a silica gel column (hexane/ethyl acetate 2:1) to afford the desired product (137 mg, 32%) as yellow solid: $R_f = 0.3$ (hexane/ethyl acetate 2:1); mp 101 °C; ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.29$ (s, 9H, CH_3), 2.62 (m, 1H, CH_2Ar), 2.74 (dd, ^{3}J(H,H) = 4.46 Hz, 9.44 Hz, 1H, CH_2Ar), 3.62 (t, ^{3}J(H,H) = 5.28 Hz, 2H, $CH_{2\ maleimide}$), 3.66 (s, 8H, CH_2CH_2Cl), 4.09 (m, 2H, CH_2O), 4.22 (m, 1H, CH), 6.63 (d, ^{3}J(H,H) = 8.52 Hz, 2H, Ar*H*), 7.02 (d, 2H+2H, Ar*H*+$H_{maleimide}$), 7.17 (d, ^{3}J(H,H) = 8.01 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ = 28.31 (CH_3), 36.64 (CH_2Ar), 36.98 ($CH_2N_{maleimide}$), 40.46 ($CH_2NAr+CH_2Cl$), 54.49 (CH), 62.11 (CH_2O), 79.84 ($C(CH_3)_3$), 112.09 ($C_{aromatic}$), 124.8 (C), 130.56 ($C_{aromatic}$), 134.25 ($CH_{maleimide}$), 145.04 ($C_{aromatic}$), 155.07 (COOBoc), 170.29 ($CO_{maleimide}$), 171.75 (COO); MS (EI, 80 eV, 300 °C) *m*/*z* (%) 527.2 (5.93) [M^+-1], 404.1 (0.36) $[M-C_6H_6NO_2]^+$, 230.3 (100) $C_{15}H_{14}NCl_2$; Anal. ($C_{24}H_{31}Cl_2N_3O_6$) C, H, N.

*O-{4-[Bis(2-chloroethyl)amino]-L-phenylalanyl}-2-hydroxyethylmaleimide Hydrochloride (***5***).* MelBoc-spacer **4** (350 mg, 0.66 mmol) was dissolved in 6 mL of THF. To this solution, 250 μL of hydrochloric acid (w = 25%) was added and the mixture was stirred for 20 h at room temperature. The solvent was removed under reduced pressure. The remaining oil was dissolved in methanol, and the product was precipitated with diethyl ether, filtered, and dried, to afford 245 mg (80%) as a colorless solid: mp 130 °C; ^{1}H NMR (400 MHz, CD_3OD) δ = 3.00 (dd, ^{3}J(H,H) = 7.89 Hz, 14.59 Hz, 1H, CH_2Ar), 3.14 (dd, ^{3}J(H,H) = 5.22 Hz, 14.60 Hz, 1H, CH_2Ar), 3.69 (m, 4H, CH_2Cl), 3.78 (m, 4H+2H, $CH_2CH_2Cl+CH_{2\ maleimide}$), 4.18 (m, 1H, CH), 4.38 (m, 1H, CH), 6.77 (d, ^{3}J(H,H) = 8.81 Hz, 2H, Ar*H*), 6.86 (s, 2H, $H_{maleimide}$), 7.11 (d, ^{3}J(H,H) = 8.75 Hz, 2H, Ar*H*); ^{13}C NMR (CD_3OD) δ = 37.55 ($CH_2N_{maleimide}$), 39.67 (CH_2Ar), 41.07 (CH_2N), 42.6 (CH_2Cl), 53.7 (CH), 60.14 (CH_2O), 119.5 ($C_{aromatic}$), 132.50 ($C_{aromatic}$), 132.54 ($C_{aromatic}$), 132.7 ($C_{aromatic}$), 135.43 ($CH_{maleimide}$), 135.68 ($C_{aromatic}$), 170.33 ($CO_{maleimide}$), 172.3 (COO); MS (ESI) calcd. for $C_{19}H_{24}N_3O_4Cl_2^+$ 428.1138, found 428.1152; Anal. ($C_{19}H_{25}Cl_3N_3O_4 \cdot HCl$) C, H, N.

*O-{4-[6-Bis(2-chloroethyl)amino-3-methylbenzimidazoyl(2)]-butanoyl}-2-hydroxyethylmaleimide (***7***).* Bendamustine free base **6** (2 g, 5.6 mmol), DMAP (35 mg, 0.26 mmol), and *N*-(2-hydroxyethyl)maleimide **3** (2.36 g, 16.7 mmol) were dissolved in 250 mL of dry dichloromethane at room temperature. DCC (1.27 g, 6.16 mmol), dissolved in 100 mL of dry dichloromethane, was added dropwise to this solution within 1 h and the solution stirred for additional 24 h. The solution was filtered and evaporated in vacuo. The residue was chromatographed on

[1]Abbreviations: Mel, Melphalan; DMAP, 4-dimethylaminopyridine; DCC, *N*,*N*-dicyclohexylcarbodiimide; DIPEA, *N*-ethyl-diisopropylamine; Benda, Bendamustine; TFA, trifluoroacetic acid; TBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; HOBT, hydroxybenzotriazole hydrate; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride.

Chart 1. Supporting Scheme for NMR Data of Compound 7

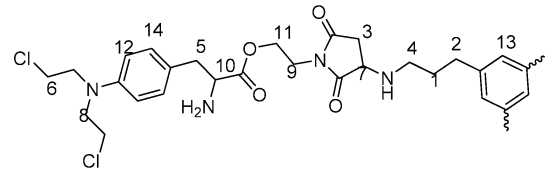


a silica gel column (dichloromethane/methanol 20:1, dichloromethane/acetone 3:1) to afford the desired product (1.89 g, 70.1%) as yellow solid: $R_f = 0.4$ (dichloromethane/acetone 3:1); mp 115 °C; ^{1}H NMR (400 MHz, DMSO) δ = 1.95 (quin, ^3J(H,H) = 7.31 Hz, 2H, CH_2), 2.39 (t, ^3J(H,H) = 7.27 Hz, 2H, CH_2CO), 2.79 (t, ^3J(H,H) = 7.37 Hz, 2H, $CH_2CO_{benzimidazole}$), 3.64 (m, 4H+2H, CH_2CH_2Cl+CH_2O), 3.70 (s, 4H+3H, CH_2CH_2Cl+$NCH_{3\ benzimidazole}$), 4.14 (t, ^3J(H,H) = 5.32 Hz, 2H, $CH_{2\ maleimide}$), 6.77 (dd, ^3J(H,H) = 2.21 Hz, 8.8 Hz, 1H, CH b), 6.91 (d, ^3J(H,H) = 2.08 Hz, 1H, CH c), 7.02 (s, 2H, $H_{maleimide}$), 7.31 (d, ^3J(H,H) = 8.77 Hz, 1H, CH a) (Chart 1); ^{13}C NMR ($CDCl_3$) δ = 25.0 (CH_2), 26.4 (CH_2), 33.0 (NCH_3), 33.9 (CH_2), 36.9 (CH_2), 40.8 (CH_2CH_2Cl), 61.4 (CH_2N), 103.5 ($C_{aromatic}$), 109.6 ($C_{aromatic}$), 110.6 ($C_{aromatic}$), 129.8 ($C_{aromatic}$), 134.6 ($C_{maleimide}$), 143.8 ($C_{aromatic}$), 154.7 ($C_{benzimidazole}$), 170.4 ($CO_{maleimide}$), 172.9 (CO); MS (EI, 80 eV, 300 °C) *m/z* (%) 479.8 (32.78) [M^+-1], 431.0 (95.45) [M-CH_2Cl]$^+$, 321.9 (100) $C_{16}H_{20}ClN_3O_2$; Anal. ($C_{22}H_{26}Cl_2N_4O_4$) C, H, N.

*1,3,5-Tris{N-[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)propanoate)-1-yl]propylamino}benzene (***8***).* MelBoc-spacer **4** (620 mg, 1.17 mmol) was dissolved in 20 mL of chloroform. To this solution was added dropwise a mixture of **G0**·3HCl (125 mg, 0.35 mmol) and DIPEA (137 mg, 1.05 mmol, 186 μL) dissolved in 2 mL of dry methanol. The reaction mixture was refluxed for 72 h and was continuously monitored by TLC. The solution was then washed once with sodium hydrogen carbonate solution and once with brine. The organic layer was dried over magnesium sulfate and the solvent evaporated in vacuo. The residue was purified by column chromatography (silica gel, dichloromethane/methanol 20:1) to afford the desired product (192 mg, 30%) as a white solid: R_f = 0.2 (dichloromethane/methanol 20:1); mp 230 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.40 (s, 27H, **1** CH_2), 1.79 (quin, ^3J(H,H) = 7.29 Hz, 6H, **2** CH_2), 2.35 (s, 3H, **1** NH), 2.49 (m, 3H, **3** CH_2), 2.61 (m, 3H+6H, **3**+**4** CH_2), 2.71 (m, 3H, **5** CH_2), 2.88−3.03 (m, 3H+6H, **5**+**6** CH_2), 3.59 (t, ^3J(H,H) = 6.30 Hz, 12H, **7** CH_2), 3.68 (t, ^3J(H,H) = 6.57 Hz, 12H, **8** CH_2), 3.75 (m, 3H+6H, **9** CH_2), 4.33 (m, 3H, **10** CH_2), 4.45 (m, 3H, **10** CH_2), 4.94 (t, ^3J(H,H) = 7.56 Hz, 3H, **11** CH), 6.59 (d, ^3J(H,H) = 8.52 Hz, 12H, **12** CH), 6.82 (s, 3H, **13** CH), 7.00 (d, ^3J(H,H) = 7.08 Hz, **14** CH); ^{13}C NMR (CD_3OD) δ = 26.79 (**2** CH_2), 28.79 (CH_3), 32.31 (**3** CH_2), 34.80 (**4**+**5** CH_2), 38.64 (**9** CH_2), 41.73 (**7**+**8** CH_2), 48.2 (**6** CH_2), 54.49 (**9**+**11** CH_2), 62.42 (**10** CH_2), 80.6 (C), 113.41 (**12** CH), 127.02 ($C_{aromatic}$), 129.26 (**14** CH), 129.97 ($C_{aromatic}$), 131.59 (**13** CH), 146.63 ($C_{aromatic}$), 159.90 (CONH), 172.9 (COO), 173.76 ($CO_{maleimide}$), 176.95 ($CO_{maleimide}$); MS (ESI) calcd. for $C_{87}H_{121}N_{12}O_{18}Cl_6$ 1835.7034, found 1835.6977; Anal. ($C_{87}H_{121}N_{12}O_{18}Cl_6$) C, H, N.

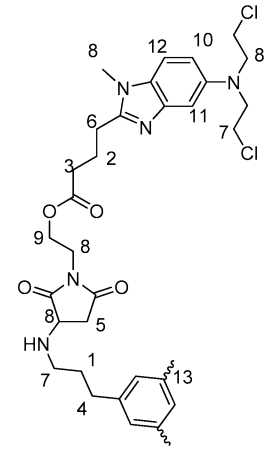
*1,3,5-Tris{N-[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-1-yl]propylamino}benzene trishydrochloride (***9***).* The G0 dendrimer **8** (280 mg, 0.15 mmol)) was dissolved in 10 mL of THF. To this solution, 173 μL of hydrochloric acid (w = 25%) was added and the mixture was stirred at room temperature. After monitoring the reaction by TLC, 400 μL of hydrochloric acid (25%) was added. After 72 h, the solvent was removed under reduced pressure to afford the deprotected product as a brown solid (240 mg, 95%) after lyophilization from water: mp 255 °C; ^{1}H NMR (400 MHz,

Chart 2. Supporting Scheme for NMR Data of Compound 9



CD_3OD) δ = 2.12 (m, 6H, **1** CH_2), 2.76 (m, 6H, **2** CH_2), 3.06−3.12 (m, 6H, **3** CH_2), 3.18−3.26 (m, 6H+6H, **4**+**5** CH_2), 3.68 (m, 12H+6H, **6**+**9** CH_2), 3.82 (m, 12H, **8** CH_2), 4.29 (m, 3H, **7** CH), 4.42 (m, 3H, **11** CH_2), 4.52 (m, 3H, **11** CH_2), 4.61 (m, 3H, **10** CH), 6.89 (d, ^3J(H,H) = 8.37 Hz, 6H, **12** CH), 7.07 (s, 3H, **13** CH), 7.23 (d, ^3J(H,H) = 8.37 Hz, 6H, **14** CH) (Chart 2); ^{13}C NMR (CD_3OD) δ = 28.84 (**1** CH_2), 33.52 (**3** CH_2), 34.22 (**2** CH_2), 36.45 (**9** CH_2), 39.08 (**5** CH_2), 41.12 (**8** CH_2), 41.19 (**6** CH_2), 47.42 (**4** CH_2), 55.21 (**10** CH), 59.29 (**7** CH), 63.83 (**11** CH_2), 115.32 (**12** CH), 127.97 ($C_{aromatic}$), 131.95 ($C_{aromatic}$+**14** CH), 132.02 ($C_{aromatic}$), 145.95 ($C_{aromatic}$), 169.91 (COO), 171.34 ($CO_{maleimide}$), 173.80 ($CO_{maleimide}$); MS (ESI): calcd. for $C_{72}H_{97}N_{12}O_{12}Cl_6$ 1535.5445, found 1535.5312.

Chart 3. Supporting Scheme for NMR Data of Compound 10



*1,3,5-Tris{N-[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoate)-1-yl]propylamino}benzene (***10***).* Benda-spacer **7** (470 mg, 0.97 mmol) was dissolved in 20 mL of chloroform. To this solution, a mixture of **G0**·3HCl (77.8 mg, 0.216 mmol) and 116 μL of DIPEA dissolved in 2 mL of dry methanol was added dropwise. The reaction mixture was refluxed for 72 h, being continuously monitored by TLC. The reaction mixture was then washed once with sodium hydrogen carbonate solution and once with brine. The organic layer was dried over magnesium sulfate and the solvent was evaporated in vacuo. The residue was purified by column chromatography (silica gel, chloroform/methanol 20:1) to afford the desired product (290 mg, 79%) as an ochre solid: R_f = 0.44 (dichloromethane/methanol 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.79 (quin, ^3J(H,H) = 7.22 Hz, 6H, **1** CH_2), 2.17 (m, 6H, **2** CH_2), 2.48 (m, 6H, **3** CH_2), 2.60 (m, 6H+3H, **4**+**5**CH_2), 2.69−2.75 (m, 3H, **5** CH_2), 2.93 (m, 6H, **6** CH_2), 3.64 (m, 12H+6H, **7** CH_2), 3.71−3.79 (m, 12H+3H+6H+9H, **8** CH_2), 4.23 (m, 6H, **9** CH_2), 6.79 (dd, ^3J(H,H) = 2.17 Hz, 8.75 Hz, 3H, **10** CH), 6.83 (s, 3H, **13** CH), 7.08 (d, ^3J(H,H) = 1.96 Hz, 3H, **11** CH), 7.19 (d, ^3J(H,H) = 8.76 Hz, 3H, **13** CH) (Chart 3); ^{13}C NMR ($CDCl_3$) δ = 22.2 (**2** CH_2), 26.3 (**6** CH_2), 29.4 (**1** CH_2), 31.9 (**8** CH_2), 32.9 (**3** CH_2), 33.1 (**5** CH_2), 33.3 (**4** CH_2), 37.8 (**8** CH_2), 40.86 (**7**+**8** CH_2), 47.1 (**7** CH_2), 54.6 (**8** CH_2), 60.8 (**9** CH_2), 102.6 ($C_{aromatic}$), 110.0 ($C_{aromatic}$), 110.9 ($C_{aromatic}$), 126.1 ($C_{aromatic}$), 129.1 ($C_{aromatic}$), 141.8 ($C_{aromatic}$), 142.9

Chart 4. Supporting Scheme for NMR Data of Compound 11

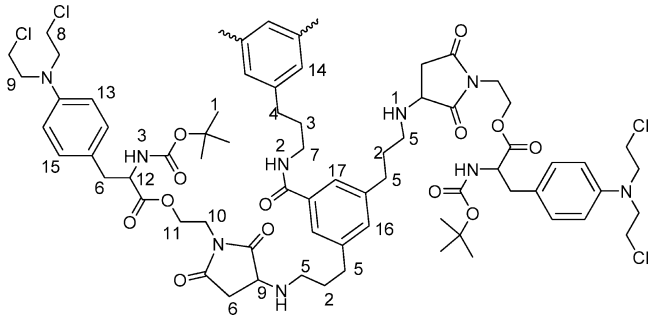
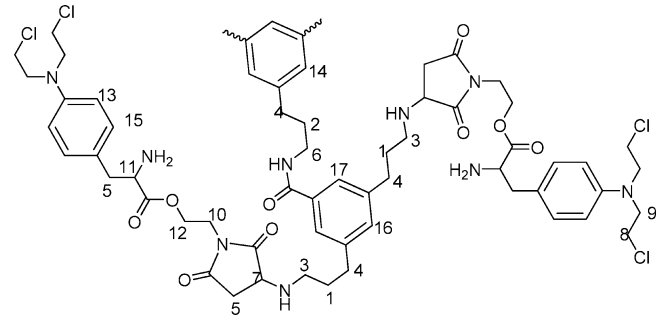


Chart 5. Supporting Scheme for NMR Data of Compound 12



($C_{aromatic}$), 154.2 ($C_{benzimidazole}$), 173.02 (CO), 175.06 (CO), 177.6 (CO); MS (ESI): calcd. for $C_{81}H_{106}N_{15}O_{12}Cl_6$ 1692.6258, found 1692.6193; $C_{81}H_{106}N_{15}O_{12}Cl_6^{2+}$ 846.8165, found 846.8170.

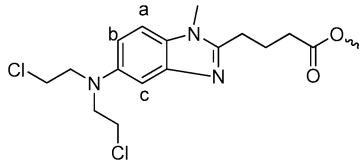
*1,3,5-Tris{3,5-bis-[N-[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)-propanoate)-1-yl]propylamino]-N-propylbenzamide}benzene (***11***).* To a solution of MelBoc-spacer **4** (700 mg, 1.32 mmol) in 50 mL of chloroform was added dropwise a solution of **G1**·CF_3COOH (320 mg, 0.2 mmol) and DIPEA (156 mg, 0.12 mmol, 115 μL) in dry methanol. The reaction mixture was refluxed for 70 h. After complete reaction (TLC), the solution was washed once with sodium hydrogen carbonate solution and once with brine. The organic layer was dried over magnesium sulfate; the solvent was evaporated in vacuo and the residue purified through column chromatography (silica gel, dichloromethane/methanol 20:1 and chloroform/methanol 10:1) to afford the compound as a yellow oil (200 mg, 24.5%): R_f = 0.26 (chloroform/methanol 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.32 (s, 54H, **1** CH_2), 1.71 (m, 12H, **2** CH_2), 1.86 (m, 6H, **3** CH_2), 2.40 (m, 6H, **4** CH_2), 2.56 (m, 24H, **5** CH_2), 2.80−2.95 (m, 12H+12H, **6** CH_2), 3.35 (m, 6H, **7** CH_2), 3.52 (m, 24H, **8** CH_2), 3.61 (m, 24H+6H, **9** CH_2), 3.68 (m, 12H, **10** CH_2), 4.09−4.18 (m, 6H, **11** CH_2), 4.27 (m, 6H, **11** CH_2), 4.36 (m, 6H, **12** CH), 4.93 (m, 6H, **1** NH), 6.52 (d, ^3J(H,H) = 8.03 Hz, 12H, **13** CH), 6.74 (s, 3H, **2** NH), 6.82 (s, 3H, **14** CH), 6.92 (d, ^3J(H,H) = 7.38 Hz, 12H, **15** CH), 7.04 (s, 6H, **3** NH), 7.06 (s, 3H, **16** CH), 7.33 (s, 6H, **17** CH) (Chart 4); ^{13}C NMR (CD_3OD) δ = 29.22 (CH_3+**2**+**3** CH_2), 31.16 (CH_2 $_{maleimide}$+CH_2Ar+CH_2Ar), 39.9 (**10** CH_2+ArCH_2 $_{melphalan}$), 42.17 (CH_2NHCO+*C*H_2*C*H_2Cl), 44.23 ($CH_2NH_{maleimide}$), 54.86 (**12** CH), 56.28 ($CH_{maleimide}$+**11** CH_2), 81.07 (C), 113.82 (**13** CH), 126.69 ($C_{aromatic}$), 131.92 ($C_{aromatic}$), 132.28 ($C_{aromatic}$), 140.13 ($C_{aromatic}$), 143.95 ($C_{aromatic}$), 150.3 ($C_{aromatic}$), 158.15 (NH-*C*OO), 174.21 (CO), 175.25 ($CO_{maleimide}$); MS (ESI) calcd. for $C_{198}H_{269}N_{27}O_{39}Cl_{12}^{2+}$ 2037.307, found 2037.3243.

*1,3,5-Tris{3,5-bis-[N-[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-1-yl]-propylamino]-N-propylbenzamide}benzene Hexatrifluoroacetate (***12***).* The G1 dendrimer **11** (180 mg, 0.044 mmol) was dissolved in 10 mL of dichloromethane and treated with 2.8 mL of TFA (w = 95%). The mixture was stirred for 24 h at room temperature and continuously monitored by TLC. The solvent was then evaporated in vacuo to afford the deprotected dendrimer as brown oil (170 mg, 93%): ^{1}H NMR (CD_3OD) δ = 1.92 (m, 12H, **1** CH_2), 2.09 (s, br, 12H, **2** CH_2), 2.65 (m, 12H, **3** CH_2), 2.77 (m, 18H, **4** CH_2), 2.99−3.05 (m, 6H+12H, **5** CH_2), 3.21 (m, 6H+6H, **5** CH_2+**6** CH_2), 3.40 (m, 6H, **7** CH_2), 3.67 (m, 24H, **8** CH_2), 3.74 (m, 24H, **9** CH_2), 3.84 (m, 12H, **10** CH_2), 4.19 (m, 6H, **11** CH), 4.37 (m, 12H, **12** CH_2), 6.76 (d, ^3J(H,H) = 8.13 Hz, 12H, **13** CH_2), 6.93 (s, 3H, **14** CH_2), 7.14 (d, ^3J(H,H) = 8.19 Hz, 12H, **15** CH_2), 7.32 (s, 3H, **16** CH_2), 7.55 (s, 6H, **17** CH_2) (Chart 5); ^{13}C NMR (CD_3OD) δ = 27.77 (**1**+**2** CH_2), 28.84 (**4** CH_2), 31.17 (**4** CH_2), 33.31 (**5** CH_2), 36.29 (**5** CH_2), 36.44 (**10** CH_2), 39.01 (**6** CH_2), 41.67 (**9** CH_2), 47.6 (**3** CH_2), 54.32 (**8** CH_2), 55.52 (**11** CH), 63.85 (**7**+**12** CH_2), 113.85 (**13** CH), 118.45 ($C_{aromatic}$), 121.22 ($C_{aromatic}$), 126.47 ($C_{aromatic}$), 128.85 ($C_{aromatic}$), 131.7 (**16** CH), 142.54 ($C_{aromatic}$), 143.29 ($C_{aromatic}$), 147.55 ($C_{aromatic}$), 160.34 (CO), 160.70 (CO), 170.11 (CO), 174.64 (CO); MS (ESI) calcd. for $C_{168}H_{219}N_{27}O_{27}Cl_{12}Na_2^{2+}$ 1760.1307, found 1760.1456.

*1,3,5-Tris[3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)-N-propylpropionamide]benzene (***13***).* A solution of **2** (910 mg, 2.5 mmol) in a mixture of dry dichloromethane/DMF (14:1/v:v) was cooled to −20 °C. A solution of TBTU (740 mg, 2.35 mmol) in dry DMF was added slowly and the reaction mixture was allowed to warm to room temperature. The reaction mixture was stirred at room temperature for additional 30 min, cooled to −20 °C again, followed by the dropwise addition of DIPEA (1040 μL, 6 mmol) and a solution of **G0**·3HCl (179.39 mg, 0.5 mmol) in dry methanol. The mixture was allowed to warm to room temperature and was stirred for additional 20 h. After complete reaction (TLC), the mixture was washed twice with sodium hydrogen carbonate solution, then once with brine, and then dried over magnesium sulfate. The solvent was removed under reduced pressure and column chromatography (silica gel, dichloromethane/methanol 20:1) afforded the desired product (400 mg, 0.28 mmol, 57%) as a brown oil: R_f = 0.36 (dichloromethane/methanol 24:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.36 (s, 27H, CH_3), 1.7 (quin, ^3J(H,H) = 6.892 Hz, 6H, CH_2), 2.55 (t, ^3J(H,H) = 6.9 Hz, 6H, CH_2Ar), 2.68 (dd, ^2J(H,H) = 13.46 Hz, 3H, $CH_2Ar_{melphalan}$), 2.83 (dd, ^2J(H,H) = 13.46 Hz, 3H, $CH_2Ar_{melphalan}$), 3.1 (m, 6H, CH_2N), 3.71 (s, 24H, C*H*$_2$C*H*$_2$Cl), 4.08 (m, 3H, C*H*$CH_2Ar_{melphalan}$), 6.68 (d, ^3J(H,H) = 8.168 Hz, 6H, Ar*H*$_{melphalan}$), 6.82 (d, ^3J(H,H) = 8.45 Hz, 3H, N*H*-Boc), 6.86 (s, 3H, G0-Ar*H*), 7.12 (d, ^3J(H,H) = 8.19 Hz, 6H, Ar*H*$_{melphalan}$), 7.9 (s, br, 3H, NH); ^{13}C NMR ($CDCl_3$) δ = 28.3 (CH_2), 30.5 (Ar*C*H_2), 37.6 ($NHCH_2$), 40.4 (*C*H_2*C*H_2Cl), 54.5 ($CHNH_2$), 79.8 (*C*-$(CH_3)_3$), 112.1 ($C_{aromatic}$), 124.9 ($C_{aromatic}$), 126.4 ($C_{aromatic}$), 130.6 ($C_{aromatic}$), 144.9 ($C_{aromatic}$), 155.7 ($C_{aromatic}$), 171.6 (CO); MS (ESI) calcd. for $C_{69}H_{99}N_9O_9Cl_6Na^+$ 1432.5572, found 1432.5566.

*1,3,5-Tris[3-(4-(bis(2-chloroethyl)amino)phenyl)-2-amino-N-propylpropionamide]benzene Tristrifluoroacetate (***14***).* A solution of **13** (590 mg, 0.42 mmol) in 10 mL of TFA (w = 95%) was stirred for 4 h at room temperature. After complete reaction (TLC), the solvent was repeatedly removed in vacuo to afford the deprotected dendrimer (580 mg, 0.39 mmol, 95%) as brown solid: mp 150 °C; ^{1}H NMR (400 MHz, CD_3OD) δ = 1.63 (quin, ^3J(H,H) = 7.09 Hz, 6H, CH_2), 2.44 (t, ^3J(H,H) = 7.28 Hz, 6H, CH_2Ar), 2.84 (dd, ^2J(H,H) = 12.85 Hz, 3H, C*H*$_2Ar_{melphalan}$), 2.91 (dd, ^2J(H,H) = 12.84 Hz, 3H, C*H*$_2Ar_{melphalan}$), 3.02 (m, 3H, CH_2N), 3.15 (m, 3H, CH_2N), 3.65 (s, 24H, C*H*$_2$C*H*$_2$Cl), 3.83 (m, 1H, C*H*$CH_2Ar_{melphalan}$), 6.69 (d, ^3J(H,H) = 8.58 Hz, 6H, Ar*H*$_{melphalan}$), 6.8 (s, 3H, G0-Ar*H*), 7.05 (d, ^3J(H,H) = 8.43 Hz, 6H, Ar*H*$_{melphalan}$), 8.1 (s, br, 6H, NH_2), 8.4 (t, ^3J(H,H) = 5.24 Hz, 3H, NH); ^{13}C NMR (CD_3OD) δ = 31.9 (CH_2), 34.0 (Ar*C*H_2), 40.2 ($NHCH_2$), 41.6 (*C*H_2*C*H_2Cl), 56.0 ($CHNH_2$), 113.6 ($C_{aromatic}$), 123.8 ($C_{aromatic}$), 127.3 ($C_{aromatic}$), 131.7 ($C_{aromatic}$),

Chart 6. Supporting Scheme for NMR Data of Compound 15

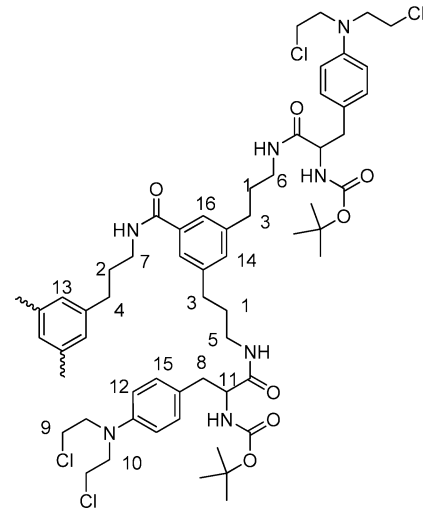


143.0 ($C_{aromatic}$), 147.2 ($C_{aromatic}$), 169.6 (CO); MS (ESI); Anal. ($C_{60}H_{78}Cl_6N_9O_9F_9$) C, H, N.

*1,3,5-Tris[4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)-N-propylbutyramide]benzene (***15***).* A solution of bendamustine free base **6** (430 mg, 1.2 mmol) in a mixture of dry dichloromethane/DMF (7 mL/2 mL) was cooled to −20 °C. A solution of TBTU (352 mg, 1.128 mmol) in dry DMF was added slowly, and the reaction mixture was allowed to warm to room temperature. The reaction mixture was stirred at room temperature for additional 30 min and cooled to −20 °C again, followed by the dropwise addition of DIPEA (502 μL, 2.88 mmol) and a solution of **G0**·3HCl (85.4 mg, 0.24 mmol) in dry methanol. The mixture stirred was allowed to warm to room temperature and was stirred for additional 20 h. After complete reaction (TLC), the mixture was washed twice with sodium hydrogen carbonate solution, then once with brine, and dried over magnesium sulfate. The solvent was removed under reduced pressure, and column chromatography (silica gel, chloroform/methanol 20:1) afforded the desired product (130 mg, 1.02 mmol, 42%) as a brown oil: R_f = 0.5 (chloroform/methanol 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.71 (quin, ^3J(H,H) = 6.91 Hz, 6H, CH_2), 2.05 (quin, ^3J(H,H) = 6.85 Hz, 6H, CH_2 bendamustine), 2.23 (t, ^3J(H,H) = 6.66 Hz, 6H, $CH_2CO_{bendamustine}$), 2.48 (t, ^3J(H,H) = 7.01 Hz, 6H, CH_2ArG0), 2.85 (t, ^3J(H,H) = 7.08 Hz, 6H, CH_2 benzimidazole), 3.08 (m, 6H, CH_2N), 3.50−3.67 (m, 24H, CH_2CH_2Cl), 3.50−3.67 (m, 9H, NCH_3 bendamustine), 6.71 (dd, ^3J(H,H) = 2.30 Hz, 8.86 Hz, 3H, CH b), 6.73 (s, 3H, Ar*H*G0), 6.93 (d, ^3J(H,H) = 2.09 Hz, 3H, CH c), 7.1 (d, ^3J(H,H) = 8.8 Hz, 3H, CH a) (Chart 6); ^{13}C NMR (CD_3OD) δ = 26.0 (CH_2 benzimidazole), 27.29 (CH_2CH_2CO), 29.33 (CH_2), 31.91 (CH_3), 32.76 (CH_2Ar), 38.69 (CH_2CO), 40.02 (CH_2NHCO+CH_2N), 42.1 (CH_2Cl), 104.1 (CH c), 112.92 (CH a+b), 125.85 ($C_{aromatic}NCH_3$+$C_{aromatic}$), 131.2 ($C_{aromatic}NC$+$C_{aromatic}$), 146.2 ($C_{aromatic}$+$CNCH_3$), 163.91 (CO); MS (ESI) calcd. for $C_{63}H_{84}N_{12}O_3Cl_6Na^+$ 1291.4800, found 1291.4634.

*1,3,5-Tris{3,5-bis-[3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)-N-propylpropionamide]-N-propylbenzamide}benzene (***16***).* MelBoc **2** (472 mg, 1.16 mmol) was dissolved in 15 mL of dry dichloromethane, HOBT (165 mg, 1.22 mmol) was added, and the solution was stirred for 30 min at room temperature. Afterward, the orange mixture was cooled to −20 °C, EDC (245.3 mg, 1.28 mmol) was added, and the mixture was subsequently stirred for additional 2 h. During that time, the reaction mixture was allowed to warm up to room temperature. After complete reaction (TLC), the mixture was cooled to −30 °C. **G1**·CF_3COOH (440 mg, 0.3 mmol) dissolved in 10 mL of dry methanol and DIPEA (1.22 mL, 7 mmol) were added dropwise slowly. The solution was stirred for additional 16 h and during that time allowed to warm up to room temperature. After complete reaction, the solution was washed twice with sodium hydrogen carbonate solution and once with brine. The organic layer was dried over magnesium sulfate, the solvent was removed in vacuo, and the crude product was purified by column chromatography (silica gel, dichloromethane/methanol 20:1) to afford the **G1** dendrimer (30 mg, 3.1%) as a brown oil: R_f = 0.33 (dichloromethane/methanol 10:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.37 (s, 54H, CH_3), 1.66 (m, 18H, **1** CH_2), 1.97 (m, 6H, **2** CH_2), 2.43 (m, 12H, **3** CH_2), 2.66 (m, 6H, **4** CH_2), 2.93 (m, 6H+6H, **5**+**8** CH_2), 3.08 (m, 6H+6H, **6**+**8**

Chart 7. Supporting Scheme for NMR Data of Compound 16



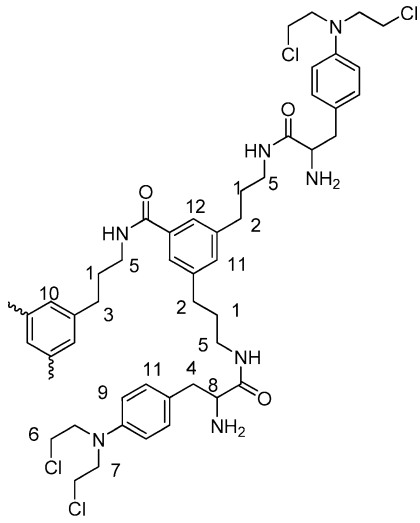
CH_2), 3.41 (m, 6H, **7** CH_2), 3.53 (m, 24H, **9** CH_2), 3.64 (m, 24H, **10** CH_2), 4.32 (m, br, 6H, **11** CH), 6.55 (d, ^3J(H,H) = 8.14 Hz, 12H, **12** CH), 6.92 (s, 3H, **13** CH), 6.98 (s, 3H, **14** CH), 7.06 (d, ^3J(H,H) = 8.28 Hz, 12H, **15** CH), 7.32 (s, 6H, **16** CH) (Chart 7); ^{13}C NMR (CD_3OD) δ = 27.33 (CH_3), 30.34 (**1** CH_2), 30.85 (**2** CH_2), 32.38 (**3** CH_2), 32.98 (**4** CH_2), 36.63 (**8** CH_2), 38.48 (**5**+**6** CH_2), 38.67 (**7** CH_2), 40.16 (**9**+**10** CH_2), 54.12 (**11** CH), 79.3 (C), 113.69 (**12** CH), 123.38 ($C_{aromatic}$), 124.77 ($C_{aromatic}$), 128.65 ($C_{aromatic}$), 130.09 ($C_{aromatic}$), 131.62 ($C_{aromatic}$), 134.54 ($C_{aromatic}$), 135.82 ($C_{aromatic}$), 141.83 ($C_{aromatic}$), 156.2 (NHCOO), 167.75 (NHCO), 167.92 (NH*C*OCH); MS (ESI) calcd. for $C_{162}H_{225}N_{21}O_{21}Cl_{12}^{+2}$ 1615.1781, found 1615.1794.

*1,3,5-Tris{3,5-bis-[3-(4-(bis(2-chloroethyl)amino)phenyl)-2-amino-N-propylpropionamide]-N-propylbenzamide}benzene Hexahydrochloride (***17***).* The **G1** dendrimer **16** (100 mg, 0.031 mmol) was dissolved in 5 mL of THF. To this solution, hydrochloric acid (w = 25%) was added dropwise and the mixture stirred for 3 days at room temperature. The reaction was continuously monitored by TLC. The solvent was then removed under reduced pressure to afford the deprotected product as a brown oil (85 mg, 96%) after lyophilization from water: ^{1}H NMR (400 MHz, CD_3OD) δ = 1.75 (m, 18H, **1** CH_2), 2.49 (m, 12H, **2** CH_2), 2.65 (m, 6H, **3** CH_2), 3.02 (m, 12H+18H, **4**+**5** CH_2), 3.54 (s, br, 24H, **6** CH_2), 3.65 (s, br, 24H, **7** CH_2), 3.8 (s, 6H, **8** CH), 6.75 (m, 12H, **9** CH), 6.89 (s, 3H, **10** CH), 7.15 (m, 12H+3H, **11** CH), 7.44 (s, 6H, **12** CH) (Chart 8); ^{13}C NMR (CD_3OD) δ = 27.78 (**1** CH_2), 35.12 (**2**+**3** CH_2), 40.21 (**4**+**5**+**7** CH_2), 42.32 (**6** CH_2), 53.00 (**8** CH), 112.50 (**9** CH), 127.70 (**10**+**12** CH), 128.66 (**11** CH), 129.16 ($C_{aromatic}$), 130.45 (**11** CH), 131.45 ($C_{aromatic}$), 134.53 ($C_{aromatic}$), 140.4 ($C_{aromatic}$), 146.2 ($C_{aromatic}$), 165.87 (CO), 170.2 (CO); MS (ESI) calcd. for $C_{132}H_{177}N_{21}O_9Cl_{12}Na_2^{+2}$ 1333.005, found 1333.0546.

HPLC Analytic Procedure. The stability of the compounds was determined in aqueous solution at a concentration of 1 mg/mL. Degradation was followed by HPLC analytic using a Kontron HPLC system: HPLC-pump 420; HPLC-autosampler 460; UV-Detector 430; Kontron Data System Kroma 2000. An RP 18 column (250 × 6 × 4 mm Nucleosil 100−5 C18 (Macherey-Nagel)) was used. The solutions were held at 37 °C, and every hour, 20 μL was injected onto the column and isocratically analyzed using a methanol/H_2SO_4 (0.001 N, 20 mmol Na_2SO_4) mixture (50:50). A wavelength of λ = 254 nm was selected for UV-detection.

The stability in PBS (pH 5 and 7.4) was measured at a concentration of 0.1 mg/mL. For protein binding, 40 mg/mL HSA and 0.1 mg/mL compound were incubated at 37 °C. The

Chart 8. Supporting Scheme for NMR Data of Compound 17



protein was separated by ethanolic precipitation. After centrifugation, the supernatant was evaluated for drug content.

Conformational Analysis for Molecular Extent Prediction. Conformational analysis was performed using the program *OMEGA* (*20*) from OpenEye Scientific Software (www.eyesopen.com). The maximum possible number of conformations was set to 10 000, and the MMFF94 force field was used for conformational search and subsequent energy minimization with a dielectric constant of 80 simulating a water environment. For all other parameters, default values were kept. This setup resulted in 918 conformations for **12** and 6005 for **10**. Subsequently, all conformations were converted and analyzed using a custom routine implemented in the LigandScout framework (*21, 22*) for determining the maximum extent of each conformation.

Biological Methods. *Cell Culture.* The human MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). The MCF-7 breast cancer cell line originated from a 69-year-old Caucasian woman and is a well-characterized estrogen receptor (ER) positive control cell line (cells are positive for cytoplasmic estrogen receptors). The human cell line MDA-MB-231 is a prototype for the study of hormone-independent breast cancer. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay (*23*). Both cell lines were maintained in L-glutamine and sodium pyruvate containing DMEM High Glucose (4.5 g/L) supplemented with 5% fetal calf serum (FCS, Gibco) using 25 cm^2 culture flasks in a humidified atmosphere (5% CO_2) at 37 °C. The cell lines were passaged weekly after treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (EDTA, 0.02%, Boehringer). Mycoplasma contamination was regularly monitored, and only mycoplasma-free cultures were used.

In Vitro Chemosensitivity Assays. Briefly, 100 μL of a cell suspension of 7500 cells mL^{-1} culture medium were plated into each well of a 96-well microtiter plate and incubated at 37 °C for 3 days in a humidified atmosphere (5% CO_2). By adding an adequate volume of a stock solution of the appropriate compound (solvent: H_2O, DMF, DMSO, MeOH) to the medium, the desired test concentration was obtained. After the proper incubation time, the medium was removed and the cells were fixed with a glutardialdehyde solution and stored under phosphate buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining technique described previously (*24, 25*). The efficiency of the compounds is expressed as corrected $\%T/C_{corr}$ values according to the following equations:

$$\text{cytostatic effect: } \%T/C_{corr} = [(T - C_0)/(C - C_0)] \times 100 \quad (1)$$

$$\text{cytocidal effect: } \%\tau = [(T - C_0)/C_0] \times 100 \quad (2)$$

in which T (test) and C (control) are the optical density values at 578 nm of the crystal violet extract of the cells in the wells (that is, the chromatin-bound crystal violet extracted with 70% ethanol), and C_0 is the density of the cell extract immediately before treatment. A microplate reader at 590 nm (Flashscan Analytik Jena AG) was used for the automatic estimation of the optical density of the crystal violet extract in the wells. The calculated $\%T/C$ values can be interpreted as follows:

$T/C > 80\%$ no antiproliferative effect

$80\% > T/C > 20\%$ antiproliferative effect

$20\% > T/C > 0\%$ cytostatic effect

$\tau = T/C < 0\%$ cytocidal effect

RESULTS

Synthesis. The synthesis first implicated the building of the drug−spacer derivatives which were then attached to the dendrimers.

Melphalan hydrochloride was Boc-protected in a first step and then reacted in a second step with *N*-(2-hydroxyethyl)maleimide using a Steglich reaction to give the ester **4** which was purified by column chromatography. Treatment of **4** with aqueous HCl yielded **5** as a colorless solid (Scheme 1).

Bendamustine hydrochloride was initially transformed to the free base **6** (*26*) and then converted to **7** through a similar approach as described for **4**.

The binding of the drug−spacer to the dendrimers was achieved in the last step of the synthetic route. Due to their nucleophilic properties, the terminal amine groups of the dendrimers were able to bind the maleimide residue. For this purpose, the reactants were dissolved in chloroform and heated to reflux. Cleavage of the Boc groups in melphalan derivatives was performed with 25% HCl. The analytically pure products were obtained after column chromatography. Schemes 2 and 3 depict the synthesis of the **G0** generation dendrimers conjugates **9** and **10**.

The melphalan-spacer **4** was bound to the **G1** dendrimer via the same synthetic route (see Scheme 4).

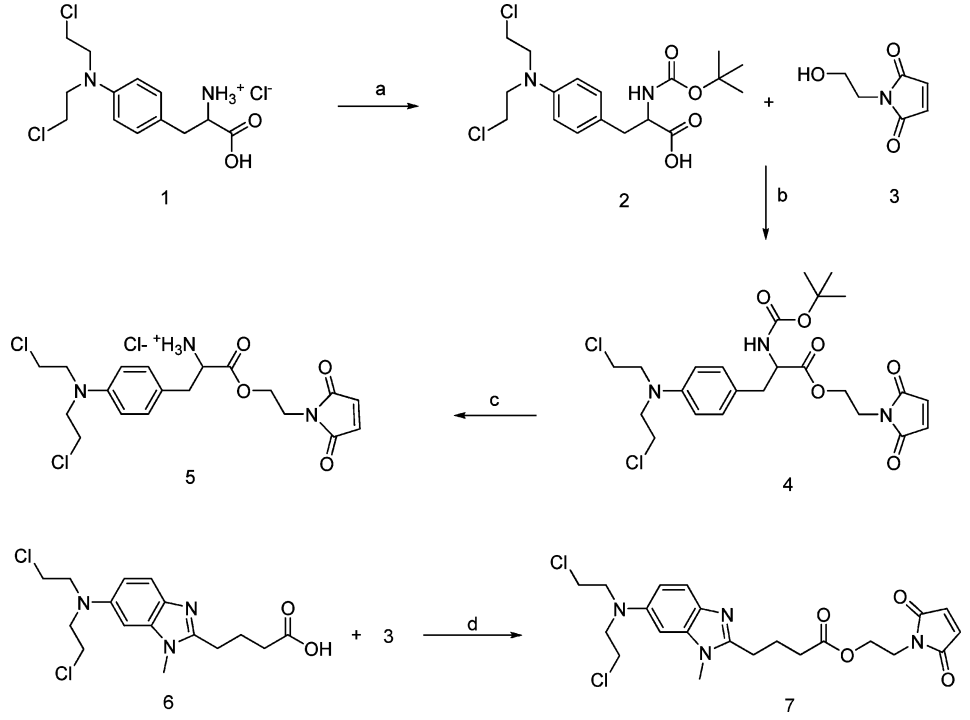
In order to evaluate the significance of the spacer **3** for the mode of action, the cytostatics were also directly bound to the dendrimer by formation of an amide bond using the standard peptide coupling reactions (*27*−*29*) (Schemes 5 and 6).

For this reaction, it is necessary to activate the carboxyl group of melphalan and bendamustine with peptide coupling agents, which led to the formation of reactive anhydrides or ester intermediates (for the mechanism of the reaction and the methods used, see ref *17*). The syntheses of **G0** dendrimer−drug conjugates were then carried out as depicted in Scheme 5 using TBTU as coupling agent.

Structural Characterization. All products and those compounds used in biological tests were characterized by their fully assigned ^1H and ^{13}C NMR spectra and molecular ion peaks in the mass spectra. Their purity was proven by high-resolution ESI-TOF mass spectrometry. All deprotected dendrimers were fully soluble in water and in buffered cell culture media as judged by visual examination.

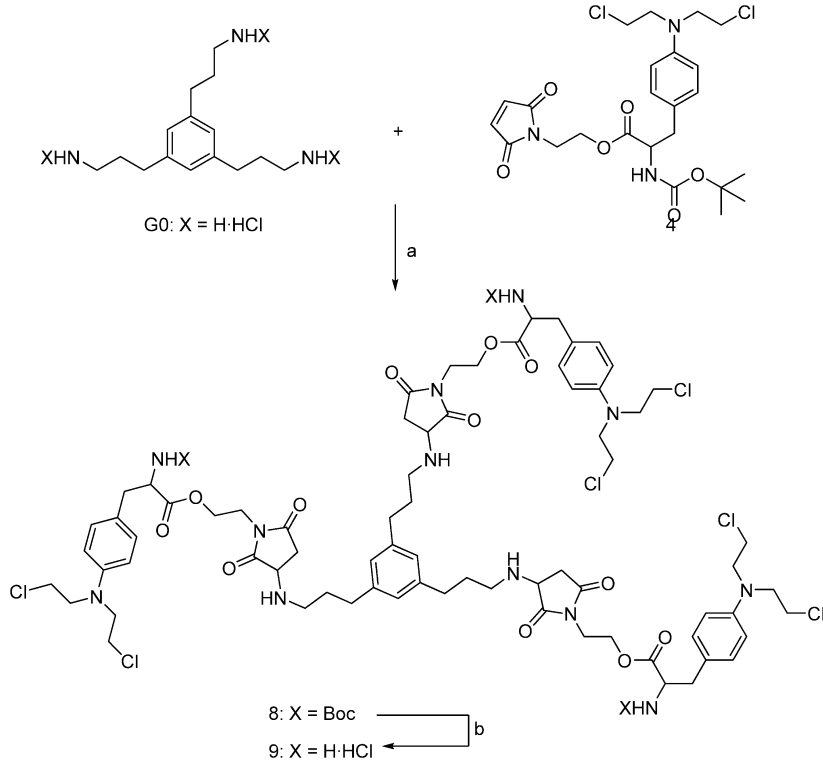
Computational Molecule Size Estimation for Assessing Enhanced Permeation and Retention. In order to estimate the molecule size in solution, the maximum extent of the

Scheme 1. Synthesis of the Drug−Spacer[a]



[a] Reagents and conditions: (a) Boc_2O, TEA, methanol, RT, 1 h; (b) DCC, DMAP, CH_2Cl_2, RT, 24 h; (c) aqueous HCl, THF, RT, 24 h; (d) DCC, DMAP, CH_2Cl_2, RT, 20 h.

Scheme 2. Synthesis of the Mel-spacer-G0 Conjugates[a]

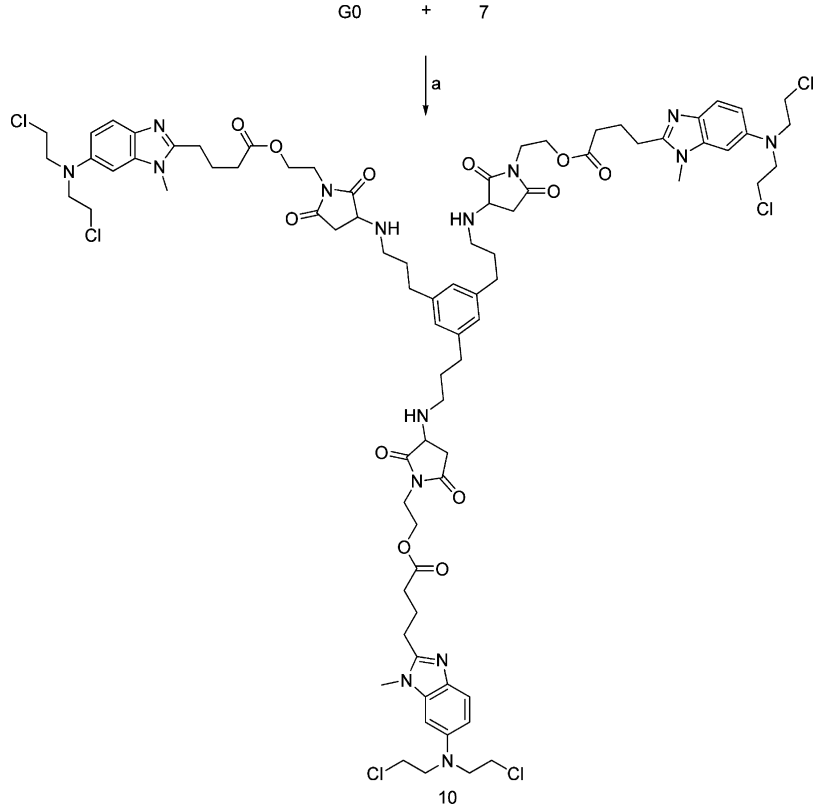


[a] Reagents and conditions: (a) chloroform, DIPEA, reflux, 72 h; (b) aqueous HCl, THF, RT.

compounds was calculated by performing an exhaustive conformational analysis using the software *OMEGA* and the cheminformatics framework of the software package *LigandScout* (*21, 22*). Figure 2 shows representative conformations of **10** and **12**, respectively, while Figure S1 (see Supporting Information) shows a histogram of the maximum extent distributions over the calculated three-dimensional conformational space for the two compounds. The relative extent frequencies reach their maxima near the average values of about 31 Å for compound **12** and at about 38 Å for **10** (Table S1, see Supporting Information).

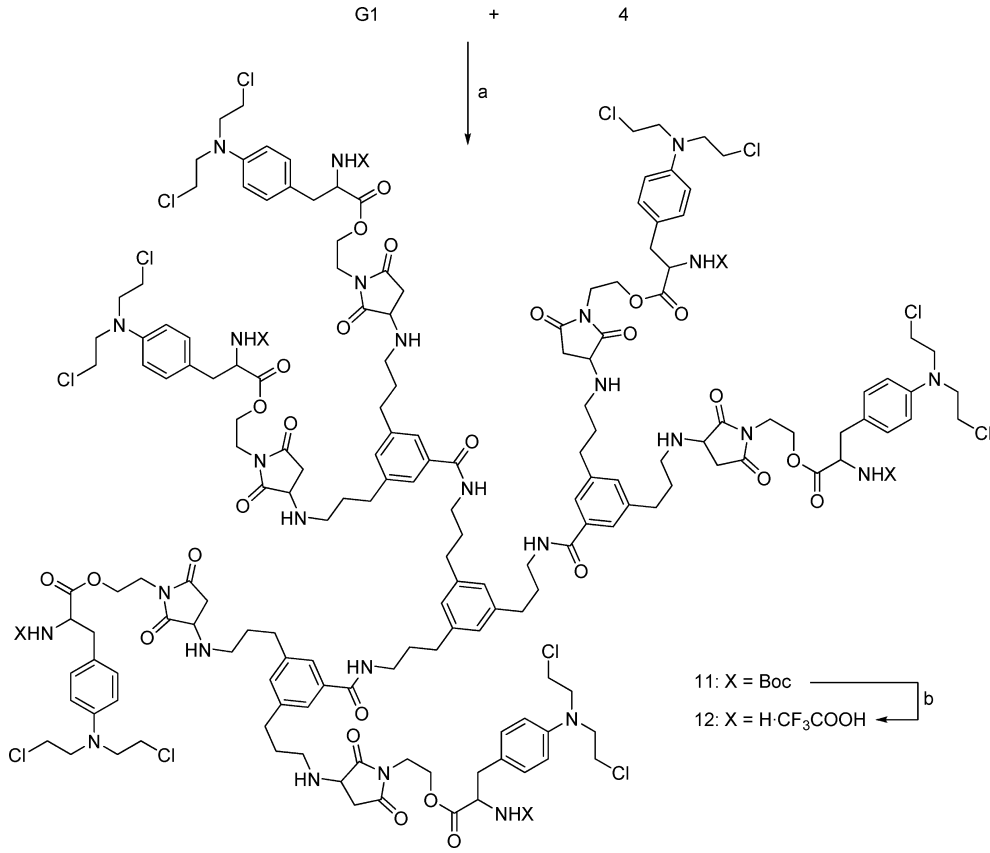
Antitumor Activity. The antiproliferative activity of the synthesized drug−dendrimer conjugates was determined in time-dependent cytotoxicity tests on the human MCF-7 and MDA-

Scheme 3. Synthesis of the Benda-spacer-G0 Conjugates[a]



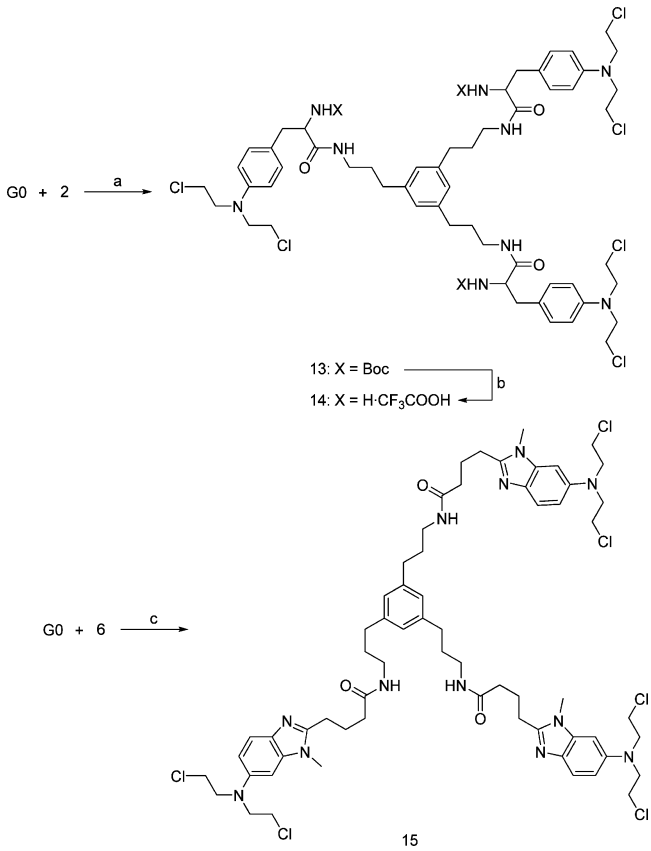
[a] Reagents and conditions: (a) DIPEA, chloroform, reflux, 72 h.

Scheme 4. Synthesis of the Mel-spacer-G1 Conjugates[a]



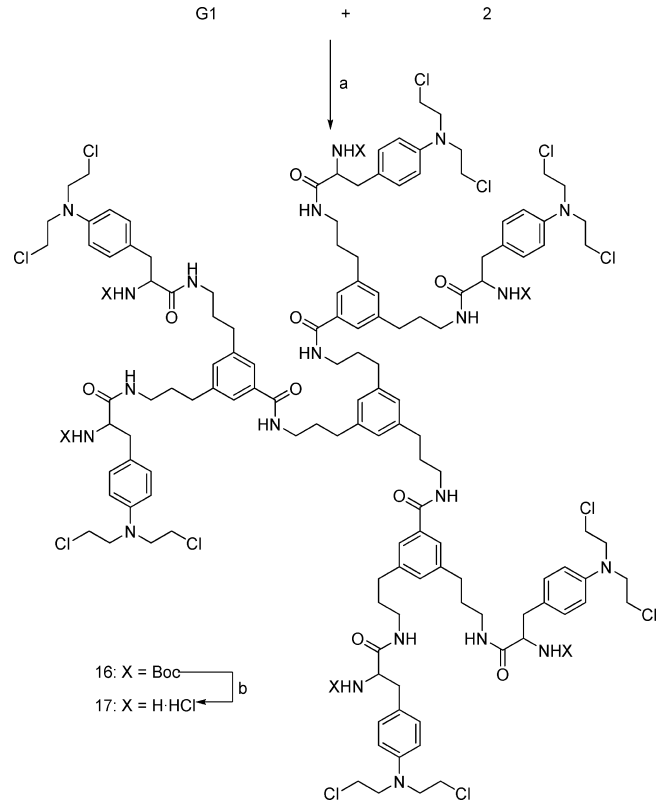
[a] Reagents and conditions: (a) chloroform, DIPEA, reflux, 70 h; (b) aqueous HCl, THF, RT.

Scheme 5. Synthesis of the Drug−G0 Conjugates[a]



[a] Reagents and conditions: (a) CH_2Cl_2, DMF, −20°C, TBTU/30 min, **G0**, DIPEA, methanol, RT, 20 h; (b) TFA (95%), RT, 4 h; (c) CH_2Cl_2, DMF, −20°C, TBTU/30 min, **G0**, DIPEA, methanol, RT, 20 h.

Scheme 6. Synthesis of the Mel−G1 Conjugates[a]



[a] Reagents and conditions: (a) EDC, HOBt, CH_2Cl_2, −20°C/1 h, RT/2 h, **G1**, DIPEA, −30°C/1 h, RT/16 h; (b) aqueous HCl, THF, RT, 3 h.

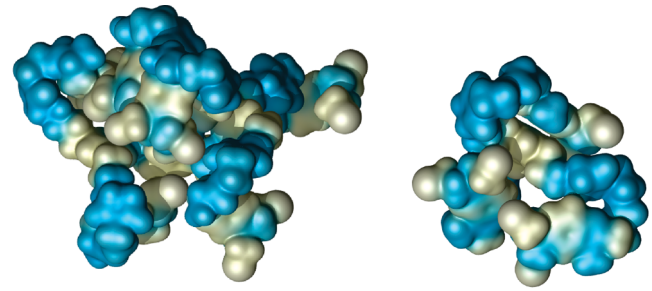


Figure 2. Representative examples of minimized 3D conformations for compounds **12** (left, extent: 38.3 Å) and **10** (right, extent: 30.6 Å). Both molecules are shown as their Van-der-Waals surfaces colored by hydrophobicity (blue: hydrophilic, gray: hydrophobic).

MB-231 breast cancer cell lines. From the graphs, antiproliferative and cytostatic effects (80% > T/C_{corr} > 0%), as well as cytocidal effects ($\tau = T/C_{corr} < 0\%$), can be deduced.

Cisplatin (DDP) was used as positive control. It influenced the cell growth in a concentration-dependent manner and caused almost 100% growth inhibition at both cell lines at a concentration of 5 μM. The two basic dendrimers **G0** and **G1** were previously tested at concentrations of 1, 5, and 10 μM (*17, 30*). **G0** was completely inactive, while the **G1** dendrimer marginally reduced cell proliferation at 10 μM ($T/C_{corr} = 60\%$).

It should be mentioned that the cytotoxic properties of the tested compounds were similar at the MCF-7 and MDA-MB-231 cell lines (except where noted; MDA-MB-231 graphs, see Supporting Information).

Melphalan hydrochloride **1** exhibited cytotoxic properties at 10 μM (Figure 3A). Boc protection led to the inactive compound **2** (data not shown) whose activity increased by esterification with the maleimide **3**. The resulting compound **4** showed cytocidal characteristics at concentrations of 5 and 10 μM after an incubation time of 48 h (Figure 3B).

Cleavage of the Boc group increased the cytotoxicity further. The maleimide ester **5** was cytostatic at the lowest concentration used ($T/C_{corr} \approx 0\%$; conc. 1 μM), however, not before the end of the test (incubation time 144 h, see Figure 3C). The comparison of the time response curves in Figure 3A and C clearly documented an ∼10-fold higher activity of **5** compared to that of Melphalan **1**.

Binding of **4** to **G0** (→ **8**) decreased the activity at the MCF-7 cell line (compare Figures 3B and 4A) but marginally enhanced the antiproliferative effects at the MDA-MB-231 compared to the MCF-7 cell line. The activity of the **G0**-bound compound **5** (→ **9**) remained nearly unchanged (compare Figures 3C and 4B). Only at 1 μM was a somewhat lower growth inhibition observed.

The increased generation of the dendrimer reduced the cytotoxicity of the Boc-protected compound further. Compound **11** showed only weak activity ($T/C_{corr} \approx 40\%$) even at the highest concentration used (10 μM). In contrast, the activity of **5**, **9**, and **12** is nearly independent of the presence of a dendrimer and its generation (compare Figures 3C, 4B, and 5B).

The same trend was determined for bendamustine (*6*), its maleimide derivative (*7*), and its **G0** conjugate (*10*). Bendamustine was inactive at the MCF-7 and the MDA-MB-231 cell lines (Figure 6A and Supporting Information). Esterification with the maleimide **3** (→ **7**, Figure 6B) increased the antiproliferative effects (cytostatic effects ($T/C_{corr} = 0\%$) at 5 μM and cytocidal effects ($T/C_{corr} < 0\%$) at 10 μM). Binding to **G0** (→ **10**) reduced the activity slightly (compare Figures 6B and 7A).

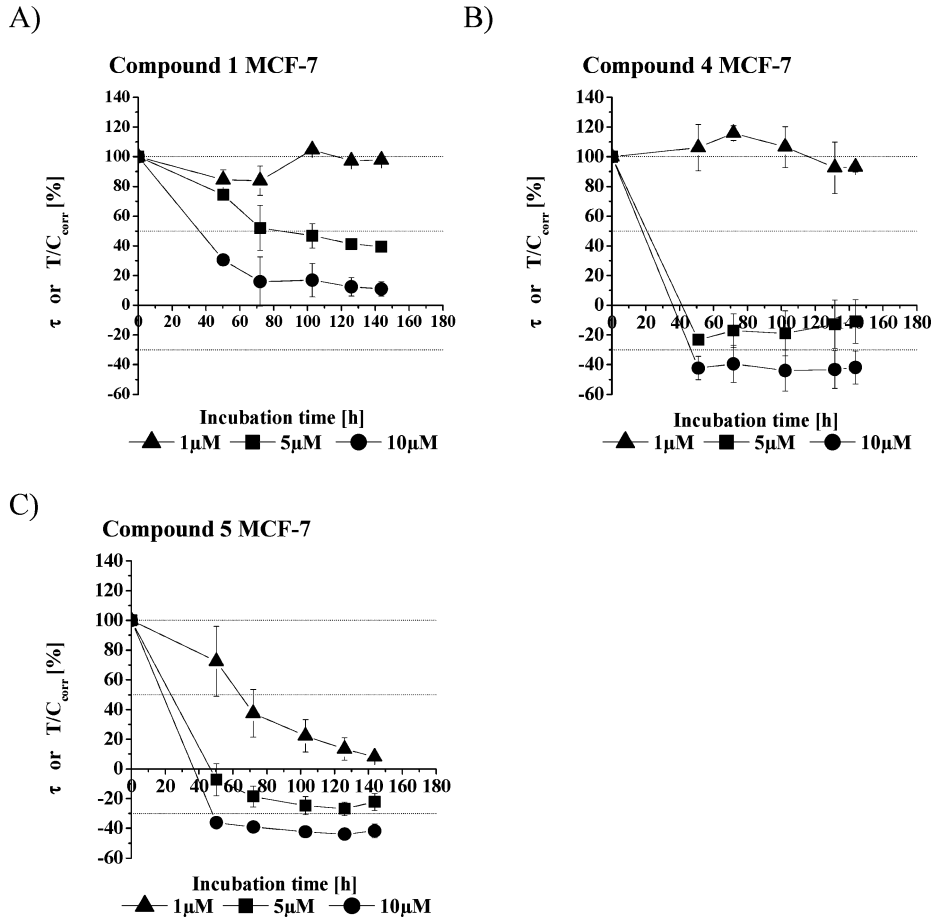


Figure 3. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) Mel, (B) MelBoc-spacer **4**, and (C) Mel-spacer **5**. For detailed information on the investigation of the T/C_{corr} [%] and τ[%] values, see Experimental Section.

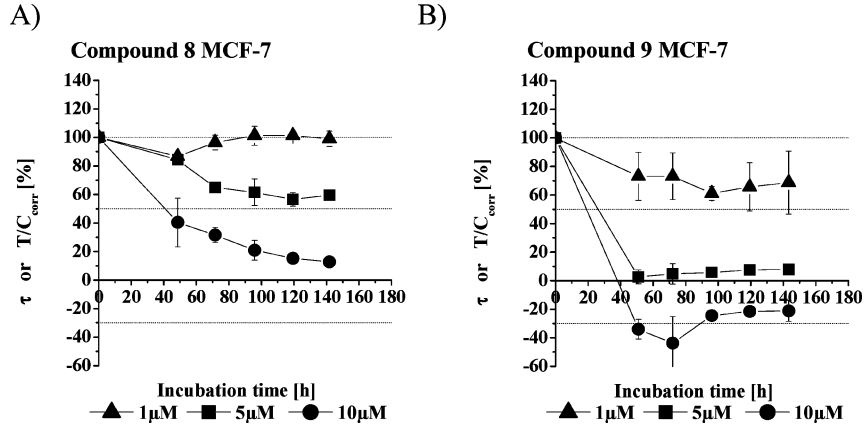


Figure 4. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) MelBoc-spacer-**G0 8**, (B) Mel-spacer-**G0 9**.

In order to estimate the significance of the maleimide spacer for the cytotoxicity of bendamustine, the drugs were directly bound to the dendrimers via an amide bond. This binding led to a complete inactive compound (→ **15**, see Figure 7B). This means that in vitro cytotoxicity of bendamustine needs binding to a maleimide spacer.

In contrast to this, the amide binding of melphalan to **G0** only slightly reduced the activity. The MelBoc-spacer-**G0** derivative **8** reached the $T/C_{corr} = 50\%$ at 5 μM, its congener without spacer **13** (MelBoc-G0) at 10 μM. The cleavage of the Boc groups resulted in the compounds **9** and **14**, which showed similar antiproliferative effects at the low concentration of 1 μM. At 5 and 10 μM, **14** distinctly reached cytocidal effects (see Figure 8B).

If dendrimers with higher generation were used, the effects were independent of the presence of a maleimide spacer (compare Figure 5 with Figure 9).

Investigations into the Hydrolysis of Bendamustine and Melphalan. Bendamustine belongs to the class of alkylating agents of the N-Lost type. Binding to the DNA occurs by attachment to guanine bases. In aqueous solution, the drug underwent hydrolysis into the hydroxyl (**HP1**, Figure 10) or dihydroxyl species (**HP2**, Figure 10). This aquation is accompanied by an inactivation of the drug.

In order to investigate the stability of bendamustine in aqueous solution, it was incubated in water for 24 h at 37 °C and the degradation was followed by HPLC analysis (RP 18; methanol/H_2SO_4 (0.001 N, Na_2SO_4 (20 mM)) and UV-detection

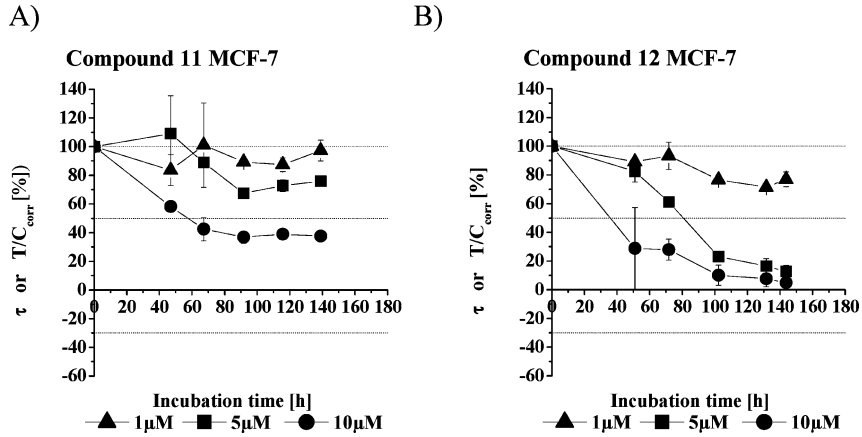


Figure 5. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) MelBoc-spacer-**G1 11**, (B) Mel-spacer-**G1 12**.

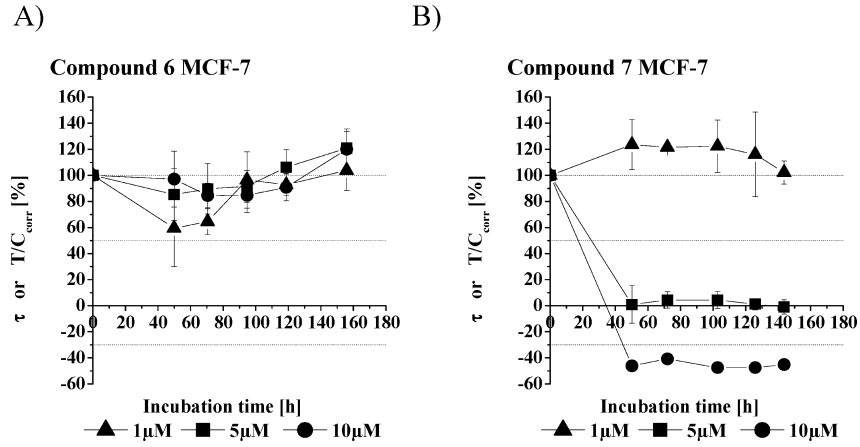


Figure 6. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) Benda **6**; (B) Benda-spacer **7**.

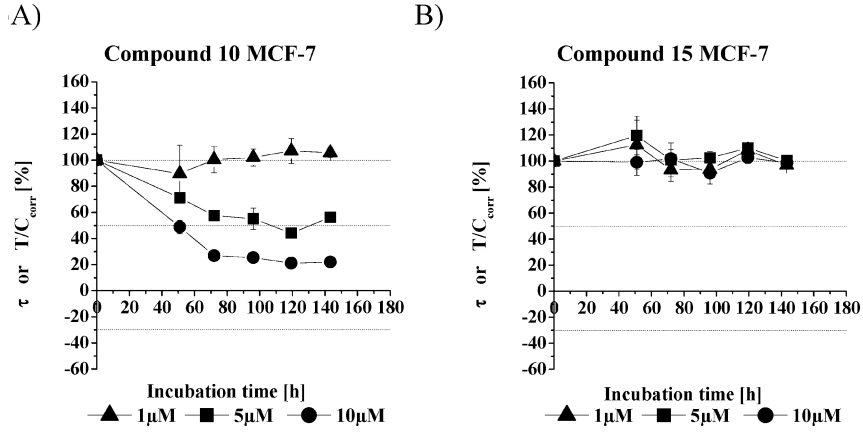


Figure 7. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) Benda-spacer-**G0 10**; (B) Benda-**G0 15**.

(254 nm). During the first 2 h, 55.6% of bendamustine hydrolyzed to **HP1** (36.8%) and **HP2** (18.8%). After 8 h, amounts of 81.6% of **HP2**, 14.7% of **HP1**, and only 3.7% of bendamustine were detected. Incubation of 24 h led to a complete hydrolysis into **HP2**.

Melphalan was distinctly less stable. An amount of 88.1% hydrolyzed after 2 h to the monohydroxyl (24.7%) and dihydroxyl (63.5%) species, while after 8 h, only the dihydroxyl species was present.

The bendamustine-maleimide derivative **7** was tested under the same conditions. A representative of a chromatogram is depicted in Figure 11.

The maleimide residue mediated **7** high hydrophobicity resulting in an increased retention time of RT = 57.3 min (bendamustine RT = 25.7 min). It is visible that two reactions took place: (1) cleavage of the ester bond in **7** resulting in bendamustine **6** and the maleimide **3**; (2) hydrolysis of **7** to the hydroxyl and dihydroxyl compounds **7-OH** and **7-diOH**. Bendamustine itself hydrolyzed to **HP1** and **HP2** (see Figure 10).

After 8 h, 14% (sum of **6**, **HP1**, and **HP2**) of bendamustine was set free. Aquation products of **7** were detected at RT = 14.6 min (**7-OH**; 38%) and RT = 5.28 min (**7-diOH**; 7.5%). Unchanged **7** amounted to 39.5%. It is obvious that the esterification of

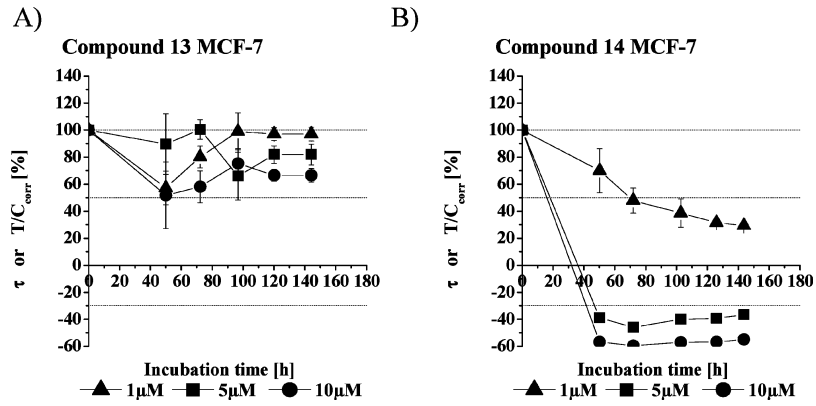


Figure 8. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) MelBoc-**G0 13**, (B) Mel-**G0 14**.

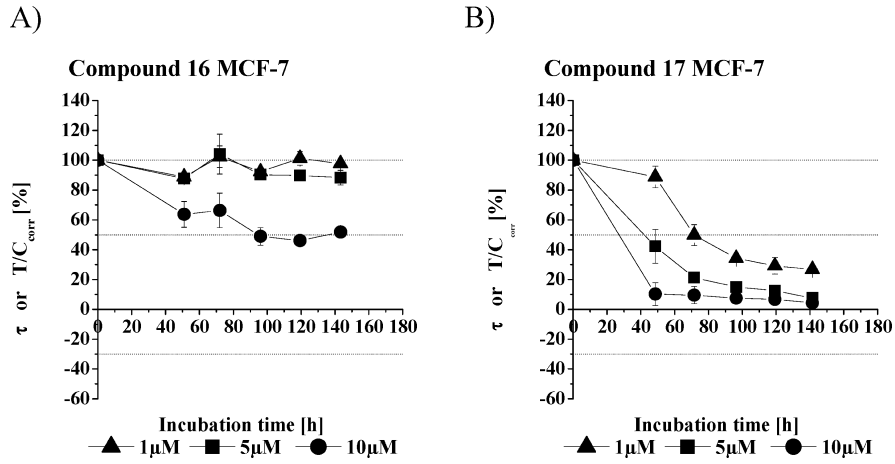


Figure 9. In vitro cytotoxicity ($\tau = T/C_{corr} < 0\%$): (A) MelBoc-**G1 16**, (B) Mel-**G1 17**.

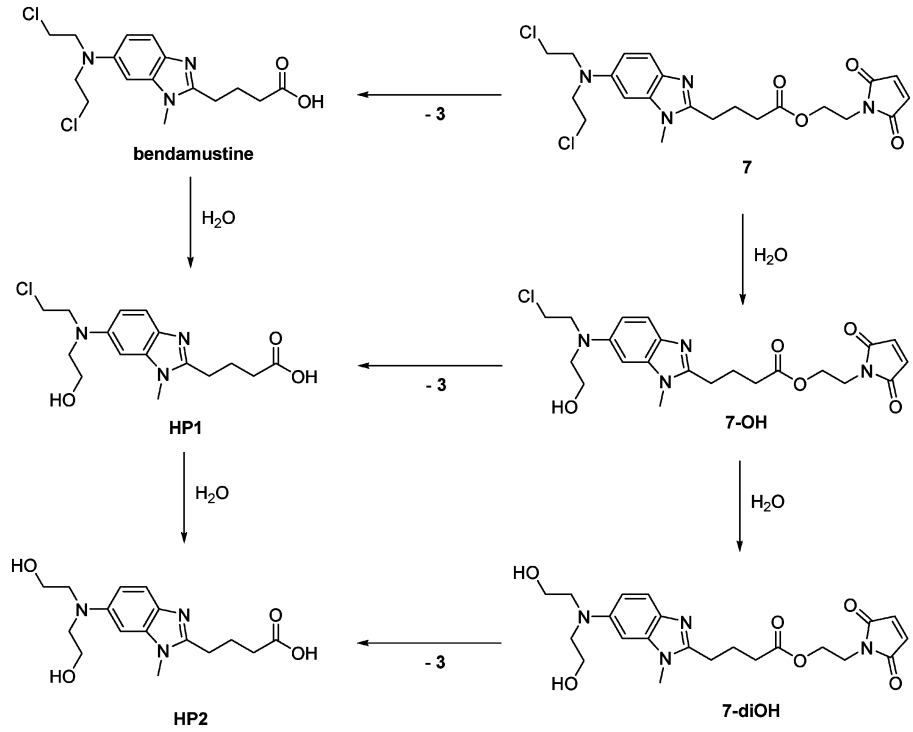


Figure 10. Hydrolysis of **7** at 37 °C in water.

bendamustine increased the stability in aqueous solution, but bendamustine was released over 36 h and held a level up to 7%. **HP1** and **HP2** are not only the hydrolysis products of bendamustine, but also the degradation products of **7-OH** and **7-diOH**.

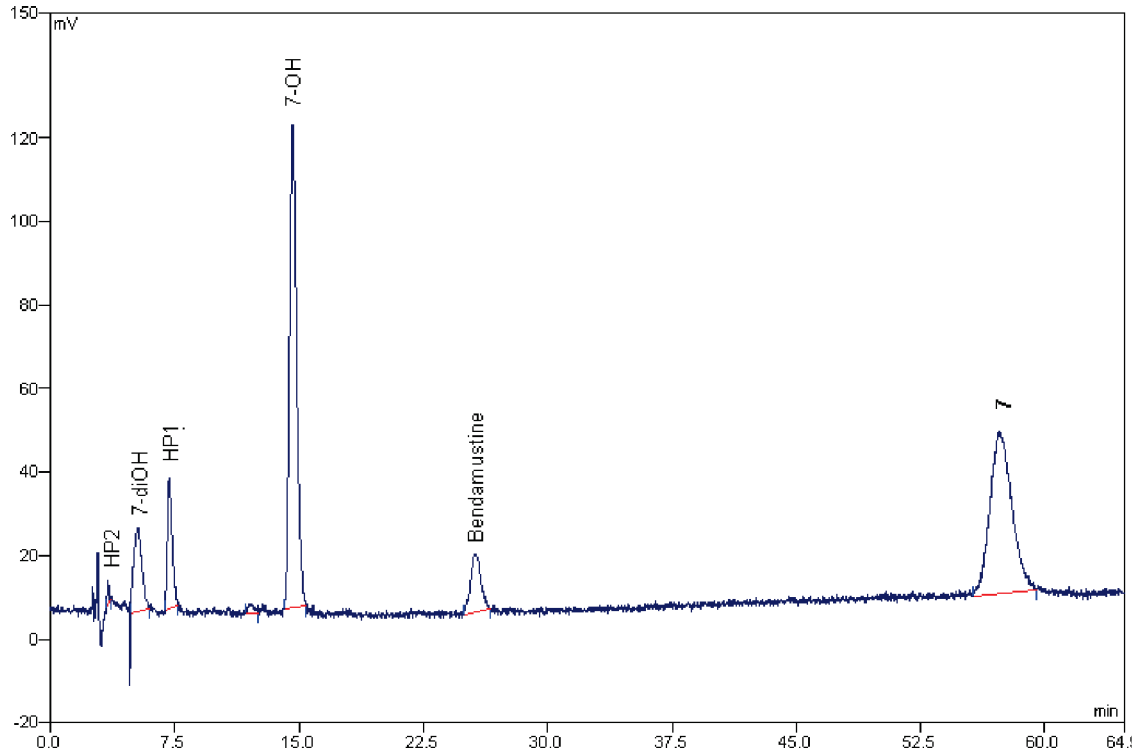


Figure 11. Chromatogram of **7** after incubation in water at 37 °C for 8 h.

The same modification done with melphalan did not increase the stability. The maleimide derivative **5** degraded ($t_{1/2}$ = 37 min) comparable to melphalan ($t_{1/2}$ = 30 min). It should be mentioned that **5** showed the same degradation profile as **7**.

In order to model blood circulation (buffer pH 7.4) and intracellular/endosomal environment (buffer pH 5), the same hydrolysis experiments were performed with bendamustine and **7** in PBS at relevant pH.

Bendamustine showed pH-sensitive degradation. It was completely hydrolyzed to **HP2** during 2 h at pH 7.4, while at pH 5, after 4 h only 50% and after 24 h 92% of bendamustine degraded. Compound **7** showed distinctly increased stability. The half-life in PBS was 8.5 h at pH 5 and 10 h at pH 7.4.

The hydrolysis rates at pH 5 and pH 7.4 were not as different as observed for bendamustine. The half-lives was 120 and 98 min, respectively. Interestingly, after derivation to **5**, a complete degradation (100%) occurred at pH 7.4 and a slightly increased stability at pH 5 ($t_{1/2}$ = 154 min).

HSA solution (40 mg/mL) was used to study protein binding. Ethanolic precipitation indicated protein binding of bendamustine. After incubation for 2 h, 50% were protein-bound and 6% hydrolyzed to **HP1** and **HP2**, respectively. In contrast, during the same incubation time only 25% of **7** were HSA-bound and 18% hydrolyzed to **7-OH**.

Melphalan was strongly HSA-bound. After 2 h, 14.3% of hydroxyl derivatives and 18.9% of melphalan could be detected. The amount of 66.8% was bound to the protein. A still faster interaction with HSA was found for **5**. No drug was identified in the chromatograms after 30 min.

DISCUSSION

The preliminary in vitro tests on MCF-7 and MDA-MB-231 breast cancer cells confirmed the existing literature data about the antitumor activity of melphalan hydrochloride (*1*). It reduced the concentration-dependent growth of MCF-7 and MDA-MB-231 breast cancer cells. At 10 μM, cytostatic effects ($T/C_{corr} \approx$ 0%) were determined (Figure 3), which depended on the presence of a free amino group. The Boc-protected melphalan derivative **2** was inactive. Interestingly, the esterification with *N*-(2-hydroxyethyl)maleimide **3** strongly increased the activity. The melphalan-Boc-protected spacer analogue **4** was proven to exhibit cytocidal effects on both cell lines. As expected, the deprotection leading to compound **5** increased the influence on the cells. Already, at a concentration of 1 μM cytostatic effects were achieved.

A possible explanation could be the binding of the spacer derivative to human serum albumin (HSA) present in the cell culture media. In some previous work, Kratz et al. synthesized such conjugates of the nitrogen mustard chlorambucil (*14*). They also demonstrated an HSA binding of the spacer derivative in situ after iv administration. Due to the considerable uptake of the macromolecule-bound drug in tumor cells, this concept has a promising future as a drug carrier system (*31*).

This mode of action can principally be realized for maleimide derivatives **4**, **5**, and **7**. HSA binding was confirmed on the examples of **5** and **7**. Fast binding also occurred with bendamustine and melphalan. After 2 h of incubation, more than 50% were bound.

Besides HSA or transferrin as carrier molecules (*32*−*34*), some synthetic polymers were designed to bind drugs for selective accumulation in tumors and tumor cells (*35, 36*). In previous studies (*17*), we used the 1,3,5-tris(3-aminopropyl)benzene (**G0**) and its **G1** analogue 3,5-bis(3-aminopropyl)-*N*-(3-{3,5-bis[3-{3,5-bis(3-aminopropyl)benzoylamino}-propyl]phenyl}propyl)benzamide for the transport of drugs into MCF-7 and MDA-MB-231 cells. The concept was very successful in the case of platinum(II) complexes (*30*).

Already, in the 1990s covalent conjugation of melphalan to macromolecular carriers was described (*37*). It was, e.g., linked to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers via enzymatically cleavable peptide side chains. In 2008, Shukla et al. published poly(ethylene glycol) conjugates of melphalan demonstrating enhanced dose-dependent in vitro cytotoxicity (*38*).

Therefore, we decided to bind melphalan to **G0** or **G1** dendrimers via the maleimide spacer (**8**, **9** and **11**, **12**) or directly

via an amide bond (**13**, **14** and **16**, **17**) to evaluate their cytotoxic potency in vitro. The BOC-protected conjugates **8**, **11**, **13**, and **16** were only marginally active at MCF-7 and MDA-MB-231 cells. Only the drug-spacer-**G0** conjugate **8** showed low cytostatic properties at the highest concentration. The **G1**-conjugates with free NH_2 groups (**12** and **17**) only slightly differ in their activity. They showed cytostatic properties at 5 and 10 μM.

These results indicated a relatively high stability of the conjugates. We assume that melphalan interacted after transport into the tumor cells in free form and also dendrimer-bound with the DNA via the N-lost group. The resulting cross-links led to cell death. Bendamustine belongs to the same class of antitumor agents. However, its cytotoxic behavior in free form, maleimide bound, or as dendrimer conjugate differs from that of melphalan.

Melphalan and bendamustine are sensitive to hydrolysis in water and were degraded at 37 °C to the bis(hydroxyethyl)amino compounds over 24 h. The results of Chang et al. demonstrated for melphalan a retarded hydrolysis in the presence of bovine serum albumin or human plasma proteins (*39*). In a Roswell Park Memorial Institute Medium containing 10% fetal bovine serum, the half-life of melphalan at 37 °C was 1.13 ± 0.10 h and was only slightly higher compared to that in pure water (*40*). In our experiments, besides strong HSA binding a reduced amount of hydrolysis was detected, too.

Bendamustine showed a half-life of about 100 min in pure water and increased stability in physiological NaCl solution (*41*). This correlates very well with the hydrolysis of melphalan (*39*). However, the attempt failed to stabilize the aqueous solution of bendamustine by HSA. A drug amount of 50% was HSA-bound during an incubation time of 2 h and an amount of 12% was hydrolyzed. In PBS buffer at pH = 7.4, fast hydrolysis took place. After 2 h, only **HP2** was present. Therefore, we assume that under cell culture conditions not enough active drug was present to reduce the growth of MCF-7 and MDA-MB-231 cells.

The mode of action of bendamustine is discussed in very contradictory terms. However, it is still accepted that DNA-alkylation is the main cause of cell death in vivo. Preclinical data demonstrate that bendamustine induces rapid, sustained single- and double-strand DNA damage, which results in apoptosis, or programmed cell death, in the tumor. It also induces mitotic checkpoint inhibition, which results in nonapoptotic cell death (*42*−*44*).

Such a mode of action requires an intact N-lost moiety. The dihydroxybendamustine derivative **HP2** is inactive in cell culture experiments. The participation of the partially hydrolyzed **HP1** to the antiproliferative effects is still unknown. This assumption is confirmed by the fact that in in vitro assays bendamustine is inactive (*45*) (see also Figure 6A). If it were possible to prevent hydrolysis in aqueous solution, antiproliferative properties should be observed. The stability is enhanced in alcoholic solution (unpublished results) which is not usable in in vitro conditions. However, in this SAR study it was at first demonstrated that esterification of the carboxylic group increased the water stability.

The maleimide moiety of compound **7** is attached to bendamustine by a hydroxyethyl chain. The resulting ester was incubated at 37 °C in water and the degradation was followed by HPLC.

After 8 h of incubation, degradation to bendamustine followed by hydrolysis to **HP1** was observed. Furthermore, besides intact **7**, its monohydroxyl derivative **7-OH** was determined, too. Interestingly, the stability of **7** in PBS was higher than that of bendamustine and independent of the pH. The half-life of **7** at pH 7.4 was 10 h, and guaranteed higher amounts of active compounds resulting in increased antiproliferative effects in cell culture experiments.

The binding of **7** to **G0** through the maleimide spacer slightly reduced the activity from cytocidal to cytostatic. In contrast, the direct binding of bendamustine to **G0** via an amide bond terminated the cytotoxic effects. The higher stability of the amide bond prevents a release of bendamustine from the molecule. Therefore, it is very likely that it was not **10** itself causing cell death but the hydrolytically cleaved bendamustine **6**. This contrasts with the results obtained with melphalan, which was active in free as well as dendrimer bound via the spacer and the amide bond.

Our concept to increase the activity of melphalan and bendamustine by an adequate carrier (dendrimer) was successful. Through the binding to the dendrimer via spacer, not only can it be assumed that the new bendamustine derivatives accumulated in the tumor cells probably through endocytosis, but it can be also presumed that the hydrolysis of bendamustine is suppressed.

The investigations pointed, on one hand, to a participation of the butyric acid in the hydrolytic processes; on the other hand, Pencheva et al. achieved for bendamustine immobilized onto polyphosphoester a higher stability by the formation of molecular associates (*46*). If this were true, the stability of bendamustine should increase using polymers such as poly(ethylene glycol)s. In our lab, however, we cannot find increased stability with PEG 400/water mixtures.

Hopwood and Stock demonstrated that macromolecules with lipophilic moieties are capable of diminishing the hydrolysis of drugs (*47*). Albumin proved to be the most efficient at protecting the anticancer agents. This may also be true for the bendamustine maleimide derivative **7**. However, the binding mode at HSA is yet unknown.

CONCLUSIONS

A new set of dendrimer and dendrimer−spacer conjugates of melphalan and bendamustine have been successfully synthesized. The antiproliferative activity of these compounds and their maleimide precursors has been determined against MCF-7 and MDA-MB-231 cell lines. No selectivity for one of the cell lines was detected. The cytotoxicity of melphalan depended on a free amino group and was independent of the kind of bond to the spacer or the dendrimer.

The antiproliferative activity of bendamustine was strongly increased due to reduced hydrolytic processes after esterification with the *N*-(2-hydroxyethyl)maleimide. An amide bond to 1,3,5-tris(3-aminopropyl)benzene terminated the activity. Therefore, it is very likely that the cell death promoting effects were caused after transport into the tumor cells and release of the intact drug from the core molecule **G0**.

In a following study, we will investigate the hydrolysis reactions and the release of bendamustine from dendrimers of higher generation. Furthermore, we will evaluate the binding mode at the carboxylic acid necessary for increased activity.

ACKNOWLEDGMENT

The presented study was supported by grants Gu285/4-1, Gu285/5-1, and Gu285/5-2 from the Deutsche Forschungsgemeinschaft.

Supporting Information Available: Elemental analyses of the new compounds, their results (cytotoxicity tests) on the MDA-MB-231 cell line, the cytotoxicity of **G0** and **G1** on the MCF-7 cell line, a histogram of the maximum extent distributions over the calculated three-dimensional conformational space for **10** and **12** and median, average and maximum values for molecule sizes of **10** and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC900453F