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Remarkable drug-release enhancement with an elimination-based AB_3 self-immolative dendritic amplifier

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Abstract—Self-immolative dendritic prodrugs, activated through a single catalytic reaction by a specific enzyme, could offer significant advantages in inhibition of tumor growth relative to monomeric prodrug, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. We have designed and synthesized new AB_3 self-immolative dendritic prodrug system that releases three active drugs by a single cleavage of the enzyme penicillin-G-amidase. The cleavage signal is transferred from the dendron focal point to its periphery through fast elimination reactions and the design leads to three-fold signal amplification. In cell-growth inhibition assays, the elimination-based AB_3 self-immolative dendritic prodrug was significantly more effective than a cyclization-based AB_3 dendritic prodrug.

1. Introduction

Self-immolative dendrimers are novel class of molecules that can amplify a single cleavage event received at the focal point into multiple release of tail groups at the periphery.[1–3] These dendrimers can be used for the construction of dendritic prodrugs, if a specific trigger is attached to the focal point and drug molecules are linked to the periphery.[4,5] Self-immolative dendritic prodrugs, activated through a single catalytic reaction by a specific enzyme, could offer significant advantages in inhibition of tumor growth relative to monomeric prodrug, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. We have shown that single-triggered dendritic prodrugs significantly inhibit tumoral cell growth compared to classic monomeric prodrugs.[6] The dendritic platform was also used for the synthesis of a single-triggered hetero-dimeric prodrug derivatized with the anticancer agents doxorubicin and camptothecin.[7] This prodrug made it possible to release two different chemotherapeutic drugs simultaneously at the same location. In another report, we designed and synthesized fully biodegradable dendrimers that disassemble through multi-enzymatic triggering followed by self-immolative chain fragmentation.[8] A practical application for such multi-triggered self-immolative dendrons was recently demonstrated by the concept of prodrug activation through a molecular OR logic trigger.[9,10]

Rapid release of tail-drug units from the dendritic platform is essential in order to achieve maximal drug concentration at a specific location. Therefore, self-immolative dendrons with a fast disassembly mechanism should have a significant advantage in a dendritic prodrug system.[11] Here we report the design and synthesis of a fast AB_3 self-immolative dendritic prodrug system, activated through a single enzymatic cleavage by penicillin-G-amidase (PGA).[12]

2. Results and discussion

Recently, we reported the synthesis and activation of dendritic prodrug similar to **1**.[6] This prodrug releases three molecules of active drug upon single cleavage by

Abbreviations: ACN, acetonitrile; DCM, dichloromethane; DIPEA, diisopropylethyleneamine; DMF, dimethyl formamide; DMAP, dimethylaminopyridine; EtOAc, ethylacetate; Et_3N, triethylamine; Hex, hexane; MeOH, methanol; Mel, melphalan; PNP, *p*-nitrophenol; PNA, *p*-nitroaniline; TBSCl, *tert*-butyldimethylsillyl chloride; THF, tetrahydrofuran; Trp, tryptophan.

Keywords: Prodrug; Dendrimer; Enzyme; Self-immolative.

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PGA (Fig. 1). The disassembly pathway is initiated by removal of phenylacetic acid, elimination of azaquinone-methide, and decarboxylation to generate amine-intermediate **1a**. The latter cyclizes to release dimethylurea derivative and phenol **1c**, which rapidly undergoes triple elimination to release the three drug units. The rate-determining step was found to be the cyclization of **1a**–**1c**.

In order to enhance the release-rate of the peripheral drug units, we sought to design a system that would disassemble without the slow cyclization step. Taking into consideration this objective, we designed AB_3-dendritic molecule **2**. The first step in the reaction is catalytic cleavage of phenylacetic acid by PGA,[13] followed by elimination of azaquinone-methide and decarboxylation to release amine-intermediate **2a**. This amine intermediate further disassembles through triple elimination to release the three drug units (Fig. 2). The disassembly of this molecule after the enzymatic cleavage is solely based on elimination reactions and therefore is expected to occur very rapidly.

Although molecule like **2a** was reported before,[2] there is no published procedure for its synthesis. We developed a simple and efficient new synthetic pathway for dendritic compounds like **2**, reported here. To compare the disassembly rate of the above dendritic systems, we synthesized AB_3 molecules **3** and **4** (Fig. 3). Both molecules are designed for activation by PGA and have three units of tryptophan as a model drug. Tryptophan was used for initial evaluation as it contains a strong UV chromophore, allowing us to monitor the disassembly reaction.

The synthesis of compounds **3** and **4** was performed as presented in Figures 4 and 5. Compound **5** was

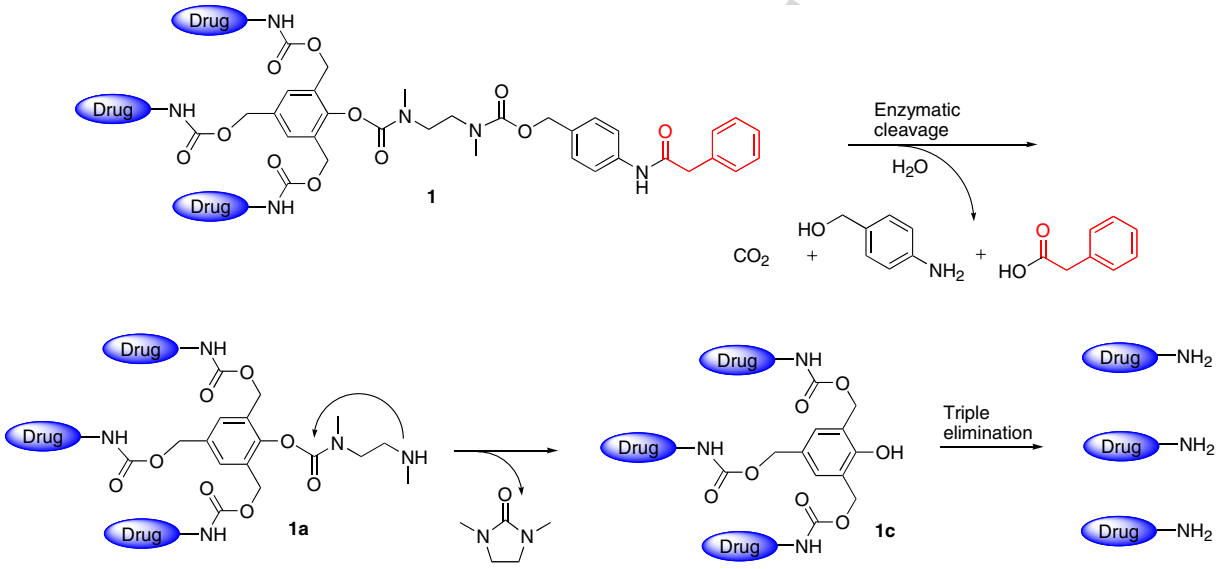

Figure 1. Disassembly mechanism of AB_3 self-immolative dendritic molecule **1**.

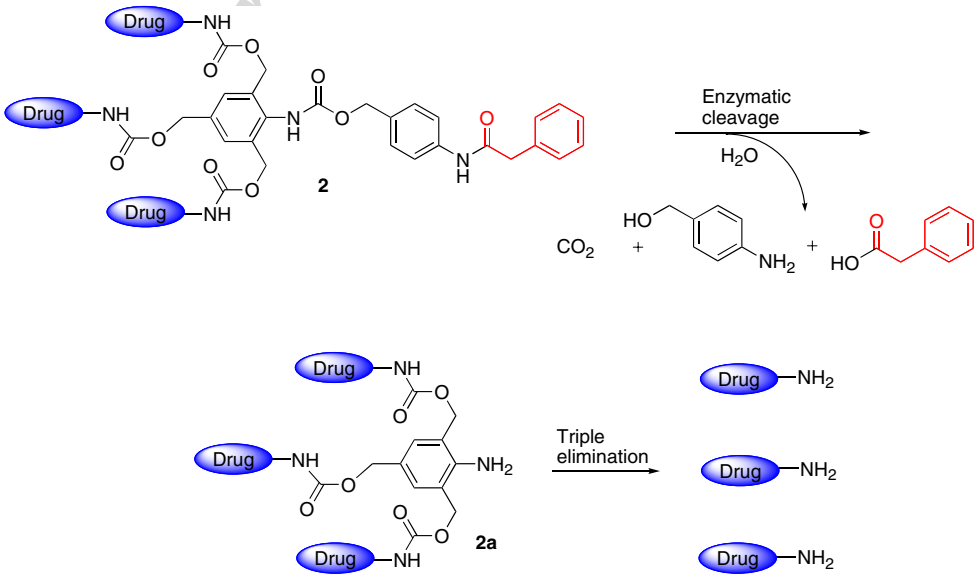

Figure 2. Disassembly mechanism of AB_3 self-immolative dendritic molecule **2**.

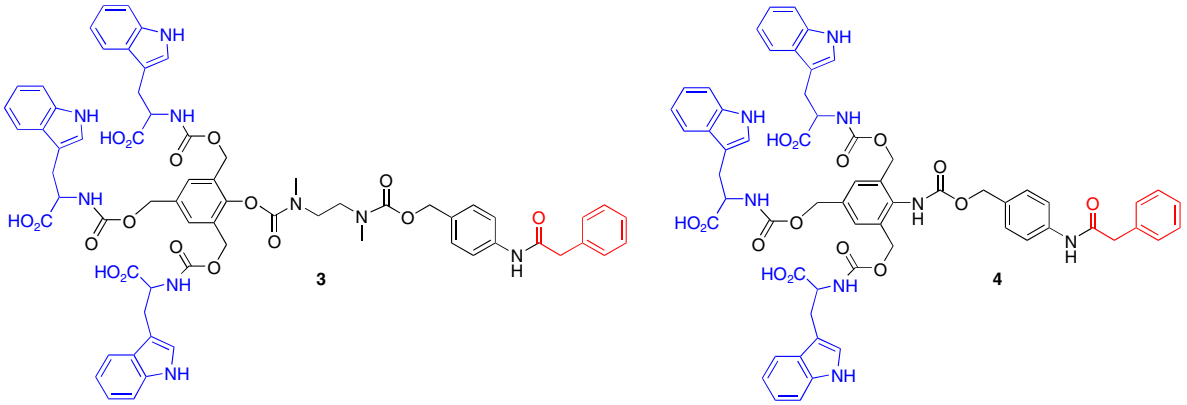

Figure 3. Chemical structures of AB_3 self-immolative dendritic molecules with tryptophan tail units and a trigger that is activated by PGA.

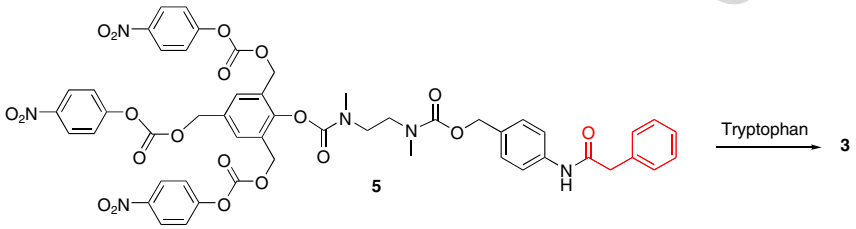

Figure 4. Chemical synthesis of dendritic molecule **3**.

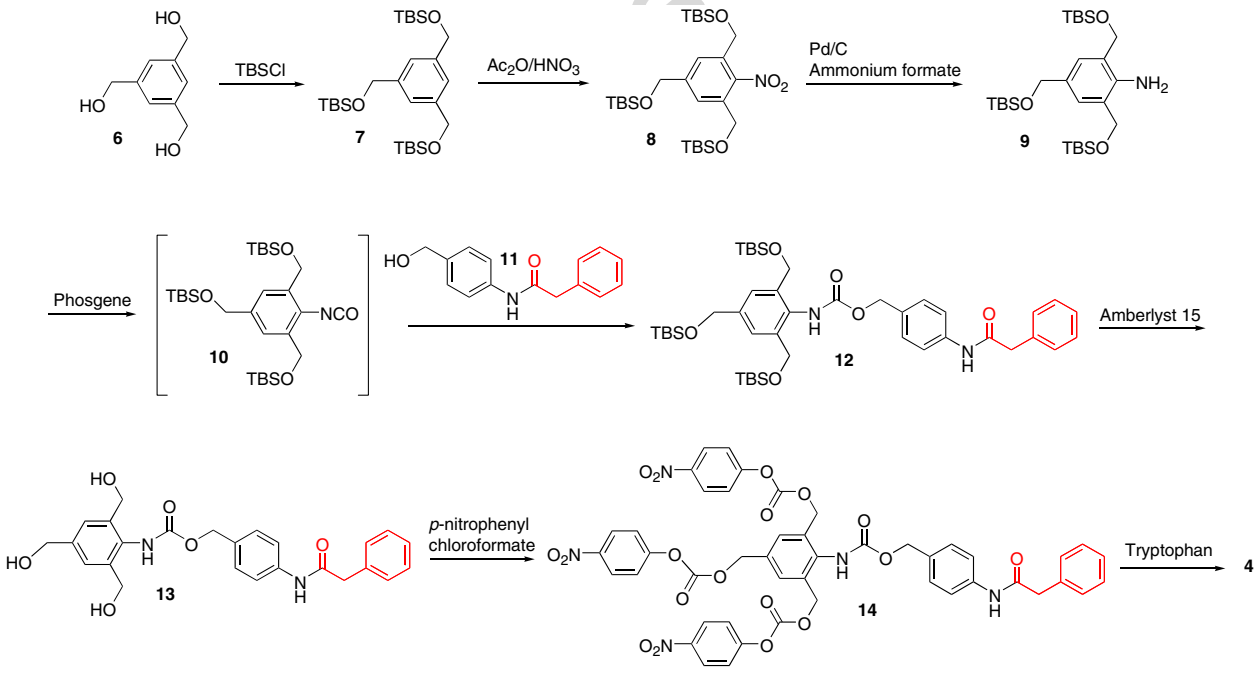

Figure 5. Chemical synthesis of dendritic molecule **4**.

synthesized as previously described.[6,14] Three equivalents of tryptophan was reacted with tricarbonate **5** to generate dendritic molecule **3** (Fig. 4).

Dendritic molecule **4** was prepared starting from tri(hydroxymethyl)-1,3,5-benzene **6**. Thus, triple protection with *tert*-butyldimethylslyl chloride of the hydroxy groups of **6** gave compound **7** and reaction with nitric acid and acetic anhydride afforded compound **8**. The latter was reduced with palladium on carbon and ammonium formate to give amine **9**. Reaction of phosgene with **9** in situ generated isocyanate **10**, which was immediately reacted with alcohol **11** to give compound **12**. Deprotection of the *tert*-butyldimethylslyl groups of **12** with amberlyst-15 afforded triol **13**, which was activated with 4-nitrophenyl-chloroformate to give tricarbonate

14. The latter was reacted with three equivalents of tryptophan to generate AB_3 dendritic molecule **4**.

The disassembly reactions of dendritic molecules **3** and **4** were evaluated in phosphate-buffered saline (PBS, pH 7.4) in presence and absence of PGA. The release of tryptophan was monitored by a reverse-phase HPLC at a wavelength of 320 nm. The results are presented in Figures 6 and 7. No disassembly of either system was observed in the buffer without PGA (data not shown). In the presence of PGA, dendritic molecule **3** disassembled to release tryptophan within approximately four days (Fig. 6), whereas dendritic molecule **4** released its tryptophan tail-units within 40 min (Fig. 7).

Under the experiment conditions the enzymatic cleavage occurs within seconds. Therefore, the observed release time of the tryptophan is also the actual disappearance time of the intermediate forms after the enzymatic cleavage. This dramatic enhancement of tail-unit release with the elimination-based system (dendritic molecule **4**) compared to the cyclization-based system (dendritic molecule **3**) is best viewed by superimposition of the graphs (Fig. 8).

We decided to apply the elimination-based dendritic system to the synthesis of an anticancer prodrug and to evaluate it in a tumor cell cytotoxicity assay. Dendritic prodrugs **15** and **16** were synthesized with the chemotherapeutic drug melphalan as a tail unit and a trigger that is activated by PGA (Fig. 9). Three equivalents of melphalan was coupled with tricarbonates **5** and **14** to afford AB_3 self-immolative dendritic prodrugs **15** and **16**, respectively.

In order to evaluate the in vitro antitumor activity of the prodrugs, compounds **15** and **16** were incubated at varied concentrations with human T-lineage acute lymphoblastic leukemia MOLT-3 cells in the presence or absence of 1 μM PGA. The data from the cell proliferation assays are presented in Figure 10. A colorometric assay based on the tetrazolium salt XTT was used to evaluate the cytotoxicity of the compounds.

Melphalan prodrugs **15** and **16** exhibited significantly (about 100-fold) reduced toxicity than free melphalan in the absence of PGA. XTT cytotoxicity assays showed a decrease of 17-fold in IC_{50} for prodrug **15** (100 vs 6 μM with PGA) and 200-fold for prodrug **16** (100 vs 0.5 μM with PGA). In the presence of PGA, prodrug **15** showed some increased cytotoxicity but still notably less than that of free melphalan (6 and 0.3 μM, respectively). However, when prodrug **16** was activated by PGA, the cytotoxicity was almost identical to that of free melphalan (IC_{50} values of free melphalan and prodrug **16** in the presence of PGA were 0.3 and 0.5 μM,

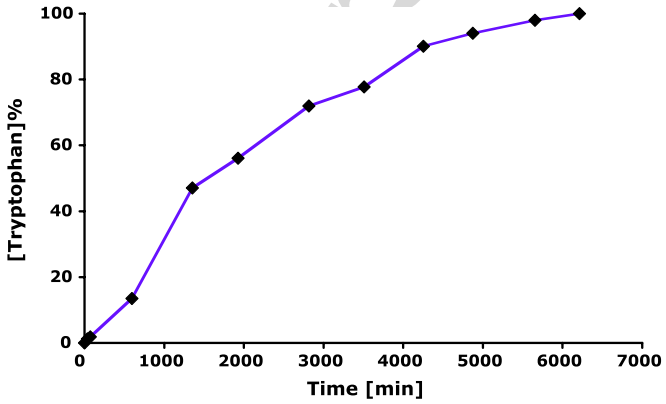

Figure 6. PGA-catalyzed release of tryptophan from dendritic compound **3** (compound **3** [500 μM] in PBS, PGA [1 mg/mL]).

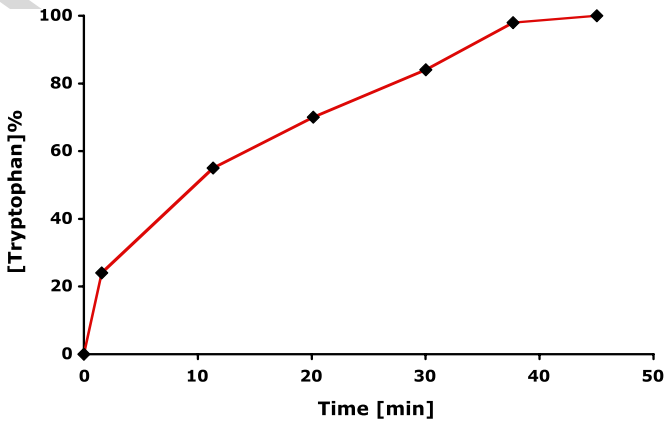

Figure 7. PGA-catalyzed release of tryptophan from dendritic compound **4** (compound **4** [500 μM] in PBS, PGA [1 mg/mL]).

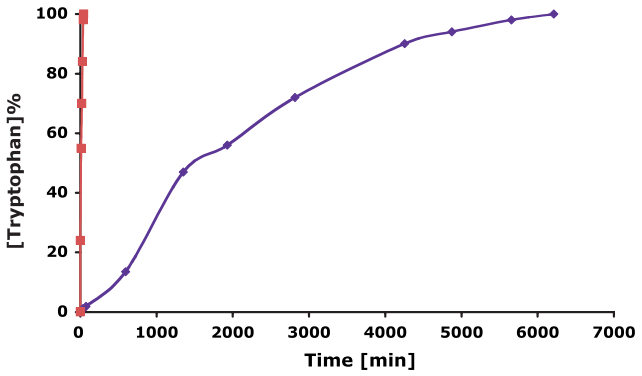

Figure 8. PGA-catalyzed release of tryptophan from dendritic compound **3** (purple) with $t_{1/2}$ of 1400 min versus release from dendritic compound **4** (red) with $t_{1/2}$ of 10 min.

respectively). Interestingly, in the absence of PGA both prodrugs were relatively not toxic even at very high doses of 100 μM. No toxicity was observed for the platform building blocks within the activity range of melphalan or melphalan prodrug.[6]

Site-specific drug delivery via a prodrug approach has generated considerable interest for enhancing the potency and for diminishing the side effects of a drug.[15] Rapid release of chemotherapeutic drugs from a prodrug system is important if a high concentration of active drug is needed at the tumor site. The described elimination-based AB_3 self-immolative dendritic prodrug system had a rapid activation pathway. Furthermore, a single cleavage by PGA was efficiently amplified to release three active drug molecules. The results shown in this study provide a proof of concept for this amplifying approach. Similar dendritic prodrugs with triggers that are activated by specific endogenous tumoral enzymes could be applied for selective chemotherapy. For example, legumain, a recently identified lysosomal protease, is a promising candidate target for prodrug therapy since it is overexpressed in the majority of human solid tumors.[16] Legumain promotes cell migration and its overexpression is associated with enhanced tissue invasion and metastasis. The enzyme cleaves the amide linkage of the tri-peptide Asn-Ala-Ala.[17] Linking this peptide, instead of the PGA substrate, to the elimination-based dendritic system described here will generate a promising self-immolative AB_3 prodrug that is suitable for site-specific drug delivery.

Additional potential application for these single-triggered dendritic systems could be in the amplification of signals in diagnostic assays. For example, chromogenic amines can be used as tail units. The amine is colorless when attached to the dendritic platform, but upon release a distinct new chromophore can be detected by spectrophotometry.[18] Introduction of phenylacetamide as a trigger will generate a molecular sensor for the enzyme PGA. A single cleavage by PGA will result in release of three reporter units and generation of a strong chromophore. There are several examples for reporter molecules with primary or secondary amine groups that can generate UV, UV–vis, or fluorescence signals.

3. Conclusions

In summary, we have designed and synthesized new AB3 self-immolative dendritic prodrug system that releases three active drugs upon a single cleavage by the model enzyme PGA. The cleavage signal is transferred from the dendron focal point to its periphery through fast elimination reactions and is amplified three-fold. The elimination-based AB_3 dendritic prodrug showed significant enhancement of drug release in comparison to a cyclization-based AB_3 dendritic prodrug. This difference was noticeably reflected in a cytotoxicity assay. Our new trimeric prodrug system could offer significant advantages in inhibition of tumor growth relative to regular monomeric prodrugs, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue.

4. Experimental

4.1. General

All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC) was performed on silica gel plates Merck 60 F_{254}; compounds

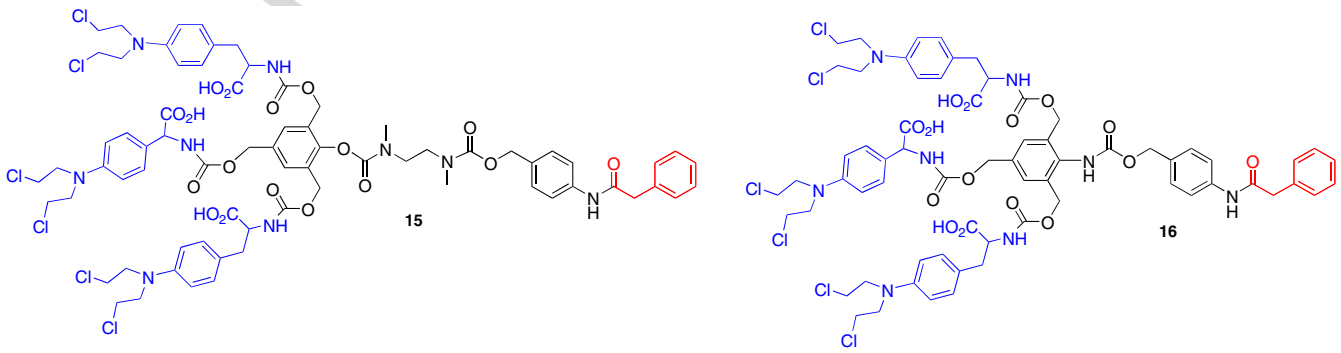

Figure 9. Chemical structures of AB3 self-immolative dendritic prodrugs with melphalan tail units and a trigger that is activated by PGA.

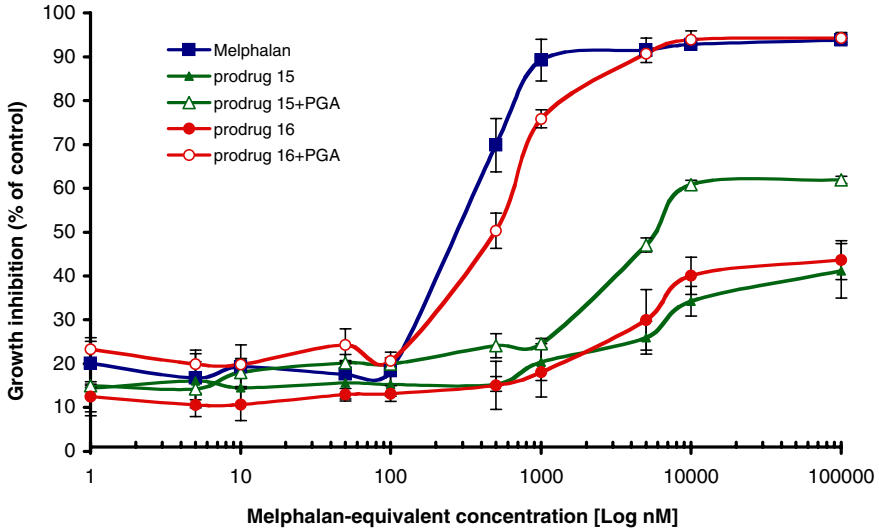

Figure 10. Growth inhibition assay of leukemia MOLT-3 cell line with dendritic prodrug **15** or **16** in the presence or absence of PGA. Cells were incubated for 72 h. Full blue squares represent melphalan, full green triangles prodrug **15**, empty green triangles prodrug **15** with PGA, full red circles prodrug **16**, empty red circles prodrug **16** with PGA. Symbols represent mean ± SD.

were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (25 g), $Ce(SO_4)_2{\cdot}H_2O$ (10 g), concd H_2SO_4 (60 mL), and H_2O (940 mL), followed by heating. Flash chromatography was performed by using silica gel Merck 60 (particle size 0.040–0.063 mm) and the eluent given in parentheses. ^{1}H NMR spectroscopy was performed by using a Bruker AMX 200 or 400 instrument. The chemical shifts are expressed in δ relative to tetramethylsilane (TMS) (δ = 0 ppm) and the coupling constants J in Hz. The spectra were recorded in $CDCl_3$ or CD_3OD as a solvent at room temp. HR-MS: liquid secondary ionization (LSI-MS): VG ZAB-ZSE with 3-nitrobenzyl-alcohol matrix. All reagents, including salts and solvents, were purchased from Sigma–Aldrich (Milwaukee, MN).

4.2. Cell culture

Tumor cells (Molt-3 human acute lymphoblastic leukemia cells) were a kind gift from Holger Lode (Charlté Children's Hospital, Berlin). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2.

4.3. Compound 3

Compound **5** (20 mg, 0.019 mmol) was dissolved in DMF. Tryptophan (15 mg, 0.076 mmol) was added, followed by the addition of Et_3N (150 μL, 1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase chromatography (HPLC) with C-18 semi-preparative column, to give compound **3** (23 mg, 75%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{66}H_{65}N_9O_{17}Na$: 1278.4355; found: 1278.4390 $[M+Na]^+$.

4.4. Compound 7

Compound **6**[19] (2.95 g, 17.53 mmol) was dissolved in DMF and cooled to 0 °C. Imidazole (4.77, 70.16 mmol) and TBSCl (10.57 g, 70.16 mmol) were added. The reaction was allowed to warm to room temperature and was stirred for additional 3 h. The reaction was monitored by TLC (EtOAc/Hex 5:95). After completion, the reaction was diluted with EtOAc and washed with NH_4Cl solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 5:95) to give compound **7** (6.09 g, 68%) in the form of colorless oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.12 (3H, s); 4.69 (6H, s); 0.92 (27H, s); 0.06 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 143.27, 124.34, 67.00, 27.94, 20.39, −3.28. MS (FAB): *m/z*: 509.4 $[M+H]^+$.

4.5. Compound 8

Acetic anhydride (30 mL) was cooled to 5 °C and nitric acid (2 mL, 71%) was added dropwise. After the addition was completed, the mixture was stirred for 15 min at room temperature and then cooled to −20 °C. A solution of compound **7** (5.85 g, 11.46 mmol) in 10 mL of acetic anhydride was added dropwise. The reaction mixture was allowed to warm to 0 °C and was stirred for additional 30 min. After completion, the reaction was diluted with EtOAc and was washed with $NaHCO_3$ solution followed by brine. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAC/Hex 5:95) to give compound **8** (4.78 g, 75%) in the form of a yellow oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.49 (2H, s); 4.75 (6H, s); 0.92 (27H, s); 0.06 (18H, s). ^{13}C NMR (100 MHz,

$CDCl_3$): δ = 144.64, 141.23, 122.30, 64.97, 61.39, 25.93, 18.36, −5.29. MS (FAB): *m/z*: 556.4 $[M+H]^+$.

4.6. Compound 9

Compound **8** (5.43 g, 9.76 mmol) was dissolved in a 50:50 THF/MeOH solution. A catalytic amount of palladium was added to the mixture, followed by the addition of ammonium formate (1 g, 15.8 mmol). The reaction mixture was stirred in room temperature for 2.5 h and monitored by TLC (EtOAc/Hex 5:95). After completion, the salts were filtered out and the solvent was removed under reduced pressure. The residue was diluted with EtOAc and washed with brine. The organic layer was dried over magnesium sulfate, and the crude product was purified by column chromatography on silica gel (EtOAc/Hex 5:95) to give compound **9** (4.39 g, 85%) in the form of a yellow oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.9 (2H, s); 4.66 (6H, s); 4.56 (2H, s); 0.87 (27H, s); 0.03 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 146.93, 131.40, 128.51, 126.96, 67.02, 66.88, 27.79, 20.15, −3.26. MS (FAB): *m/z*: 525.3 $[M+H]^+$.

4.7. Compound 12

Toluene was heated to reflux (110 °C) and a solution of 20% phosgene in toluene (9.8 mL, 19 mmol) was added. Then, a solution of compound **9** (1 g, 1.9 mmol) in toluene was slowly added dropwise with a syringe. The reaction mixture was stirred for 30 min at reflux and monitored by ^{1}H NMR. After the isocyanate derivative was observed, the solvent was removed under reduced pressure. A solution of compound **11**[14] (596 mg, 2.47 mmol)in DMF, followed by 0.5 mL Et_3N, was added to the isocyanate residue. The reaction mixture was stirred for 1 h and monitored by TLC (EtOAc/Hex 30:70). After completion, the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 20:80) to give compound **12** (751 mg, 50%) in the form of a white solid.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.41–7.29 (11H, m); 5.11 (2H, s); 4.71 (2H, s); 4.65 (2H, s); 3.74 (2H, s); 0.94 (27H, s); 0.08 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.96, 156.20, 139.49, 138.00, 136.29, 131.44, 131.19, 130.79, 129.65, 126.69, 124.34, 121.59, 68.46, 66.70, 64.87, 46.79, 27.92, 20.21, −3.42. MS (FAB): *m/z*: 525.3 $[M+H]^+$.

4.8. Compound 13

Compound **12** (286 mg, 0.36 mmol) was dissolved in MeOH/DCM 1:1 and Amberlyst 15 was added. The reaction mixture was stirred in room temperature for 2 h and monitored by TLC (EtOAc/Hex 9:1). After completion, the Amberlyst 15 was filtered out and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 9:1) to give compound **13** (105 mg, 65%) in the form of a white solid.

^{1}H NMR (200 MHz, MeOD): δ = 7.53–7.29 (11H, m); 5.09 (2H, s); 4.58 (2H, s); 4.54 (4H, s); 3.64 (2H, s). ^{13}C NMR (400 MHz, MeOD): δ = 170.86, 155.88, 140.33, 138.34, 135.28, 128.61, 128.08, 126.45, 124.73, 119.62, 66.18, 63.60, 63.43, 60.05, 29.22. MS (FAB): *m/z*: 451.2 $[M+H]^+$.

4.9. Compound 14

Compound **13** (236 mg, 0.523 mmol) was dissolved in dry THF and a catalytic amount of pyridine was added. The solution was cooled to −20 °C and a solution of PNP-chloroformate (1.6 g, 7.8 mmol) in dry THF was added dropwise. The temperature was not allowed to exceed −10 °C. The mixture was stirred for 6 h and monitored by HPLC and by TLC (EtOAc/Hex 50:50). After completion, the reaction mixture was diluted with EtOAc and washed with saturated NH_4Cl solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 50:50) to give compound **14** (395 mg, 80%) in the form of a pale yellow powder.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.26 (6H, m); 7.65 (2H, s); 7.52–7.33 (15H, m); 5.36 (4H, s); 5.27 (2H, s); 5.14 (2H, s); 3.70 (2H, s). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 175.54, 160.46, 160.32, 157.56, 150.53, 143.45, 139.91, 138.37, 136.61, 134.21, 133.79, 132.21, 130.45, 128.91, 124.99, 75.29, 72.87, 72.34, 72.03, 49.05, 35.2. HRMS (MALDI-TOF): *m/z* calcd for $C_{46}H_{35}N_5O_{18}Na$: 968.1846; found: 968.1869 $[M+Na]^+$.

4.10. Compound 4

Compound **14** (20 mg, 0.021 mmol) was dissolved in DMF. Tryptophan (17.3 mg, 0.085 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase HPLC with C-18 semi-preparative column (H_2O/CH_3CN gradient 10%–100%), to give compound **4** (18 mg, 75%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{61}H_{56}N_8O_{15}Na$: 1163.3647; found: 1163.3757 $[M+Na]^+$.

4.11. Compound 15

Compound **5** (20 mg, 0.019 mmol) was dissolved in DMF. Melphalan (15.5 mg, 0.076 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase HPLC with C-18 semi-preparative column (H_2O/CH_3CN gradient

10%–100%), to give compound **15** (20 mg, 70%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{72}H_{83}N_9O_{17}Cl_6Na$: 1578.3945; found: 1578.3930 $[M+Na]^+$.

4.12. Compound 16

Compound **14** (20 mg, 0.021 mmol) was dissolved in DMF. Melphalan (17.3 mg, 0.085 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred over night and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase chromatography (HPLC) with C-18 semi-preparative column (H_2O/CH_3CN gradient 10%–100%), to give compound **16** (21 mg, 70%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{67}H_{74}N_8O_{15}Cl_6Na$: 1463.3262; found: 1463.3297 $[M+Na]^+$.

4.13. Cell proliferation assay with XTT reagent

Molt-3 cells were harvested from culture flasks, resuspended in cell culture medium, and plated at a density of 5×10^3 cells/well in 100 μL onto 96-well culture plate in RPMI 1640 medium supplemented with 10% FBS. Cells were challenged with prodrug 15 or prodrug 16 (1–100,000 nM) in the presence or absence of PGA enzyme (1 μM) and incubated for 72 h (5% CO_2). Control cells were grown with 10% FBS.

Activation solution (100 μL) was added to 5 mL XTT reagent. The reaction solutions (50 μL) were added to each well. The plate was incubated for 2 h, shaken gently to evenly distribute the dye in the wells. Absorbance was measured at a wavelength of 450–500 nm. In order to measure reference absorbance (to measure nonspecific readings), a wavelength of 630–690 nm was used.

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