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A Chemical Adaptor System Designed to Link a Tumor Targeting Device with a Prodrug and an Enzymatic Trigger.

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General methods

Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}: compounds were visualized by irradiation with UV light and/or by treatment with a solution of 25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2 \cdot H_2O$, 60 mL conc. H_2SO_4 and 940 mL H_2O followed by heating and/or by staining with a solution of 12 g 2,4-dinitrophenylhydrazine in 60 mL conc. H_2SO_4, 80 mL H_2O and 200 mL 95% EtOH followed by heating and/or immersing into an iodine bath (30 g I_2, 2 g KI, in 400 mL EtOH/H_2O 1:1) and warming. – Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. ^{1}H NMR spectra were measured in CDCl3 using Bruker AMX 200 MHz. The chemical shifts are expressed in relative to TMS (δ = 0 ppm) and the coupling constants *J* in Hz. The spectra were recorded in $CDCl_3$ as solvent at room temperature unless stated otherwise. All general

reagents, including salts and solvents, were purchased from Aldrich (Milawaukee, MN). HPMA copolymer-Gly-Gly-ONp (4.4 mole %), (ONp is *p*-nitrophenyl), M.W 30,000D was obtained from Polymer Laboratories (Church Stratton, UK).

Abbreviations. Boc- t-buthyl carbonate, DMAP- Dimethyl aminopyridine, DMF- Dimethyl formamide, EDC- 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide, EtOAc- Ethyl acetate, Et3N- Triethyl amine, He- n-Hexane, HOBT- 1-hydroxybenzotriazole, MeOH- Methanol, PNP- 4-nitrophenyl, THF- Tetrahydrofuran

Compound 5

The commercially available 4-hydroxymandelic acid monohydrate (1 g, 5.4 mmol) and mono-Boc-N,N-dimethylethylenediamine (1.2 g., 6.4 mmol) were coupled in DMF in the presence of EDC (1.2 g, 6.4 mmol) and HOBT (365 mg, 2.7 mmol). The reaction was stirred for 48h at room temperature. The solvent was removed under reduced pressure and the product was purified by column chromatography on silica gel (EtOAc: He= 3:1) to give compound **5** (900 mg, 49%) in the form of white powder. MS (FAB): C17H26N2O5 $[M+H]^+$ 339.1; ^1H NMR (200MHz, $CDCl_3$): δ=7.2 ppm (2H, d, J=4.45); 6.8 (2H, d, J=4.4); 5.15 (1H, d, J=2.28); 3.5-3.28 (4H, m); 3.08 (3H, s); 2.83 (3H, s); 1.49 (9H, the signal of the Boc group appears as two singlets, the sum of their integrations corresponded to 9 hydrogens)

Compound 6

Compound **5** (890 mg, 2.6 mmol) was dissolved in dried THF. Triethylamine (1 ml) was added. The reaction was cooled to 0ºC and PNP-chloroformate (633 mg, 3.14 mmol) dissolved in THF was added dropwise. The reaction was stirred at room temp' for 1 hr and monitored by TLC (EtOAc:He=3:1). After completion of reaction the precipitation was filtered out and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc: He= 1:1) to give compound **6** in the form of the white powder (890 mg, 67%).

MS (FAB): $C_{24}H_{29}N_3O_9$ $[M+H]^+$ 504.1, ^{1}H NMR (200MHz, $CDCl_3$): δ =8.3 ppm (2H, d, J=7); 7.5 (2H, d, J=7); 7.4-7.3 (4H, m); 4.7 (1H, d, J=3.4); 3.57-3.22 (4H, m); 3.06 (3H, s); 2.85 (3H, s); 1.45 (9H, see details above)

Compound 7

Antibody 38C2 substrate[1] (313 mg, 0.87 mmol) was deprotected with 1 ml TFA for 2 min at 0˚C, to remove the Boc group. The excess of the acid was removed under reduced pressure and the amine salt was dissolved in 3 ml of DMF. Compound **6** (480 mg, 0.95 mmol) was added in with 1 ml of triethylamine, and the solution was stirred for 10 minutes. The reaction was monitored by TLC (EtOAc:MeOH=9:1). After completion the DMF was removed under reduced pressure and the crude

product was purified by flash chromatography (ethyl acetate 100%) to give pure compound **7** in the form of white powder (483 mg, 90%).

MS (FAB): $C_{30}H_{48}N_4O_{10}$ $[M+Na]^+$ 647.3, ^1H NMR (200MHz, $CDCl_3$): δ=7.35(2H, d, J=8); 7.14 (2H, d, J=8); 5.19 (1H, d, J=8); 4.24(2H, m); 3.51-3.48 (4H, m); 3.13 (2H, s); 3.04 (2H, s); 2.97-2.95 (3H, m); 2.90-2.82 (5H, m); 2.65 (2H, d, J=4); 2.17 (3H, s); 1.88 (2H, m); 1.46 (9H); 1.23 (3H, s)

Compound 8

Compound **7** (167 mg, 0.27 mmol) was dissolved in 2 ml DMF and cooled to 0°C. Triethylamine (75 l, 0.53 mmol), DMAP (33 mg, 0.27 mmol) and PNP-chloroformate (107 mg, 0.53 mmol) were added and the mixture was stirred in room temp for 20 min. Then, the reaction mixture was diluted with EtOAc, washed with HCl 0.1N and brine, dried over sodium sulfate, and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc 100%) to give compound **8** in the form of white powder (154 mg, 72%).

MS (FAB): $C_{37}H_{51}N_5O_{14}$ $[M+H]^+$ 790, ^1H NMR (200MHz,$CDCl_3$): δ=8.26 (2H, d, J=8); 7.51 (2H, d, J=8.4); 7.39 (2H, d, J=8.2); 7.19 (2H, d, J=8); 4.24 (2H, t, J= 6.7); 3.89 (1H, d, J=1.6); 3.5-3.4 (8H, m); 3.11 (2H, s); 3.01-2.93 (7H, m); 2.81 (3H, s); 2.62 (2H, d, J=3.6); 2.14 (3H, s); 1.89-1.83 (2H, m); 1.45 (9H); 1.2 (3H, s)

Compound 14

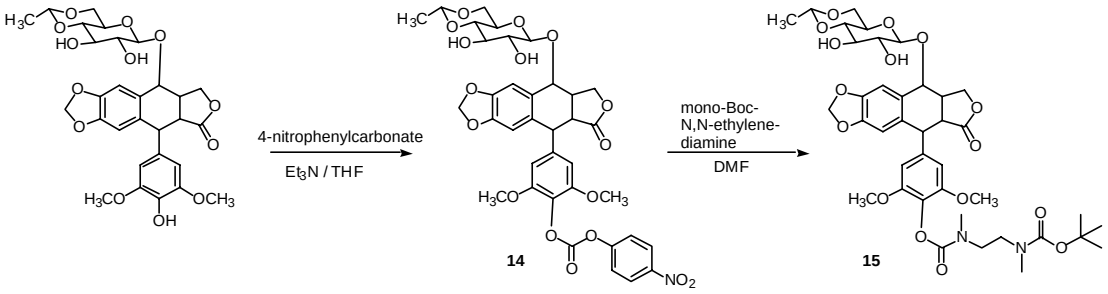
Etoposide (500 mg, 0.85 mmol) was dissolved in dried THF. Triethylamine (358 l, 2.55 mmol) and DMAP (10 mg, 0.085 mmol) were added. The reaction was cooled to 0°C and PNP-chloroformate (205.5 mg, 1.02 mmol) dissolved in THF was added dropwise. The reaction was stirred at room temp for 10 min and monitored by TLC (EtOAc:He=3:1) for completion. After completion, 1 ml of acetic acid was added and the reaction was stirred for 2 min. The precipitation was filtered and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc: He= 4:1) to give compound **14** in the form of white powder (542 mg, 72%).

^{1}H NMR (200MHz,CDCl3): δ =8.27 ppm (2H, d, J=6); 7.46 (2H, d, J=6); 6.81 (1H,s); 6.54 (1H, s); 6.31 (2H, s); 5.97-6.007 (2H, m); 4.92 (1H, m); 4.74 (1H, q); 4.57-4.66 (2H, m); 4.36-4.48 (1H, t); 4.06-4.28 (2H, q); 2.89 (m); 1.71 (broad singlet); 1.38 (3H, d)

Compound 15

Compound **14** (150 mg, 0.2 mmol) was dissolved in 1 ml DMF, mono-Boc protected N,N-dimethylethylene-diamine (41.2 mg, 0.22 mmol) was added. The reaction was stirred for 30 minutes. After completion the DMF was removed under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate 100%) to give pure compound **15,** (147 mg, 91%).

^{1}H NMR (200MHz,CDCl3): δ =6.87 ppm (1H, s); 6.61 (1H, s); 6.32 (2H,s); 6.05(2H,d); 4.96 (1H, s); 4.9 (1H, s); 4.70-4.74 (2H, m); 4.5 (1H, m); 3.17 (1H, s); 3.06 (2H, s); 2.96 (5H, m); 2.75 (1H, s); 2.4 (1H, m); 1.5 (9H, s); 1.45 (3H, m)



Compound 9

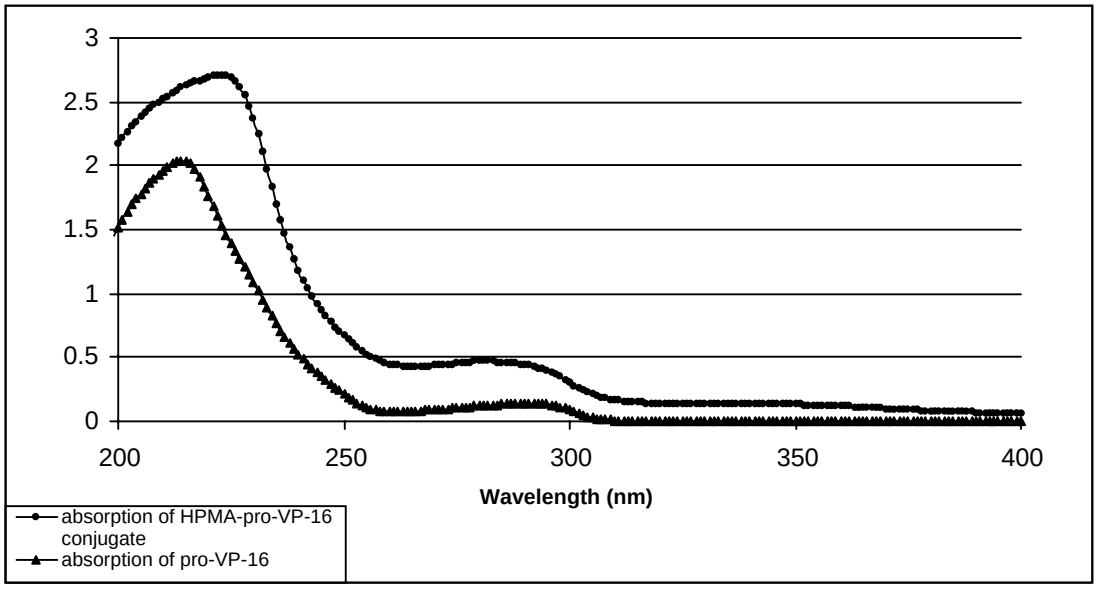
Compound **15** (50 mg, 0.062 mmol) was cooled to 0ºC and deprotected with 1 ml TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 2 ml DMF. Compound **8** (59 mg, 0.075 mmol) and 1 ml triethylamine were added and the solution was stirred for 10 minutes. After completion the DMF was removed under reduced pressure and the crude product was purified by PLC (EtOAc:MeOH=9:1) to give pure compound **9** in the form of white powder (66 mg, 77%).

MS(FAB):C66H90N6O26 $[M+Na]^+$ 1375.5

Polymer conjugate preparation (compound 10)

Compound **9** (30 mg, 0.022mmol, 22 eq.) was deprotected with 1 ml TFA. The excess of the acid was removed under reduced pressure and the residue was dissolved in 2 ml DMF. HPMA-copolymer (30 mg) and 0.5 ml triethylamine were added and the solution was stirred for 3h. The reaction was quenched with 0.5 ml ethanolamine and the solvents were removed under reduced pressure. The crude was dissolved in water and dialyzed through MWCO-10,000D membrane against water. The conjugate (26.5 mg) was obtained after lipholization of the water in the form of pinkish powder.

UV spectrum measurements.



By measuring its UV spectrum, the number of drug molecules attached to the HPMA-copolymer was determined. Based on the etoposide chromophore, which has a λmax at 280 nm, we found that on average three molecules of the drug were linked to one molecule of the HPMA-copolymer. There are approximately ten linkage sites on the HPMA-copolymer. The mild coupling's yield can be probably improved by optimizing the reaction conditions.

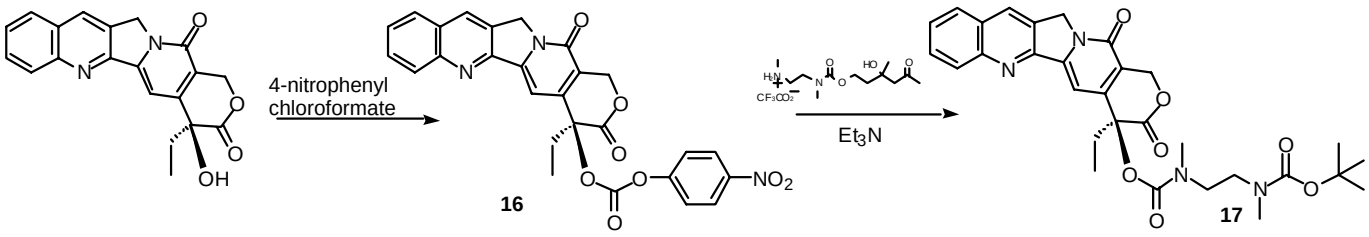
Drug Release Analysis

HPMA-drug-conjugate (1 mg) was dissolved in 100 L solution of catalytic antibody 38C2 (66) and incubated at 37˚C. The background sample was prepared similarly in PBS-7.4. 4-Nitrophenol was used as an internal standard to monitor the concentration of the released drug. The previously reported etoposide prodrug[2], was used as a positive control for monitoring the activity of catalytic antibody 38C2. Drug release was monitored by an HPLC assay using C-18 column, Wavelength; 280 nm, eluent; acetonitrile:water; 35:65, flow rate; 1 ml/min.

[1] D. Shabat, C. Rader, B. List, R. A. Lerner, C. F. Barbas, III, *Proc. Natl. Acad. Sci. U. S. A., Vol. 96* 1999, p. 6925-6930.

[2] D. Shabat, H. Lode, U. Pertl, R. A. Reisfeld, C. Rader, R. A. Lerner, C. F. Barbas, III, *Proc. Natl. Acad. Sci. U. S. A. 98* (2001) 7528-33.

Additional example I. Incorporation of Camptothecin prodrug with a retro-Michael retro-aldol trigger in the chemical adaptor.



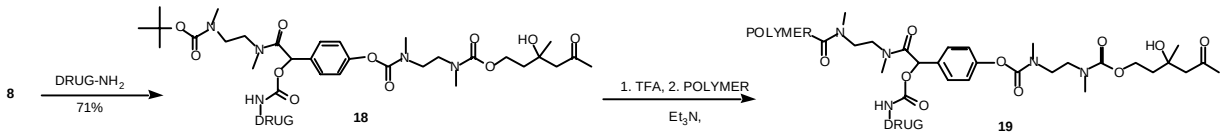
Compound 16

Camptothecin (1.5 g, 4.3 mmol), PNP-chloroformate (2.6 g, 12.9 mmol) were dissolved in methylene chloride (45 ml) at 0° C, followed by addition of DMAP (3.18 g, 25.8 mmol). The resulting clear solution was stirred at room temperature for 1 hour. The reaction was monitored by TLC (EtOAc 100 %). After completion, the reaction was diluted with 75 ml of methylene chloride and was washed with 30 ml HCl 0.1 N. The organic layer was dried over magnesium sulfate, concentrated under reduced pressure to 10 ml and precipitated with ether. The precipitated solid was filtered and dried to give crude compound **16** in the form of yellow powder (2.5 g).

^{1}H NMR (200MHz,CDCl3): δ=8.39 (1H, s); 8.25 (1H, d, J=8); 7.94 (1H, d, J=8); 7.85-7.81 (1H, m); 7.71-7.67 (2H, m); 7.48 (2H, d, J=7); 7.14 (2H, d, J=7); 5.76 (1H, d, J=16); 5.35-5.2-32 (3H, m); 1.90-2.86 (2H, m); 1.11-1.03 (3H, m).

Compound 17

Compound **16** (70 mg, 0.14 mmol) was dissolved in DMF and and mono-Boc-N,N-dimethylethylenediamine (39.44 mg,0.21 mmol) was added. The reaction was stirred in room temp for 15 min and was monitored by TLC (EtOAc:MeOH=9:1). The solvent was removed under reduced pressure and the product was purified by column chromatography on silica gel (EtOAc ^{1}H NMR (200MHz,CDCl3): δ=8.39 (1H, s); 8.23 (1H, d, J=8); 7.95 (1H, d, J=8); 7.85-7.81 (1H, m); 7.71-7.67 (2H, m); 5.7 (1H, d of d, J=16, J=2); 5.31 (3H, m); 3.62-5.96 (2H, m); 3.31-3.29 (2H, m); 3.18 (3H, s); 2.89 (3H, s); 1.87-1.97 (2H, m); 1.45 (9H, s); 1.05-1.00 (3H, m). MS(FAB):C30H34N4O7 $[M+Na]^{+}$ 585.2



Compound 18

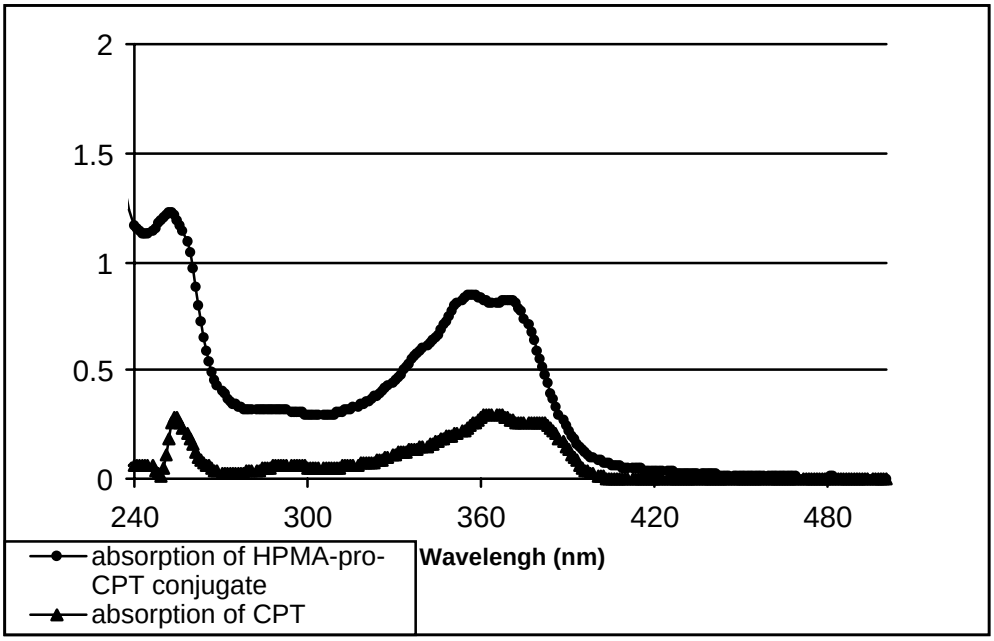
Compound **17** (40 mg, 0.071 mmol) was deprotected with 1 ml TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 1 ml DMF. Compound **8** (43.3 mg, 0.05 mmol) dissolved in 1ml DMF and 1 ml triethylamine were added and the solution was stirred for 15 minutes. The reaction was monitored by TLC (EtOAc:MeOH=9:1. After completion the DMF was removed

under reduced pressure and the crude product was purified by flash chromatography (150 ml EtOAc 100% and then EtOAc:MeOH=95:5) to give pure compound **18** (67.3 mg, 85 %). MS(FAB):C56H72N8O16 $[M+Na]^+$ 1135.2

Compound 19

Compound **18** (43 mg) was deprotected with 1 ml TFA. The excess of the acid was removed under reduced pressure and the residue was dissolved in 2 ml DMF. HPMA-copolymer (40 mg) and 0.5 ml triethylamine were added and the solution was stirred for 2h. The product was dialyzed through MWCO-10,000D membrane against water, and purified by size exclusion chromatography over PD-10 column. The product was obtained after lipholization of the water in the form of pinkish powder (39.8 mg).

UV spectrum measurements

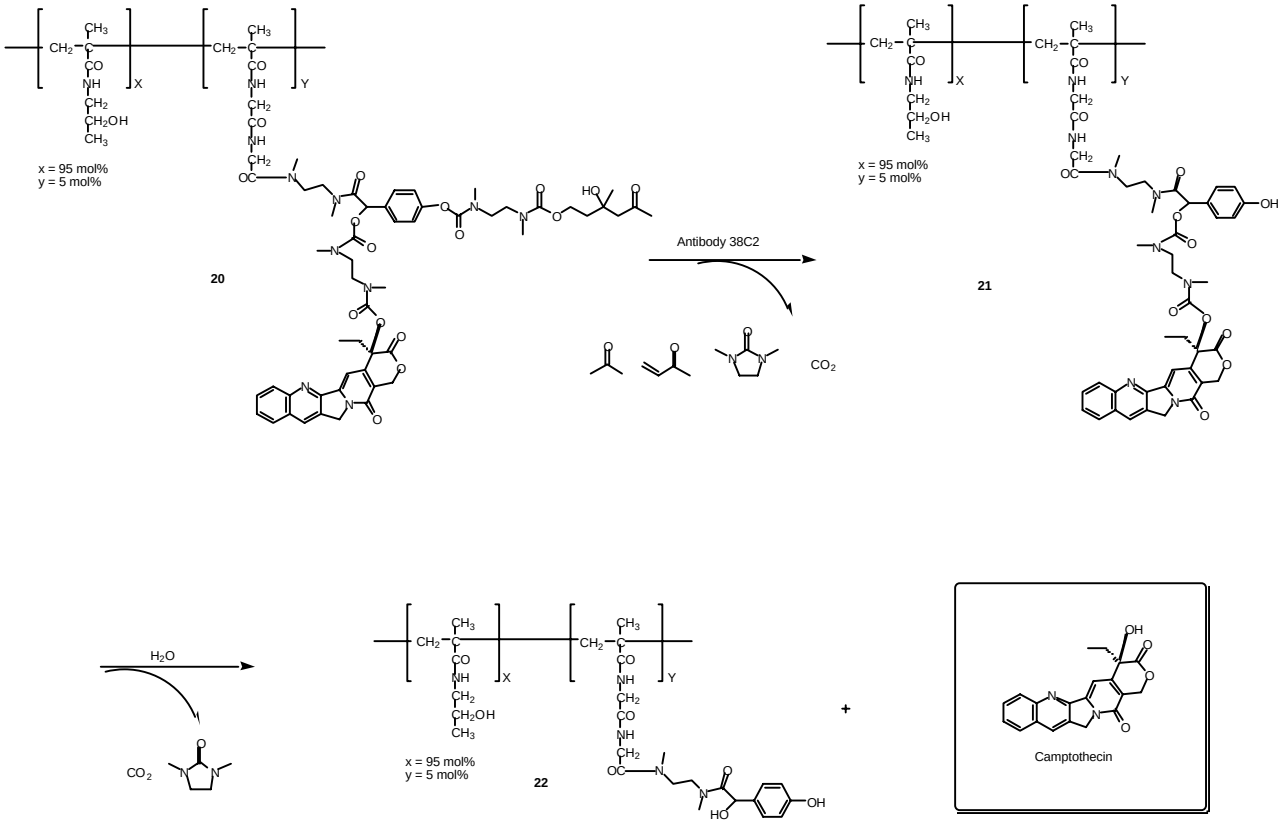


By measuring its UV spectrum, the number of drug molecules attached to the HPMA-copolymer was determined. Based on the camptothecin chromophore, which has a λmax at 360 nm, we found that on average 3 molecules of the drug were linked to one molecule of the HPMA-copolymer.

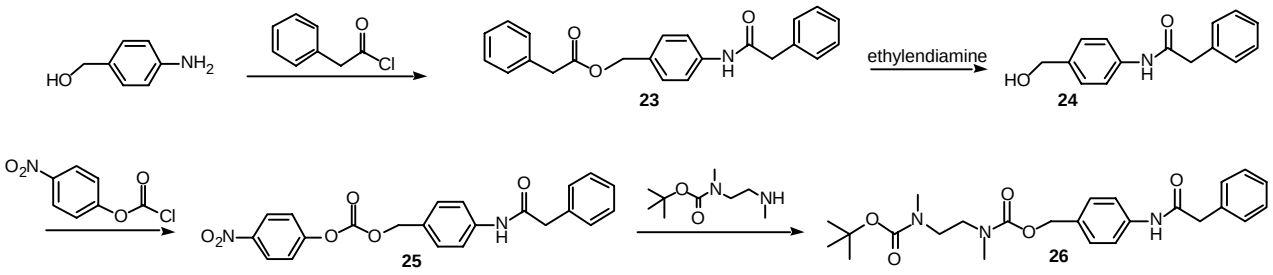
Drug Release Analysis

HPMA-drug-conjugate (1 mg) was dissolved in 100 µL solution of 38C2 (2 mg/ml) and incubated at 37˚C. The background sample was prepared similarly in PBS-7.4. Drug release was monitored by an HPLC assay using C-18 column, Wavelength; 360 nm, eluent; acetonitrile:water;

30:70, flow rate; 1 ml/min. The results (data not shown) clearly proved the release of free camptothecin drug according to the mechanism, which is illustrated below.



Additional example II. Incorporation of etoposide prodrug with a penicillin amidase substrate trigger in the chemical adaptor.



Compound 23

The commercially available phenylacetic acid (1 g, 7.3 mmol) was refluxed with 10 ml of thionyl chloride for one hour. The excess of thionyl chloride was removed under reduced pressure and the clean product phenyl acetyl chloride was obtained (1.1 g, 97.5%) in the form of yellow liquid. Then, 4-amino-benzyl alcohol (1 g, 8.12 mmol) and DMAP (2.47 g., 20 mmol) were dissolved in dry THF. The reaction mixture was cooled to 0ºC and phenylacetyl chloride dissolved in dry THF (3.14 g, 0.02 mmol) was added dropwise. The reaction was stirred overnight at room temperature. After completion the reaction mixture was filtered, diluted with methylene chloride and washed with NaOH 1M. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc:Hex 2:3) to give compound **23** in the form of yellow oil (2.1 g, 72%).

^{1}H NMR (200MHz,CDCl3):δ=7.32 (14H, m); 51 (2H, d, J=8.4); 7.39 (2H, d, J=8.2); 5.0 (2H, s); 3.74 (2H, s); 3.64 (2H,s,)

Compound 24

Compound **23** (2.1 g, 5.84 mmol) was dissolved in 4 ml ethylene diamine. The reaction mixture was heated to 80°c and was stirred for 15 min. The reaction was monitored by TLC (EtOAc:Hex=1:1). After completion the reaction mixture was cooled to room temperature, diluted with 100 ml EtOAc and washed with HCl 1M. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure to give compound **24** in the form of yellow powder (954 mg, 68%).

^{1}H NMR (200MHz,MeOD):δ=7.52 (2H, d, J=8.5); 7.3 (2H [of the AB system] + 5H, m); 4.37 (2H, s); 3.49 (2H, s).

Compound 25

Compound **24** (724 mg, 3 mmol) and DMAP (402 mg, 3.3mmole) were dissolved in dry THF. PNP-chloroformate (725 mg, 3.6 mmol) dissolved in dry THF was added drop wise and the mixture was stirred in room temp for 10 min. The reaction was monitored by TLC (EtOAc:Hex=1:1). After completion, the reaction was diluted with 100 ml of EtOAc and was washed with HCl 1M. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The

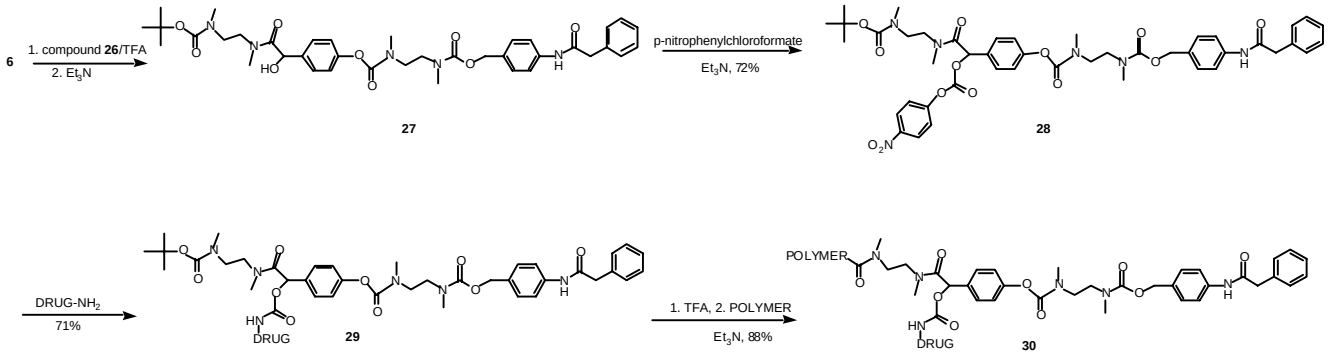
product was purified by column chromatography on silica gel (EtOAc:Hex 30:70) to give compound **25** in the form of white powder (414 mg, 34%).

^{1}H NMR (200MHz,CDCl3): δ=8.26 (2H of the AB system, d), 7.40(11H, m [2H of the AB system +9H); 5.22(2H, s); 3.76(2H, s).

Compound 26

Compound **25** (300 mg, 0.74 mmol) was dissolved in DMF and and mono-Boc-N,N-dimethylethylenediamine (200 mg,1.06 mmol) was added. The reaction was stirred in room temp for 15 min and was monitored by TLC (EtOAc:Hex=1:1). The solvent was removed under reduced pressure and the product was purified by column chromatography on silica gel (EtOAc:Hex 1:1) to give compound **26** in the form of colorless oil (252 mg, 75%).

^{1}H NMR (200MHz,CDCl3): δ=7.40(9H, m); 5.01(2H, s); 3.66(2H, s); 3.33(5H [3H of the amino methyl group +2H of the ethylene group]); 2.85(5H [3H of the amino methyl group +2H of the ethylene group], m).



Compound 27

Compound **26** (93 mg, 0.2 mmol) was deprotected with 1 ml TFA for 2 min, to remove the Boc group. The excess of the acid was removed under reduced pressure and the amine salt was dissolved in 2 ml of DMF. Compound **6** (100 mg, 0.2 mmol) was added in followed by 1 ml of triethylamine, and the solution was stirred for 15 minutes. The reaction was monitored by TLC (EtOAc:Hex=75:25). After completion, the DMF was removed under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate:Hex 80:20) to give pure compound **27** in the form of white powder (68.5 mg, 49%).

^{1}H NMR (200MHz, CDCl3): δ =7.24(13H, M); 5.06 (2H, S); 4.99 (1H, s); 3.7(2H, s); 3.59-3.25 (8H, m); 3.09-2.76 (14H, m); 1.47 (9H, s).

Compound 28

Compound **27** (66 mg, 0.09 mmol) was dissolved in 5 ml methylene chloride, DMAP (23 mg, 0.19 mmol) and PNP-chloroformate (47 mg, 0.23 mmol) were added and the mixture was stirred in room temp for 1h. The solvent was removed under reduced pressureand the product was purified by column chromatography on silica gel (100 ml EtOAc:Hex 80:20 and then EtOAc 100%) to give compound **28** in the form of white powder (51 mg, 63%).

^{1}H NMR (200MHz,CDCl3): δ=8.23 (2H, d, J=8); 7.35-7.26 (15H, m [2H of the AB system +13H]); 5.07 (2H, s); 3.51 (8H, m);3.03-2.83 (13H, m);1.45 (9H, s).

Compound 29

Compound **15** (60 mg, 0.074 mmol) was deprotected with 1 ml TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 1 ml DMF. Compound **28** (43.3 mg, 0.05 mmol) dissolved in 1ml DMF and 1 ml triethylamine were added and the solution was stirred for 15 minutes. The reaction was monitored by TLC (EtOAc:MeOH=95:5).After completion the DMF was removed under reduced pressure and the crude product was purified by flash chromatography (150 ml EtOAc 100% and then EtOAc:MeOH=95:5) to give pure compound **29** in the form of white powder (50 mg, 69%).

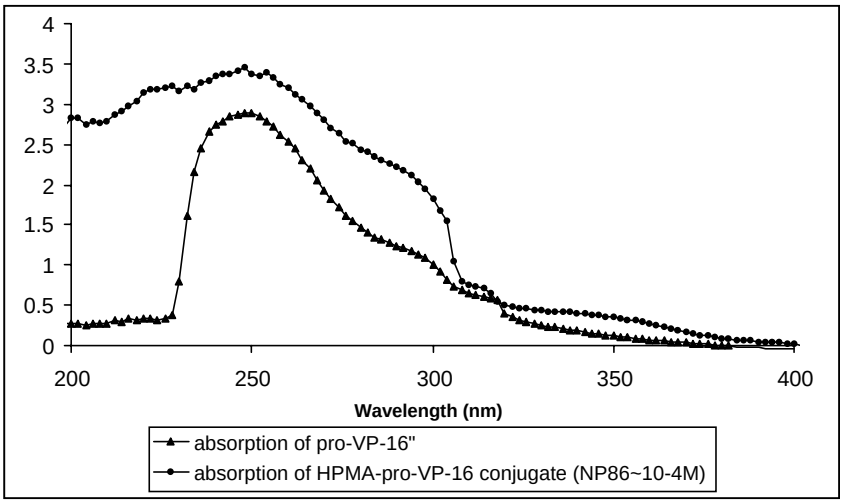
^{1}H NMR (200MHz,CDCl3): δ=7.33 (13H,m,); 7.21 (1H,s); 7.12 (1H,s); 7.02 (2H, s); 5.05 (2H, d, J= 9); 3.64 (3H, s); 3.5-3.4 (8H, m); 3.11 (2H, s); 3.01-2.93 (7H, m); 2.81 (3H, s); 2.62 (2H, d, J=3.6); 2.14 (3H, s); 1.89-1.83 (2H, m); 1.45 (9H); 1.2 (3H, s). MS(FAB):C73H89N7O24 $[M+Na]^{+}$ 1470.5

Polymer-etoposide conjugate preparation (compound 30)

Compound **29** (25 mg, 0.017mmol, 20 eq.) was deprotected with 1 ml TFA. The excess of the acid was removed under reduced pressure and the

residue was dissolved in 2 ml DMF. HPMA-copolymer (25 mg) and 0.5 ml triethylamine were added and the solution was stirred for 2h.The the reaction was quenched with a few drops of ethanolamine, and the solvents were removed under reduced pressure. The crude was dissolved in 1 ml water and was filtered through celite. The product was dialyzed through a MWCO-10,000D membrane against water and purified by size exclusion chromatography over PD-10 column. The product obtained after lipholization of the water in the form of pinkish powder (17.6 mg, 65%).

UV spectrum measurements

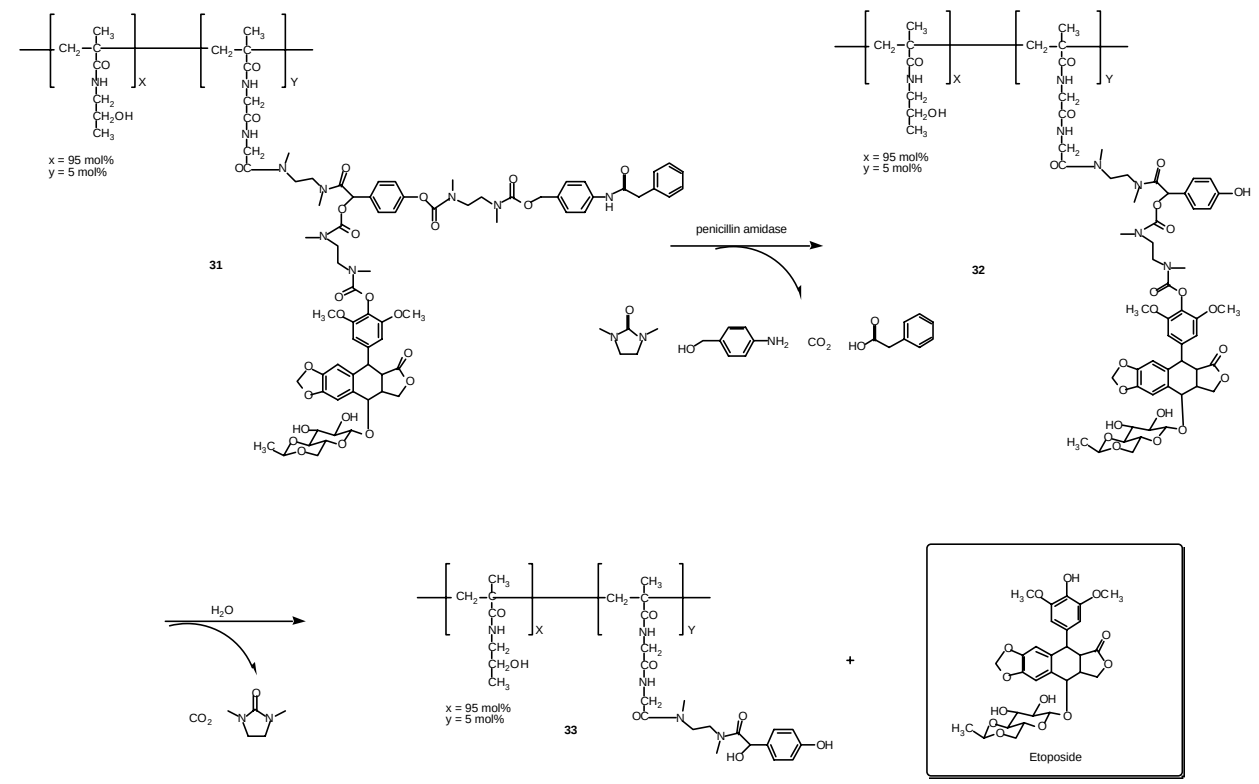


By measuring its UV spectrum, the number of drug molecules attached to the HPMA-copolymer was determined. Based on the etoposide chromophore, which has a λmax at 280 nm, we found that on average

two molecules of the drug were linked to one molecule of the HPMA-copolymer.

Drug Release Analysis

HPMA-drug-conjugate (1 mg) was dissolved in 100 L solution of penicillin G amidase (1 mg/ml) and incubated at 37˚C. The background sample was prepared similarly in PBS-7.4. 7-amino-4-methyl coumarine was used as an internal standard to monitor the concentration of the released drug. Drug release was monitored by an HPLC assay using C-18 column, Wavelength; 280 nm, eluent; acetonitrile:water; 30:70, flow rate; 1 ml/min. The results (figure not showed) clearly proved the release of free etoposide drug according to the mechanism, which is illustrated below.



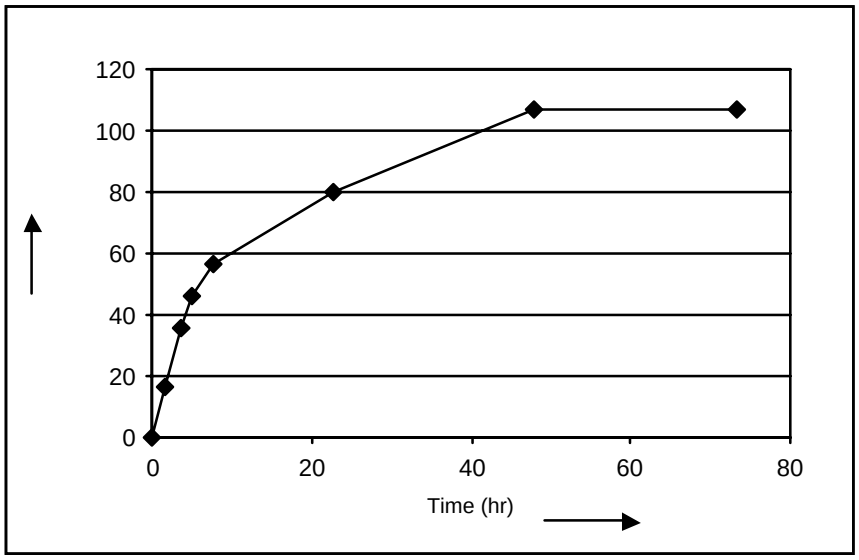


Figure **1**: Determination of etoposide release from the HPMA-copolymer (100 M) by the enzyme penicillin-G-amidase (1mg/ml).

The reaction was incubated at 37°C for the indicated time. The drug release concentration was monitored by HPLC analysis.