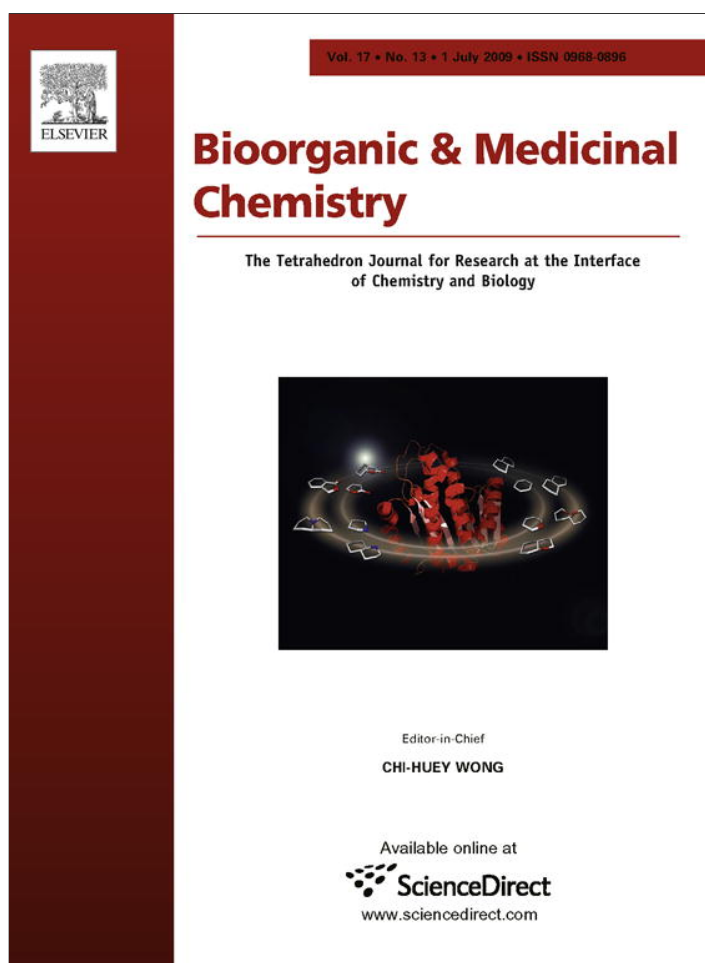


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Enhanced cytotoxicity of a polymer–drug conjugate with triple payload of paclitaxel

Rotem Erez^a, Ehud Segal^b, Keren Miller^b, Ronit Satchi-Fainaro^b, Doron Shabat^{a,*}

^a Department of Organic Chemistry, School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv 69978, Israel

^b Department of Physiology and Pharmacology, Sackler Faculty of Medical Sciences, Tel Aviv University, Tel Aviv 69978, Israel

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ABSTRACT

The development of targeting approaches to selectively release chemotherapeutic drugs into malignant tissue is a major challenge in anticancer therapy. We have synthesized an *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer–drug conjugate with an AB₃ self-immolative dendritic linker. HPMA copolymers are known to accumulate selectively in tumors. The water-soluble polymer–drug conjugate was designed to release a triple payload of the hydrophobic drug paclitaxel as a result of cleavage by the endogenous enzyme cathepsin B. The polymer–drug conjugate exhibited enhanced cytotoxicity on murine prostate adenocarcinoma (TRAMP C2) cells in comparison to a classic monomeric drug–polymer conjugate.

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1. Introduction

Selective chemotherapy remains a key issue for successful treatment of cancer. The use of targeting approaches and selective release of chemotherapeutic drugs in the malignant tissue is consequently needed.¹ We and two other groups have recently reported the design and synthesis of dendrimers that disassemble into their building blocks in a self-immolative manner to release the tail-group units after fragmentation has been initiated by a triggering reaction.^{2–4} The unique disassembly of these molecules was harnessed for the construction of self-immolative dendritic prodrugs with single or multiple triggering modes of activation.^{5,6} The basic building block of a first-generation dendrimer is usually referred to as an AB_{*n*} unit, in which A is the head and B is the tail. We have developed an efficient AB₃ self-immolative dendritic

adaptor that amplifies an enzymatic single cleavage event into the release of three reporter units.^{7–9} A compound constructed of an AB₃ dendritic system bearing three hydrophobic drug molecules as tail-groups is not soluble under aqueous conditions and, therefore, cannot function as a drug delivery system. However, when conjugated to the water-soluble *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer via an enzyme-cleavable linker, the hydrophobic dendritic platform becomes a water-soluble drug delivery system (Fig. 1, conjugate **1a**). Here we report the synthesis, characterization, and in vitro evaluation of an AB₃ dendritic paclitaxel prodrug conjugated with HPMA copolymer.

2. Results and discussion

2.1. Design of AB₃ dendritic-prodrug conjugate

HPMA copolymers are water-soluble, biocompatible, non-immunogenic, and non-toxic carriers that enable specific delivery into tumor tissue.^{10–12} These macromolecules do not diffuse through normal blood vessels but rather accumulate selectively in tumors due to the enhanced permeability and retention (EPR) effect.¹³ This phenomenon of passive diffusion through the hyper-permeable neovasculature and localization in the tumor interstitium is observed for this type of macromolecular agents and for lipids.¹⁴ Furthermore, conjugation to HPMA copolymer should restrict the passage of drug through the blood brain barrier. It should also prolong the circulating half-life of the conjugated drug

Abbreviations: AcOH, acetic acid; Alloc, allyl alcohol; Boc, butyl-*O*-carbonyl; Bu₃SnH, tributyltinhydride; DCM, dichloromethane; DIPEA, diisopropylethyleneamine; DMAP, dimethylaminopyridine; DMF, dimethyl formamide; Et₃N, triethylamine; EtOAc, ethylacetate; Gly, glycine; Hex, hexane; HPMA, *N*-(2-hydroxypropyl)methacrylamide; Leu, leucine; Lys, lysine; MeOH, methanol; NaOH, sodium hydroxide; NH₄Cl, ammonium chloride; NMM, *N*-methylmorpholine; ONp, *O*-nitrophenyl; PABC, *p*-aminobenzyl carbonate; PABOH, *p*-aminobenzyl alcohol; Pd(PPh₃)₄, tetrakis(triphenylphosphine)palladium; Phe, phenylalanine; PNP, *p*-nitrophenyl; PTX, paclitaxel; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; TRAMP, transgenic adenocarcinoma of the mouse prostate.

* Corresponding author. Tel.: +972 (0) 3 640 8340; fax: +972 (0) 3 640 9293.

E-mail address: chdoron@post.tau.ac.il (D. Shabat).

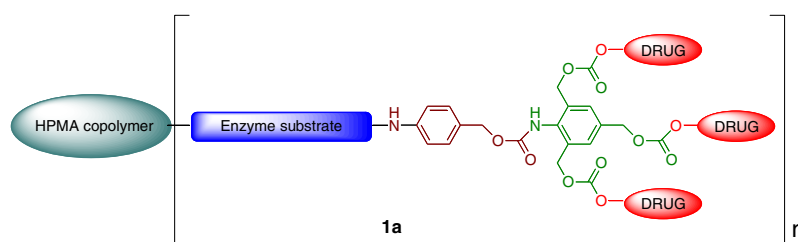


Figure 1. Schematic structure of an AB₃ dendritic prodrug conjugated with HPMA copolymer via an enzyme-cleavable linker.

compared to that of the free drug, hence more effectively inhibiting the growth of tumor endothelial and tumor epithelial cells.^{15–18}

The taxane paclitaxel (PTX) is a potent anti-neoplastic agent. PTX is highly effective as anti-neoplastic in a monotherapy dosing schedule and in combination therapy for the treatment of metastatic prostate and breast cancers.^{19–21} The primary mode of action of PTX is to promote and stabilize microtubulin assembly; this inhibition of microtubule dynamics^{19,22,23} causes impaired mitosis, leading to cell cycle arrest and finally to apoptosis. Despite its strong anticancer activity, PTX is poorly water-soluble and has serious dose-limiting toxicities and causes hypersensitivity reactions in its commercial formulation. The side effects originate from the formulating vehicle, a polyethoxylated castor oil known as cremophor EL, and the absence of selectivity for target tissue.²⁴ Therefore, we sought to construct a water-soluble, copolymer-based dendritic prodrug system for tissue-specific delivery of PTX based on the design presented in Figure 1.

Conjugate **1** (Fig. 2) is constructed of HPMA copolymer linked to an AB₃ self-immolative dendritic system with three hydrophobic PTX molecules as tail groups. The enzymatic substrate linker was designed to be cleaved by the lysosomal enzyme cathepsin B. Cleavage should occur upon internalization of the conjugate in cells and will result in release of three PTX molecules per dendrimer.

The dendritic platform is attached to the HPMA copolymer-Gly-Phe-Leu-Gly via a carbamate linkage to the dipeptide

phenylalanine-lysine-*p*-aminobenzyl linker (Phe-Lys-PABC). Cleavage at this linker by the lysosomal enzyme cathepsin B will result in disassembly (Fig. 3). The six-amino-acid peptide was used in order to provide convenient conjugation chemistry, a longer spacer, and higher probability of cleavage. Enzymatic cleavage at the Phe-Lys will trigger disassembly of conjugate **1**. The free amine intermediate (PABC-dendrimer) will undergo 1,6-elimination and decarboxylation, followed by triple elimination to release the three PTX molecules from each dendritic platform.

2.2. Synthesis of copolymer-dendritic-prodrug conjugate 1

The synthesis of conjugate **1** is outlined in Figure 4. Previously synthesized L-Boc-Phe-ONp²⁵ was reacted with L-Lys(alloc)-OH to give dipeptide **3**. The latter was conjugated with 4-aminobenzyl alcohol to generate alcohol **4**. Isocyanate **5**⁸ was reacted with benzyl alcohol **4** to give carbamate **6**, followed by deprotection with amberlyst 15 to generate triol **7**. Activation with 4-nitrophenyl chloroformate afforded tricarboxylate **8**, which was reacted with three equivalents of PTX to yield compound **9**. Deprotection of the terminal Boc group with TFA allowed the conjugation with HPMA copolymer-Gly-Phe-Leu-Gly-ONp. The remaining activated sites of the copolymer were conjugated with excess aminoethanol to generate compound **10**. Deprotection of the Lys alloc residue of **10** afforded the desired conjugate **1**.

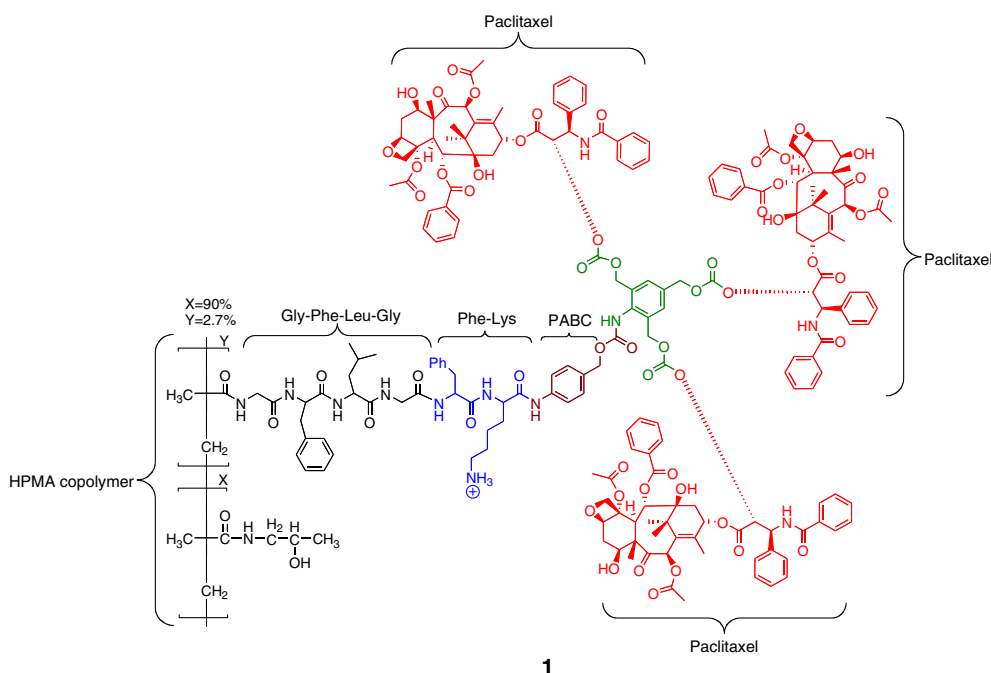


Figure 2. Chemical structure of HPMA copolymer AB₃ dendritic-prodrug conjugate **1**. The dipeptide linker (blue) is a substrate for cathepsin B. All amino acids used have natural L-configuration.

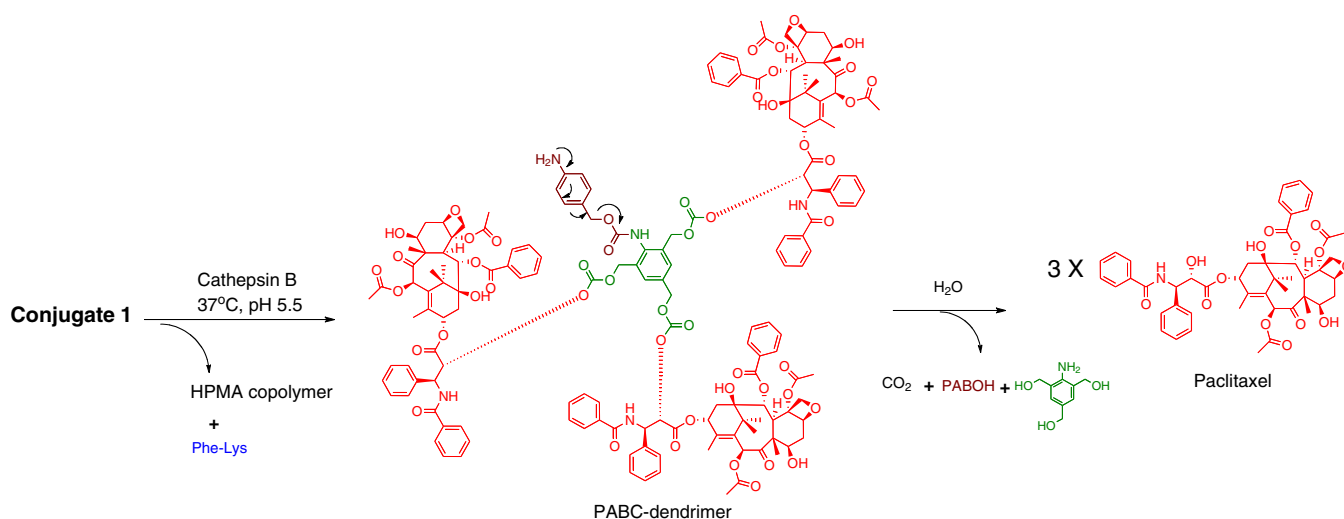


Figure 3. Disassembly mechanism of conjugate 1.

The loading of conjugate **1** with drug was determined by UV spectroscopy; the PTX-chromophore absorbs at a wavelength of 254 nm. The spectroscopic measurements indicated that at maximum loading, about 15 units of PTX (5 of AB₃ units) were attached per molecule of HPMA copolymer.

2.3. Synthesis of copolymer–drug conjugate 2

To enable comparison of the activity of the dendritic-polymeric conjugate to that of a classic copolymer–drug conjugate, we synthesized conjugate **2** bearing one unit of PTX per polymer conjugation site (Fig. 5). PTX was conjugated to HPMA copolymer-Gly-Phe-Leu-Gly through a carbonate linkage with the dipeptide Phe-Lys-PABC as previously described.²⁶ Like the release mechanism of conjugate **1**, cleavage by cathepsin B will trigger the disassembly of conjugate **2** through spontaneous 1,6-benzyl elimination and decarboxylation, leading to the release of free PTX (Fig. 5).

The synthesis of conjugate **2** was achieved as presented in Figure 6. Activation of alcohol **4** with 4-nitrophenyl chloroformate gave carbonate **11**, which was reacted with PTX to yield compound **12**. Deprotection of Boc-Phe-Lys(alloc)-PABC-PTX **12** allowed the conjugation with HPMA copolymer-Gly-Phe-Leu-Gly-ONp. The remaining activated sites of the copolymer were conjugated with excess aminoethanol to generate compound **13**. Deprotection of the Lys alloc residue of **13** afforded the desired conjugate **2**. The loading of PTX molecules in conjugate **2** was determined using UV spectroscopy via the chromophore of PTX at a wavelength of 254 nm. The UV measurements were performed in water (10% acetonitrile as a cosolvent for PTX). About seven units of PTX were attached per molecule of HPMA copolymer.

2.4. Cell-growth inhibition assays

To evaluate the ability of dendritic conjugate **1** and conjugate **2** to inhibit cell proliferation in the presence of cathepsin B, we performed a standard cell-growth inhibition assay using TRAMP C2 cells. In order to prove that the two conjugates are active only upon the cleavage by cathepsin B, we synthesized a control conjugate bearing the non-cleavable linker Gly-Gly-Gly-Gly-PABC (Fig. 7). TRAMP C2 cells were challenged for 24 h or 48 h with free PTX, conjugate **1**, conjugate **2**, or the control conjugate over a range of concentrations. Cell viability was measured using colorimetric assay based on the tetrazolium salt XTT. The data are presented in

Figure 8. Twenty-four hours post-treatment, proliferation of TRAMP C2 cells was inhibited by free PTX, conjugate **1**, and conjugate **2** (at PTX-equivalent concentrations) with IC₅₀s of 2, 10, and >10 μM, respectively (Fig. 8a). The control conjugate bearing the non-cleavable (Gly)₄-PABC linker, exhibited less than 10% inhibition at all concentrations (Fig. 8a). Forty-eight hours post-treatment, the differences among the inhibitory concentrations of PTX, conjugate **1**, and conjugate **2** were reduced significantly: IC₅₀s were 200, 300, and 350 nM, respectively (Fig. 8b). Although the trends were similar to those observed at the 24 h time point, the lower IC₅₀ was comparable amongst the different treatments due to the longer exposure of the cells to PTX. In contrast, at 48 h, the non-cleavable conjugate had an IC₅₀ of greater than 10 μM (Fig. 8b); the minor growth inhibition observed may be due to a small amount of spontaneous hydrolysis of the carbonate linkage between PTX and the PABC linker.

The AB₃ dendritic prodrug system used to link PTX to the water-soluble HPMA copolymer fulfills two major functions. First, it increases the loading of drug units onto the polymeric vehicle. The dendritic linker allowed conjugation of 15 PTX-molecules per polymer, whereas the monomeric linker permitted conjugation of only seven PTX molecules. Second, the self-immolative dendron amplifies a single cleavage event through a rapid triple-elimination reaction to release the active PTX molecules. The effect of these two functions was reflected by the IC₅₀s in cytotoxicity assays comparing the dendritic conjugate **1** and to the classic conjugate **2**. After 24 h, conjugate **1** was 10-fold more toxic than conjugate **2** in a standard cell-growth inhibition assay (IC₃₀ of conjugate **1** was 1000 nM compared with IC₃₀ of conjugate **2** of 10,000 nM, i.e., 1-order of magnitude higher).

3. Conclusions

In summary, we have synthesized a new HPMA copolymer conjugate with an AB₃ dendritic linker bearing three molecules of the highly hydrophobic drug paclitaxel. The water-soluble copolymer–drug conjugate was designed for activation by cathepsin B and released a triple payload of PTX from each cleavage site. The AB₃ self-immolative dendritic linker acts as a molecular amplifier. This strategy increased the loading capacity of drug molecules per a polymer molecule to more than twice that of the classic conjugate. The difference in cytotoxicity between the trimeric drug–copolymer conjugate and the monomeric one was reflected in a cell-growth inhibition assay. Our new dendritic drug–copolymer

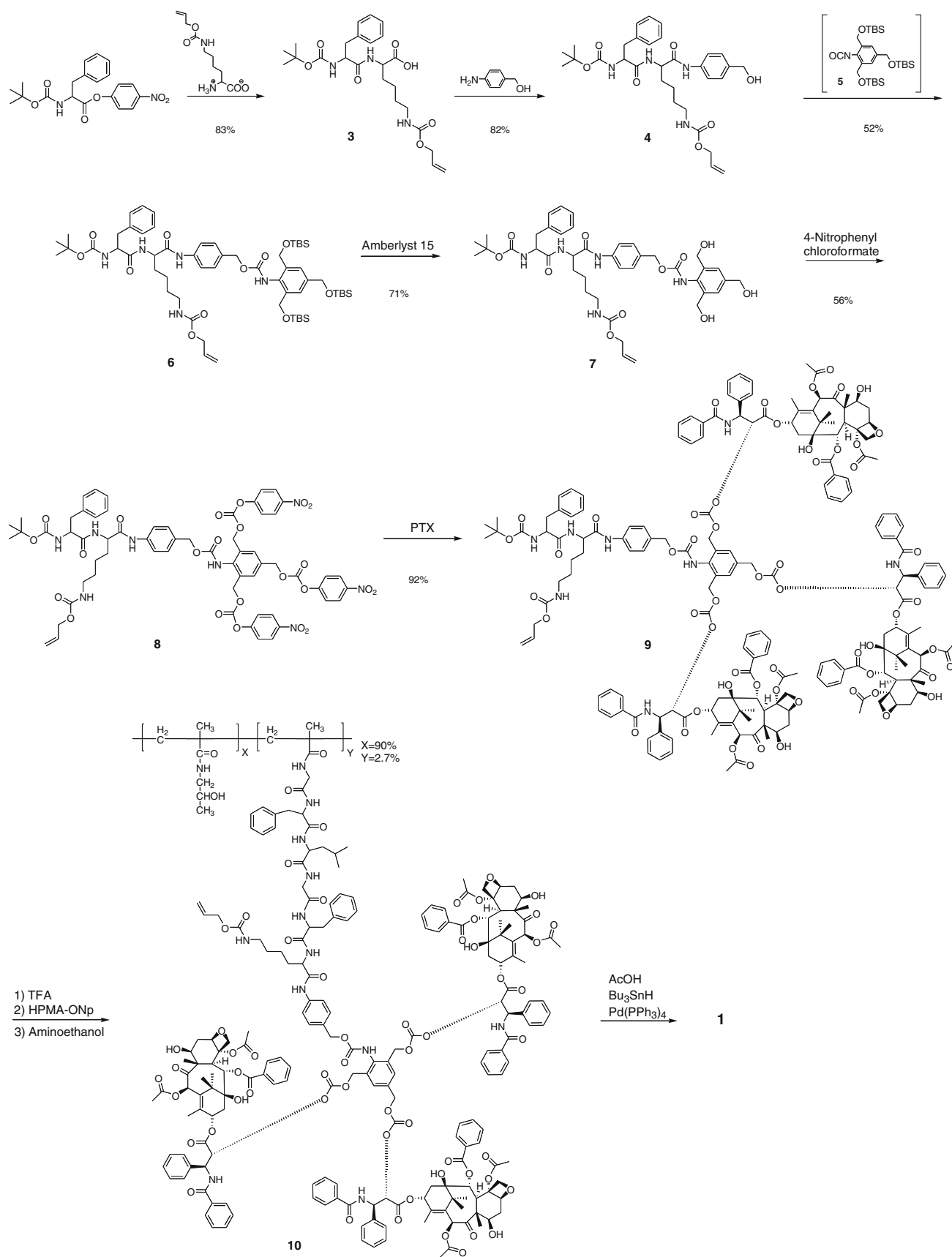


Figure 4. Synthesis of dendritic-polymeric conjugate **1**.

conjugate system may offer significant advantages in inhibition of tumor growth relative to monomeric drug-polymer conjugates,

especially if the targeted or secreted enzyme that initiates drug release is present at relatively low levels in the malignant tissue.

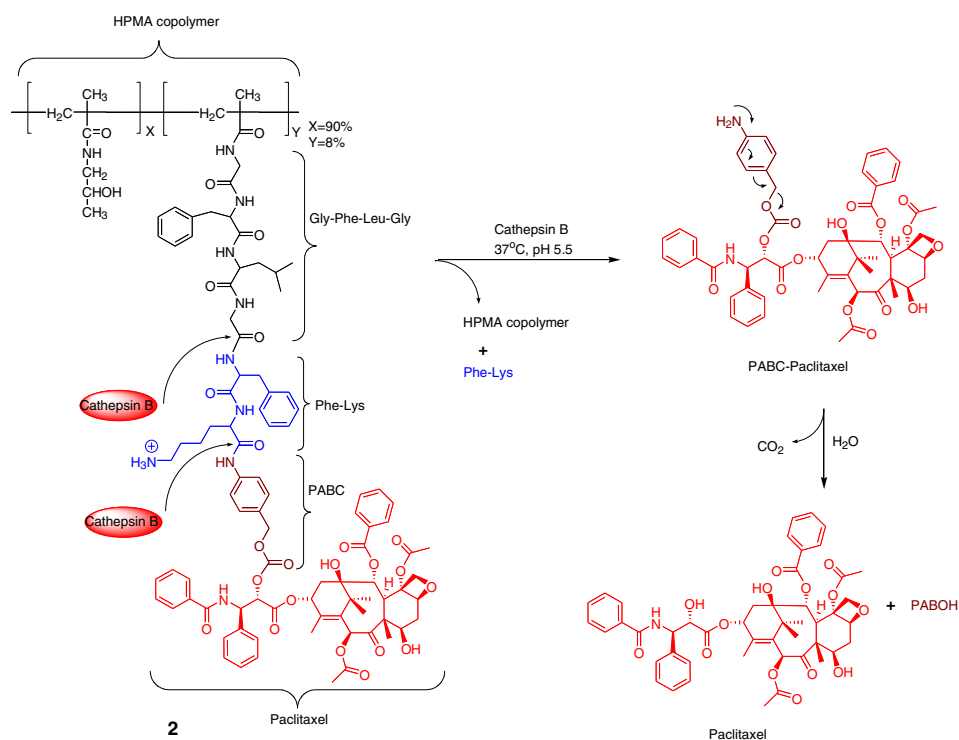


Figure 5. Disassembly mechanism of conjugate 2.

4. Experimental

4.1. General

HPMA copolymer-Gly-Gly-*p*-nitrophenol (ONp) incorporating 5 mol % of the methacryloyl-Gly-Gly-*p*-nitrophenol ester monomer units, and HPMA copolymer-Gly-Phe-Leu-Gly-ONp incorporating 10 mol % of the methacryloyl-Gly-Phe-Leu-Gly-*p*-nitrophenol ester monomer units were obtained from Polymer Laboratories (Church Stretton, UK). The HPMA copolymer-GFLG-ONp has a molecular weight of 31,600 Da and a polydispersity of 1.66.

All reactions requiring anhydrous conditions were performed under an Ar or N₂ atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques. All reagents, including salts and solvents, were purchased from Sigma-Aldrich. Thin layer chromatography (TLC) silica gel plates (Merck 60 F₂₅₄) were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20 wt % in ethanol) followed by heating. Flash chromatography (FC) was performed on silica gel (Merck 60, particle size 0.040–0.063 mm); eluent is given in parentheses in sections on individual compounds. ¹H NMR was performed on either a Bruker AMX 200 or 400 instrument. The chemical shifts are expressed in δ relative to TMS ($\delta = 0$ ppm) and the coupling constants *J* in hertz. The spectra were recorded in CDCl₃ or MeOD at room temperature. The 400 Mesh copper grid was obtained from SPI Supplies.

4.1.1. L-Boc-Phe-ONp

L-Boc-Phe-ONp was synthesized according to the procedure described previously.²⁵

4.1.2. Compound 3

L-Boc-Phe-ONp (104.3 mg, 0.27 mmol) was dissolved in 2 mL DMF. Commercially available L-Lys(alloc)-OH (62 mg, 0.27 mmol) and Et₃N (100 μ L) were added. The reaction mixture was stirred for 12 h and was monitored by TLC (AcOH/MeOH/EtOAc,

0.5:10:89.5). Upon completion of the reaction the solvent was removed under reduced pressure and the crude product was purified using column chromatography on silica gel (AcOH/MeOH/EtOAc, 0.5:10:89.5) to give compound 3 (107 mg, 83%) as a white solid.

¹H NMR (200 MHz, CDCl₃): $\delta = 7.24$ – 7.11 (5H, m); 5.83 (1H, m); 5.45–5.11 (3H, m); 4.50–4.48 (3H, m); 3.08–2.92 (4H, m); 1.84–1.64 (2H, m); 1.44–1.36 (4H, m); 1.30 (9H, s). ¹³C NMR (100 MHz, CDCl₃): $\delta = 177.09$, 164.91, 158.52, 157.61, 138.45, 134.82, 131.26, 130.50, 128.83, 120.24, 82.27, 67.51, 57.59, 53.98, 42.37, 38.60, 33.53, 33.44, 30.14, 22.63. MS (FAB): *m/z*: 478.3 [M+H]⁺, 500.3 [M+Na]⁺.

4.1.3. Compound 4

Compound 3 (832.1 mg, 1.74 mmol) was dissolved in dry THF and the solution was cooled to -15 °C. NMM (0.19 mL, 1.74 mmol) and isobutyl chloroformate (0.27 mL, 2.09 mmol) were added. The reaction was stirred for 20 min and a solution of 4-aminobenzyl alcohol (321.85 mg, 2.61 mmol) in dry THF was added. The reaction mixture was stirred for 2 h with progress monitored by TLC (100% EtOAc). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (100% EtOAc) to give compound 4 (835 mg, 82%) as a yellow solid.

¹H NMR (200 MHz, MeOD): $\delta = 7.56$ (2H, d, *J* = 8 Hz); 7.29 (2H, d, *J* = 8 Hz); 7.21–7.07 (5H, m); 5.86 (1H, m); 5.29–5.10 (2H, m); 4.83 (2H, s); 4.49–4.46 (4H, m); 3.17–3.08 (4H, m); 1.88–1.70 (2H, m); 1.44 (4H, m); 1.34 (9H, s). ¹³C NMR (100 MHz, CDCl₃): $\delta = 174.08$, 171.45, 158.61, 157.79, 139.17, 138.87, 138.03, 134.79, 131.12, 130.71, 130.49, 129.90, 129.08, 122.15, 82.71, 67.48, 66.83, 58.08, 55.74, 42.09, 39.84, 32.70, 31.31, 30.16, 24.31. MS (FAB): *m/z*: 583.3 [M+H]⁺, 605.3 [M+Na]⁺.

4.1.4. Compound 5

Compound 5 was synthesized according to the procedure described previously.⁸

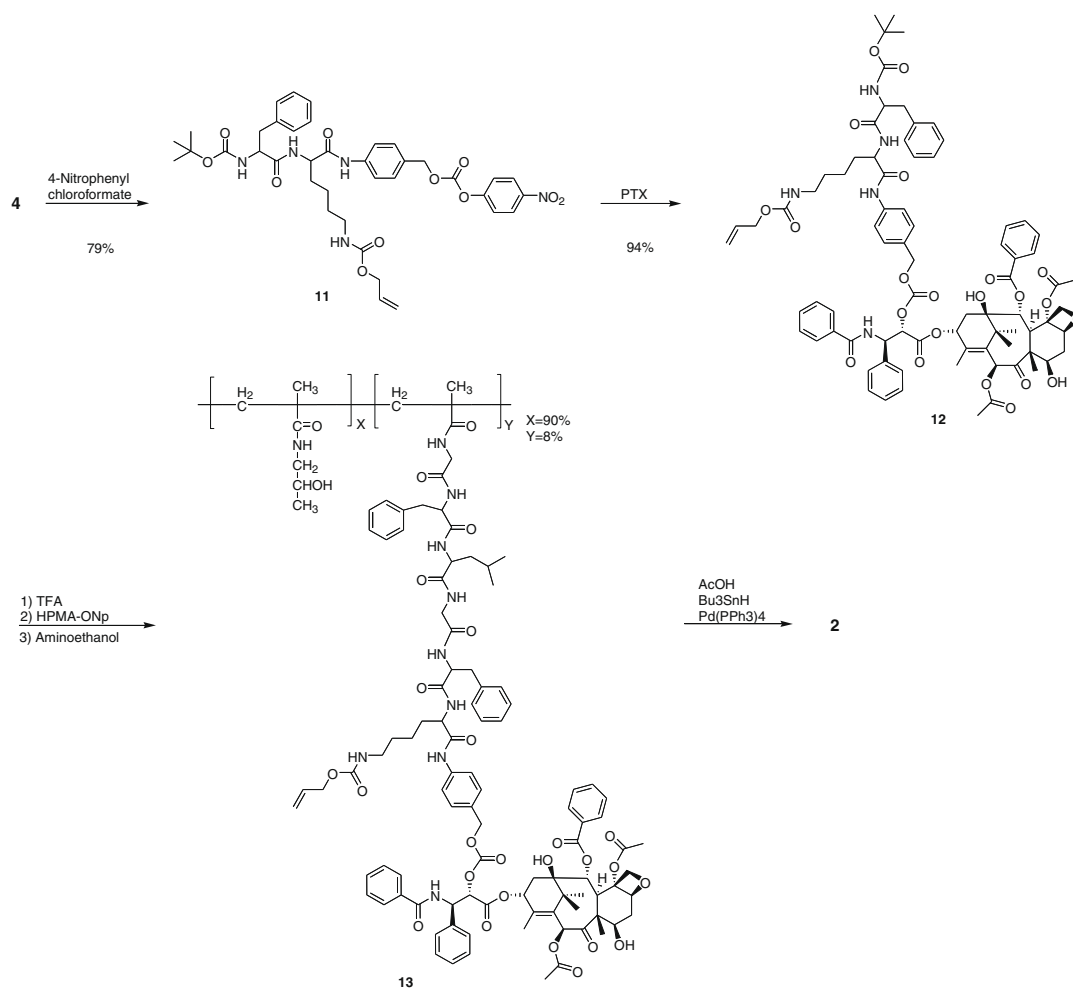


Figure 6. Synthesis of monomeric drug-copolymer conjugate **2**.

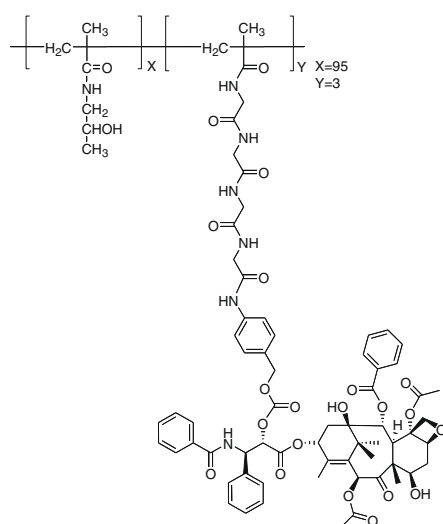


Figure 7. Structure of the non-cleavable HPMA copolymer-Gly-Gly-Gly-Gly-PABC-PTX conjugate.²⁶

4.1.5. Compound 6

A solution of compound **4** (235.6 mg, 0.40 mmol) in 3 mL THF, 100 μ L NEt₃ was added to the isocyanate residue **5** (185.74 mg, 0.33 mmol). The reaction was stirred for 24 h and was monitored

by TLC (EtOAc/Hex, 1:1). Upon completion of the reaction, the solvent was removed under reduced pressure. The crude product was purified by using column chromatography on silica gel (EtOAc/Hex, 1:1) to give compound **6** (196.7 mg, 52%) as a yellow solid.

¹H NMR (200 MHz, CDCl₃): δ = 7.58 (2H, d, *J* = 8 Hz); 7.35–7.14 (9H, m); 5.87 (1H, m); 5.34 (1H, m); 5.24 (2H, m); 5.18 (1H, m); 4.86 (1H, m); 4.70–4.67 (8H, m); 4.56 (1H, m); 3.19–3.06 (4H, m); 1.65–1.47 (6H, m); 1.25 (9H, s); 0.89 (27H, s); 0.05 (18H, s). ¹³C NMR (100 MHz, CDCl₃): δ = 176.2, 158.4, 151.7, 144.1, 139.8, 137.3, 136.5, 134.7, 130.5, 128.7, 128.4, 127.3, 126.4, 125.9, 124.7, 120.3, 116.4, 78.2, 76.5, 73.8, 73.6, 62.1, 54.7, 54.5, 40.5, 38.5, 31.0, 30.7, 25.1, 21.6, 20.3, 18.2, –6.2. MS (FAB): *m/z*: 1156.4 [M+Na]⁺.

4.1.6. Compound 7

Compound **6** (196.7 mg, 0.44 mmol) was dissolved in 6 mL MeOH and Amberlyst-15 was added. The reaction was stirred in room temperature for 1.5 h and progress was monitored by TLC (EtOAc/MeOH, 95:5). Upon completion of the reaction, Amberlyst-15 was filtered out and the solvent was removed under reduced pressure. The crude product was purified by using column chromatography on silica gel (EtOAc/MeOH 95:5) to give compound **7** (97.7 mg, 71%) as a white solid.

¹H NMR (200 MHz, MeOD): δ = 7.57 (2H, m); 7.43–7.38 (4H, m); 7.23–7.15 (5H, m); 5.86 (1H, m); 5.30 (1H, m); 5.24 (2H, m); 5.12 (1H, m); 4.87–4.63 (9H, m); 4.52 (1H, m); 3.15–2.95 (4H, m); 1.65–1.40 (6H, m); 1.28 (9H, s). ¹³C NMR (100 MHz, MeOD): δ = 172.3,

172.0, 157.3, 156.4, 155.4, 144.2, 138.4, 138.1, 137.9, 136.4, 136.2, 130.1, 128.4, 128.1, 127.6, 126.3, 125.9, 118.2, 117.1, 79.4, 67.3, 67.0, 66.7, 58.6, 55.1, 54.1, 42.3, 37.2, 31.1, 28.7, 28.2, 22.4. MS (FAB): m/z : 814.3 [M+Na]⁺.

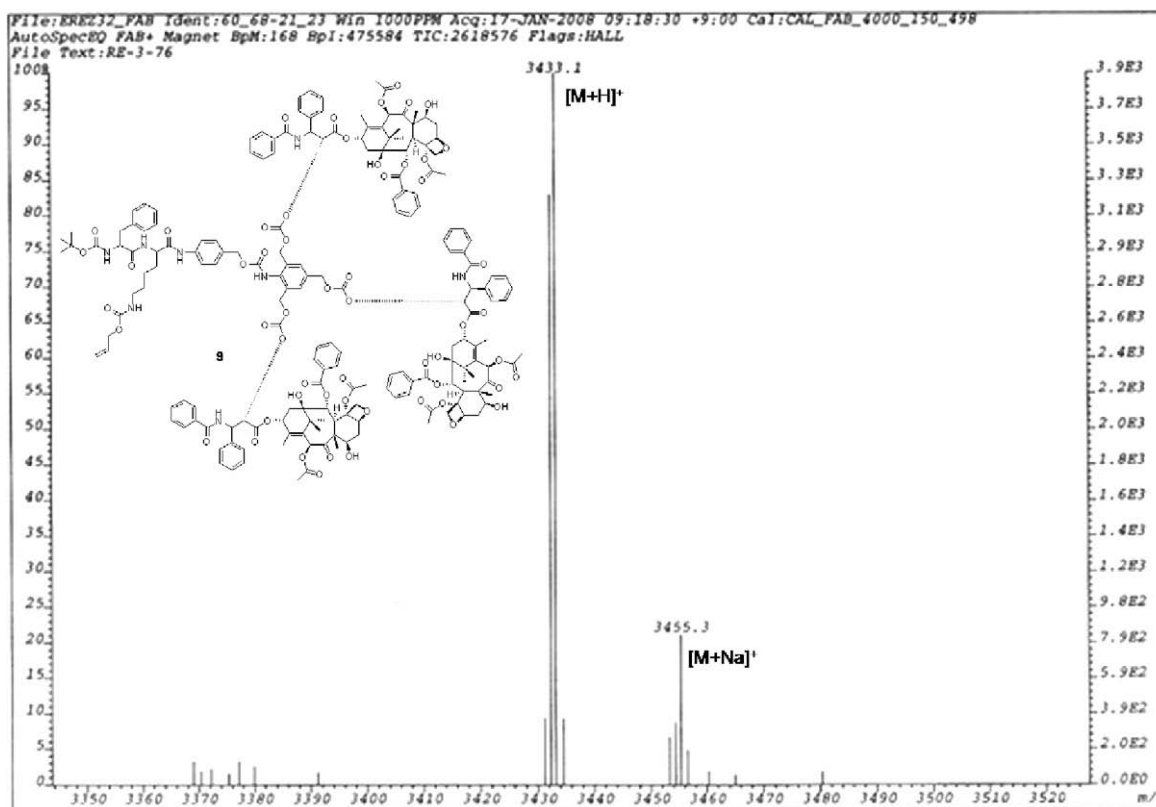
4.1.7. Compound 8

Compound **7** (97.7 mg, 0.12 mmol) was dissolved in 3 mL dry THF and a catalytic amount of pyridine (10 μ L) was added. The solution was cooled to -20 °C and a solution of PNP-chloroformate (372.9 mg, 1.85 mmol) in 2 mL of dry THF was added dropwise. The temperature was not allowed to exceed -10 °C. The mixture was stirred for 3 h and reaction progress was monitored by HPLC and by TLC (EtOAc/Hex, 2:1). Upon completion, the reaction was diluted with EtOAc and was washed with saturated NH₄Cl solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by using column chromatography on silica gel (EtOAc/Hex, 2:1) to give compound **8** (88 mg, 56%) as a white solid.

¹H NMR (200 MHz, CDCl₃): δ = 8.29–8.27 (6H, m); 7.58 (2H, m); 7.37 (6H, m); 7.24–7.14 (9H, m); 5.86 (1H, m); 5.34–5.30 (7H, s); 5.24 (2H, m); 5.18 (1H, m); 4.56–4.28 (4H, m); 3.17–3.14 (4H, m); 1.76–1.33 (6H, m); 1.28 (9H, s). ¹³C NMR (100 MHz, CDCl₃): δ = 172.0, 169.3, 156.61, 155.1, 154.4, 152.3, 145.5, 138.2, 135.8, 134.6, 132.7, 130.7, 129.0, 128.7, 127.1, 125.2, 121.6, 119.9, 117.5, 80.8, 69.5, 67.0, 65.4, 56.2, 53.8, 30.1, 29.4, 28.1, 22.3. MS (FAB): m/z : 1287.0 [M+H]⁺, 1309.0 [M+Na]⁺.

4.1.8. Compound 9

Compound **8** (22 mg, 17.09 μ mol) was dissolved in dry DCM. Paclitaxel (52.60 mg, 61.55 μ mol) and DMAP (7.52 mg, 61.55 μ mol) were added. The reaction mixture was stirred for 24 h and progress was monitored by TLC (EtOAc/Hex, 8:1). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (EtOAc/Hex, 8:1) to give compound **9** (54.6 mg, 92%) as a white solid. MS (FAB): m/z : 3432.3 [M]⁺, 3433.1 [M+H]⁺, 3455.3 [M+Na]⁺.



MS spectrum of the compound **9**.

4.1.9. Compound 10

Compound **9** (51.1 mg, 14.67 μ mol) was dissolved in 1.8 mL TFA and stirred for 2 min at 0 °C. The excess acid was removed under reduced pressure and the crude amine salt was dissolved in 2 mL DMFHPMAcopolymer-Gly-Phe-Leu-Gly-ONp (44 mg ONp = 14.67 μ mol) was added followed by the addition of Et₃N (100 μ L). The reaction mixture was stirred for 12 h and then excess aminoethanol was added. Most of the solvent was removed under reduced pressure, followed by addition of 10 mL of acetone. The precipitate was filtered out and was washed with acetone several times to give compound **10** (71.4 mg) as a white solid.

4.1.10. Compound 1

Compound **10** (71.4 mg, 23.8 μ mol) was dissolved in DMF (1.5 mL). Acetic acid (14.01 μ L, 118.9 μ mol), Bu₃SnH (76.40 μ L, 142.62 μ mol), and a catalytic amount of Pd(PPh₃)₄ were added. The reaction mixture was stirred for 2 h and was then concentrated under reduced pressure, followed by addition of 10 mL of acetone. The precipitate was filtered out and was washed with acetone several times. The crude product was purified by using Sephadex LH20 column chromatography (100% MeOH, 1 mL/min) to give compound **1** (26.9 mg) as a white solid.

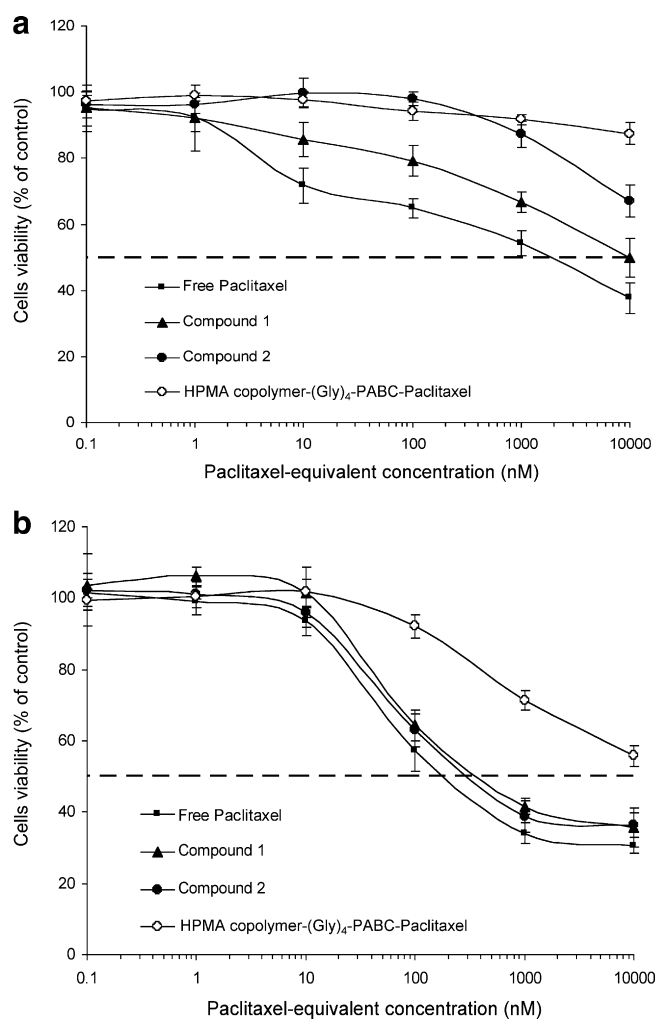


Figure 8. Inhibition of proliferation of TRAMP C2 cells by conjugate **1** and conjugate **2**. (a) At 24 h post-treatment, free PTX (closed squares), conjugate **1** (closed triangles), and conjugate **2** (closed circles) inhibited the proliferation of TRAMP C2 cells with IC₅₀s of 2, 10, and >10 μM, respectively. The conjugate bearing the non-cleavable (Gly)₄-PABC linker (open circles) showed modest growth inhibition of less than 10% inhibition at all concentrations. (b) At 48 h post-treatment, free PTX (closed squares), conjugate **1** (closed triangles), and conjugate **2** (closed circles) had IC₅₀s of 200, 300, and 350 nM, respectively, whereas the non-cleavable conjugate had an IC₅₀ of >10 μM. Data represent mean ± SD. X axis values are presented at a logarithmic scale.

4.1.11. Compound 11

Compound **4** (353.6 mg, 0.60 mmol) was dissolved in dry THF and the solution was cooled to 0 °C. DIPEA (0.42 mL, 2.42 mmol), PNP-chloroformate (367 mg, 1.82 mmol), and a catalytic amount of pyridine were added. The reaction was stirred for 2 h and progress was monitored by TLC (EtOAc/Hex, 3:1). Upon completion of the reaction, the solvent was removed under reduced pressure. The crude product was diluted with EtOAc and washed with saturated NH₄Cl. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by using column chromatography on silica gel (EtOAc/Hex, 3:1) to give compound **11** (453.2 mg, 79%) as a white solid.

¹H NMR (200 MHz, CDCl₃): δ = 8.26 (2H, d, J = 8 Hz); 7.64 (2H, d, J = 8 Hz); 7.40–7.34 (4H, m); 7.22–7.14 (5H, m); 5.83 (1H, m); 5.24 (2H, s); 5.18–5.06 (2H, m); 4.56–4.37 (4H, m); 3.19–3.05 (4H, m); 1.95–1.73 (2H, m); 1.59–1.46 (4H, m); 1.39 (9H, s). ¹³C NMR (100 MHz, CDCl₃): δ = 174.11, 171.51, 158.63, 157.60, 157.46,

154.36, 147.32, 140.70, 137.91, 134.76, 131.75, 131.53, 131.08, 130.78, 129.17, 127.21, 123.72, 122.05, 119.61, 82.86, 72.65, 67.50, 58.17, 55.84, 41.95, 39.71, 32.42, 31.40, 30.16, 24.27. MS (FAB): *m/z*: 770.4 [M+Na]⁺.

4.1.12. Compound 12

Compound **11** (360.3 mg, 0.48 mmol) was dissolved in dry DCM. Paclitaxel (494.06 mg, 0.57 mmol) and DMAP (70.61 mg, 0.57 mmol) were added. The reaction mixture was stirred for 8 h and progress was monitored by TLC (100% EtOAc). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (100% EtOAc) to give compound **12** (662 mg, 94%) as a white solid. MS (FAB): *m/z*: 1463.7 [M]⁺, 1486.9 [M+Na]⁺.

4.1.13. Compound 13

Compound **12** (29.36 mg, 20.06 μmol) was dissolved in 1.5 mL TFA and stirred for 2 min at 0 °C. The excess acid was removed under reduced pressure and the crude amine salt was dissolved in 2 mL DMF. HPMA copolymer-Gly-Phe-Leu-Gly-ONp (60.2 mg, ONp = 20.06 μmol) was added, followed by the addition of Et₃N (100 μL). The reaction mixture was stirred for 12 h and then excess aminoethanol was added. Most of the solvent was removed under reduced pressure, followed by addition of 10 mL of acetone. The precipitate was filtered out and was washed with acetone several times to give compound **13** (74.8 mg) as a white solid.

4.1.14. Compound 2

Compound **13** (74.8 mg, 24.9 μmol) was dissolved in DMF (1.5 mL). Acetic acid (14.67 μL, 124.5 μmol), Bu₃SnH (80.03 μL, 149.4 μmol), and a catalytic amount of Pd(PPh₃)₄ were added. The reaction mixture was stirred for 2 h and was then concentrated under reduced pressure, followed by addition of 10 mL of acetone. The precipitate was filtered out and was washed with acetone several times. The crude product was purified by using Sephadex LH20 column chromatography (100% MeOH, 1 mL/min) to give compound **2** (33.2 mg) as a white solid.

4.2. Cell-growth inhibition assays

TRAMP C2 cells were kindly provided by Prof. Norman Greenberg (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). Cells were cultured in complete growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, 100 U/mL streptomycin 12.5 U/mL nystatin, 1 μM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 mM androstan, and 2 mM L-glutamine (Biological Industries, Israel). Cells were grown at 37 °C in 5% CO₂.

TRAMP C2 cells were plated at 2000 cells/well in DMEM supplemented with 5% FBS and incubated for 24 h (37 °C; 5% CO₂). The medium was then replaced with complete growth medium. Cells were challenged with free PTX, compound **1**, compound **2**, or the non-cleavable HPMA copolymer-(Gly)₄-PABC-PTX over a range of concentrations (1–10,000 nM paclitaxel-equivalent concentrations) and incubated for 24 h or 48 h. Cell viability was measured using XTT reagent. Absorbance was measured at a wavelength of 450–500 nm. A wavelength of 630–690 nm was also recorded.

4.3. Non-cleavable HPMA copolymer-Gly-Gly-Gly-Gly-PABC-PTX

The non-cleavable conjugate was prepared by conjugation of HPMA copolymer-Gly-Gly-ONp with Gly-Gly-PABC-PTX. Use of the Gly-Gly rather than Phe-Lys dipeptide resulted in a conjugate that could not be cleaved by cathepsin B.

References and notes

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