SUPPLEMENTARY INFORMATION

Light-controlled Release of Caged Doxorubicin from Folate Receptor-targeting PAMAM Dendrimer Nanoconjugate

Seok Ki Choi, Thommey Thomas, Ming-Hsin Li, Alina Kotlyar, Ankur Desai, and James R. Baker, Jr.**

Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Medical School, Ann Arbor, MI 48109

Table of Contents

1. General methods for synthesis and analysis

All solvents and reagents were purchased from commercial suppliers (Aldrich, Fluka, TCI), and used without further purification including folic acid dihydrate (Sigma; purity 98%), and doxorubicin hydrochloride (AvaChem Scientific LLC, San Antonio, TX; purity 98.0%). Reactions were run under nitrogen atmosphere unless noted otherwise. Progress of reactions was monitored by thin layer chromatography on Merck® TLC plates (250 μm thick), and spots were detected by UV illumination at 254 or 365 nm or by staining with phosphomolybdic acid reagent (20% w/v in ethanol) or ninhydrin solution (5% w/v in 3% acetic acid/ethanol). Reaction mixtures were worked up as described specifically in each reaction including flash column chromatography using silica gel (200-400 mesh). Characterization of reaction products was routinely carried out by ^{1}H NMR spectroscopy and mass spectrometry. For NMR (^{1}H, ^{13}C) measurement, samples were dissolved in deuterated solvent ($CDCl_3$, CD_3OD, D_2O, DMSO-d_6), and NMR spectra were acquired with a Varian nuclear magnetic resonance spectrometer at 400 MHz or 300 MHz for ^{1}H NMR spectra, and at 100 MHz for ^{13}C NMR spectra under standard observation conditions. Mass spectrometric identification of compounds was performed by electrospray ionization mass spectrometry (ESI-MS) with a Micromass AutoSpec Ultima spectrometer. Molecular weights of PAMAM G5 dendrimer and its conjugates were measured by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) with a Waters TOfsPec-2E spectrometer. The MALDI spectra were acquired using a matrix solution of 2,5-dihydroxybenzoic acid (10 mg/ml in 50% aqueous acetonitrile) in a linear mode with a high mass detector. The spectrometer was mass calibrated with BSA in sinapinic acid, and data was acquired and processed using Mass Lynx 3.5 software. UV-vis absorption spectra were recorded on a Perkin Elmer Lamda 20 spectrophotometer.

HPLC analysis was carried out on a Waters Acquity Peptide Mapping System equipped with a Waters photodiode array detector, a column manager that facilitates 4 column housing, and a sample manager (referred to Ultra Performance Liquid Chromatography). For analysis the PAMAM dendrimer conjugates were run on a C4 BEH column (150 x 2.1 mm, 300 Å) connected to Waters Vanguard column. Elution of the conjugates was performed in a linear gradient beginning with 98:2 (v/v) water/acetonitrile (with trifluoroacetic acid at 0.14 wt % in each of the eluents) at a flow rate of 1 mL/min.

Size exclusion chromatography (SEC) was used to measure molecular weights and polydispersity index (PDI) of PAMAM G5 dendrimer. The GPC experiments were performed on an Alliance Waters 2695 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALLS) detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). Columns

employed were TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 mm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 mm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 mm), and G 4000 PW (300 mm × 7.5 mm, 17 mm). Column temperature was maintained at 25 ± 0.1 °C with a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025 wt % sodium azide, pH 2.74, at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL. The weight average molecular weight, Mw, has been determined by GPC, and the number average molecular weight, Mn, was calculated with Astra 5.3.14 software (Wyatt Technology Corporation) based on the molecular weight distribution.

2. Synthesis of **1**

Folic acid-ethylenediamine amide was prepared according to a literature procedure (Whiteley, J.M., Henderson, G. B., Russell, A., Singh, P., Zevely, E. M. *Anal. Biochem.* **1977**, *79*, 42-51). To a stirred solution of folic acid dihydrate (1.0 g, 1.94 mmol) in DMF (120 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (2.0 g, 10.4 mmol). After stirring for 1 h in the dark, 1,2-diaminoethane (1.26 g, 21.0 mmol) was added to the solution prepared early, and the final solution was stirred at rt for 6 h. The mixture was concentrated to dryness *in vacuo* yielding yellow residue. It was dissolved in water (~10 mL), loaded onto a column (silica gel, 50 g), and flash chromatographed by eluting with 50% MeOH/CH_2Cl_2, and then 10% conc. NH_4OH in 50% MeOH/CH_2Cl_2 to elute the product. After evaporation of desired fractions, the product **1** was isolated as yellow solid (0.873 g, 93%). The product material was composed of two regioisomers, α- and γ-amide, and used for next step without separation of the isomers. ^{1}H NMR (400 MHz, D_2O): δ 8.66 (s, 1H), 7.56 (d, 1H, J = 8.8 Hz), 6.58-6.56 (d, 1H, J = 8.8 Hz), 4.50 (s, 2H), 3.25 (m, 2H), 2.68-2.65 (m, 2H), 2.25-2.19 (m, 2H), 2.16-1.8 (m, 2H) ppm. ^{13}C NMR (100 MHz, D_2O): δ 181.53, 178.52, 175.77, 174.63, 172.97, 170.16, 169.26, 163.88, 155.35, 151.13, 150.93, 147.21, 147.09, 129.09, 128.85, 128.04, 121.32, 120.72, 112.47, 112.32, 55.64, 55.04, 54.97, 45.70, 43.61, 41.70, 39.65, 39.50, 33.72, 32.61, 27.72, 27.57 ppm. MS (ESI): *m/z* (relative intensity, %) = 484.19 (100) $[M+H]^+$, 327.11 (51). HRMS (ESI) calcd for $C_{21}H_{26}N_9O_5$ 484.2057, found 484.2067.

3. Synthesis of **2** to **5**

2: Step (i): 4-Formyl-2-methyoxyphenol (Vanillin) was *O*-alkylated to 2-(4-formyl-2-methoxy-phenoxy)acetic acid ethyl ester according to a literature procedure (Spurg, A., Waldvogel, S. R. *Eur. J. Org. Chem.* **2008**, *2*, 337-342). To a solution of Vanillin (10 g, 65.7 mmol) in DMF (100 mL) was added ethyl bromoacetate (10.98 g, 65.7 mmol), and potassium carbonate (18.2 g, 132 mmol). After

stirring the mixture for 17 h at rt under nitrogen atmosphere, it was evaporated to dryness *in vacuo* and the residue was partitioned between water (200 mL) and ethyl acetate (500 mL). The organic layer was collected and dried over Na_2SO_4. Evaporation of the solution afforded 2-(4-formyl-2-methoxy-phenoxy)acetic acid ethyl ester as pale yellow liquid which gradually solidified (11.8 g, 75%). R_f (hexane:EtOAc = 2:1) = 0.25. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.86 (s, 1H), 7.41-7.38 (dd, 1H, J = 2.4, 8.4 Hz), 7.37-7.22 (d, 1H, J = 2.4 Hz), 6.84 (dd, 1H, J = 8.4 Hz), 4.26-4.20 (q, 2H, J = 7.2 Hz), 3.91(s, 3H), 1.22-1.27 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.75, 167.98, 152.49, 149.94, 131.11, 126.06, 112.33, 109.84, 65.91, 61.58, 56.04, 14.08 ppm.

Step (ii): To a solution of 2-(4-formyl-2-methoxy-phenoxy)acetic acid ethyl ester (11.8 g, 49.5 mmol) in a mixture of THF (50 mL) and MeOH (100 mL) was added slowly sodium hydroxide (9.9 g, 248 mol) dissolved in water (100 mL). The mixture was stirred for 33 h at rt, and then evaporated to remove volatile organic solvents *in vacuo*. The aqueous residue was acidified to pH~3 by adding conc HCl (~21 mL) slowly and resulting precipitates were collected on a Büchner funnel. The solid was washed with water, rinsed with ethyl acetate, and dried to afford product **2**, 2-(4-formyl-2-methoxy-phenoxy)acetic acid, as white solid (8.5 g, 82%). ^{1}H NMR (300 MHz, CD_3OD): δ 9.82 (s, 1H), 7.51-7.49 (m, 2H), 7.06-7.03 (d, 1H, J = 7.8 Hz), 4.80 (s, 2H), 3.91 (s, 3H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.89, 153.04, 149.71, 130.62, 125.80, 110.85, 65.42, 56.14, 56.09, 30.93 ppm. MS (ESI, negative ion mode): *m/z* (relative intensity, %) = 209.0 (100) $[M-H]^-$.

3: Step (iii): To a stirred conc. HNO_3 (70%; 100 mL) cooled in ice bath was added acetic acid (10 mL), and 2-(4-formyl-2-methoxy-phenoxy)acetic acid **2** (6 g, 28.4 mmol) as solid. The mixture was stirred at the same temperature for 2 h, and gradually warmed to rt. After stirring for 26 h, the reaction solution was poured into ice (~500 mL), and left overnight at rt while the product was precipitated as bright yellow solid. It was collected, washed with copious volume of water, and dried to afford 2-(4-formyl-2-methoxy-5-nitrophenoxy)acetic acid (4.95 g, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.33 (s, 1H), 7.47 (s, 1H), 7.34 (s, 1H), 4.73 (s, 2H), 3.94 (s, 3H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 189.00, 169.65, 153.06, 150.66, 143.61, 125.65, 110.89, 109.12, 65.92, 56.92 ppm. MS (ESI, negative ion mode): *m/z* (relative intensity, %) = 254.0 (100) $[M-H]^-$.

Step (iv): To a cold solution of 2-(4-formyl-2-methoxy-5-nitrophenoxy)acetic acid (2.6 g, 10.2 mmol) in a mixture of $CHCl_3$ (95 mL) and DMF (5 mL) in ice bath was added 4-dimethylaminopyridine (248 mg, 2.03 mmol), 2-(N-*tert*-butoxycarbonylamino)ethylamine (1.63 g, 10.2 mmol), and finally dicyclohexylcarbodiimide (2.3 g, 11.1 mmol) as solid. The mixture was stirred at 5^{o}C for 2 h and then warmed to rt where it continued to be stirred for 36 h. The mixture was filtered to remove urea byproduct, and the filtrate was evaporated to dryness *in vacuo* yielding pale yellow residue. It was

dissolved in EtOAc (200 mL), and washed with 1M H_3PO_4, saturated $NaHCO_3$, and brine solution. The organic layer was dried over Na_2SO_4, and evaporated to afford 2-(4-formyl-2-methoxy-5-nitrophenoxy)-N-(2-(N-*tert*-butoxycarbonylamino)ethylamine)acetamide as pale yellow solid (3.55 g, 88%). R_f (5% MeOH/CH_2Cl_2) = 0.43. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.40 (s, 1H), 7.63 (s, 1H), 7.41 (s, 1H), 4.61 (s, 2H), 4.01 (s, 3H), 3.42-3.41 (m, 2H), 3.25-3.23 (m, 2H), 1.37 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 187.29, 166.97, 153.76, 127.02, 110.58, 110.15, 110.12, 68.66, 56.66, 49.11, 40.07, 33.89, 28.21, 25.57, 24.85 ppm. MS (ESI): *m/z* (relative intensity, %) = 420.1 (49) $[M+Na]^+$.

Step (v): To a solution of 2-(4-formyl-2-methoxy-5-nitrophenoxy)-N-(2-(N-*tert*-butoxycarbonylamino)ethylamine)acetamide (2.2 g, 5.52 mmol) in a mixture of THF (10 mL) and MeOH (60 mL) was added sodium borohydride (104 mg, 2.75 mmol) slowly. After stirring the mixture for 5 h, the reaction was quenched by adding water (5 mL) and stirring it for 10 min. The mixture was evaporated *in vacuo*, and the residue was dissolved in 5% MeOH in $CHCl_3$ (10 mL). The solution was loaded onto a silica flash column and eluted with MeOH/$CHCl_3$ (2 to 5%). Product **3**, 2-(4-hydroxymethyl-2-methoxy-5-nitrophenoxy)-N-(2-(N-*tert*-butoxycarbonylamino)ethylamine)acetamide, was obtained as pale yellow foam (1.56 g, 71%). R_f (5% MeOH/$CHCl_3$) = 0.37. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.73 (s, 1H), 7.33 (s, 1H), 4.98 (s, 2H), 4.55 (s, 2H), 4.00 (s, 3H), 3.45-3.40 (m, 2H), 3.29-3.25 (m, 2H), 1.40 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.97, 154.52, 145.61, 139.52, 134.34, 112.18, 111.43, 69.21, 62.37, 56.38, 56.36, 40.36, 39.68, 28.24, 24.76 ppm. MS (ESI): *m/z* (relative intensity, %) = 422.2 (100) $[M+Na]^+$, 821.3 (20) $[2M+Na]^+$, 322.1 (13) $[M+Na\text{-}Boc]^+$; HRMS (ESI) calcd for $C_{17}H_{25}N_3O_8Na$ 422.1539, found 422.1533.

4: Step (vi): To a solution of *p*-nitrophenyl chloroformate (148 mg, 0.73 mmol) in THF (5 mL) was added a solution of compound **3** (280 mg, 0.70 mmol) in $CHCl_3$ and diisopropylethylamine (256 μL, 1.47 mmol). After stirring for 12 h at rt, additional amount of *p*-nitrophenyl chloroformate (148 mg, 0.73 mmol) and 4-dimethylaminopyridine (90 mg, 0.74 mmol) were added to the reaction mixture. The mixture was stirred for 4 h more at rt, and concentrated to dryness. The residue was dissolved in EtOAc (100 mL), and washed with 1M H_3PO_4, and saturated $NaHCO_3$. After drying over Na_2SO_4, the organic solution was evaporated *in vacuo*, yielding 2-[2-methoxy-5-nitro-4-[[[(4-nitrophenoxy)carbonyl]oxy]methyl]phenoxy]-N-(2-(N-*tert*-butoxycarbonylamino)ethylamine)acetamide as pale yellow solid (390 mg, 98%). R_f (5% MeOH/$CHCl_3$) = 0.56. ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.24, 154.23, 146.38, 138.69, 126.98, 125.36, 121.66, 112.77, 111.84, 111.02, 68.80, 67.44, 56.52, 28.25 ppm. MS (ESI): *m/z* (relative intensity, %) = 587.1 (100) $[M+Na]^+$.

Step (vii): To a solution of doxorubicin hydrochloride (50 mg, 86.2 μmol) in DMF (6 mL) was added triethylamine (36 μL, 258 μmol) and 2-[2-methoxy-5-nitro-4-[[[(4-

nitrophenoxy)carbonyl]oxy]methyl]phenoxy]-N-(2-(N-*tert*-butoxycarbonylamino)ethylamine)acetamide (49 mg, 86.8 μmol) as solid. The mixture was stirred at rt for 36 h in the dark, and concentrated *in vacuo* affording dark red residue. It was dissolved in a small volume of 3% MeOH/$CHCl_3$ (~4 mL), and loaded onto a flash column (silica gel, ~15 g) followed by elution with MeOH/$CHCl_3$ (3 to 10%). Fractions containing the desired product were collected and evaporated yielding **4** as brick red solid (60 mg, 72%). R_f (10% MeOH/$CHCl_3$) = 0.19. ^{1}H NMR (400 MHz, CD_3OD): δ 7.92 (m, 1H), 7.79-7.69 (m, 2H), 7.49-7.47 (m, 1H), 7.20 (m, 1H), 5.40-5.33 (m, 2H), 5.20-5.13 (m, 1H), 4.67 (m, 2H), 4.49-4.43 (m, 1H), 4.00 (s, 3H), 3.99 (s, 3H), 3.90 (s, 1H), 3.53-3.42 (m, 1H), 3.30-3.18 (m, 4H), 3.17-3.05 (m, 1H), 2.98-2.91 (m, 1H), 2.30-2.26 (m, 1H), 2.14 (m, 1H), 2.02 (m, 1H), 1.98-1.92 (m, 1H), 1.34 (d, 3H, J = 9.6 Hz) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 213.71, 186.67, 161.02, 156.10, 155.59, 135.80, 135.80, 119.83, 118.42, 111.39, 110.73, 69.46, 67.14, 65.47, 63.42, 56.63, 35.60, 33.92, 30.13, 28.26, 18.79, 14.14 ppm. MS (ESI): *m/z* (relative intensity, %) = 991.3 (100) $[M+Na]^+$, 891.3 (15) $[M+Na-Boc]^+$; HRMS (ESI) calcd for $C_{45}H_{52}N_4O_{20}Na$ 991.3073, found 991.3080.

5: Step (viii): To **4** (25 mg, 25.8 μmol) was added a mixture of $CHCl_3$ (1.0 mL) and TFA (0.5 mL). The mixture was stirred at rt for 15 min, and evaporated to dryness *in vacuo*. The product was used immediately for the next coupling step. MS (ESI): *m/z* (relative intensity, %) = 869.2 (14) $[M+H^+]$; HRMS (ESI) calcd for $C_{40}H_{45}N_4O_{18}$ [M+H] 869.2729, found 869.2731.

4. Synthesis of **6** to **9**

6 (PAMAM G5-glutaric acid): To a solution of polyamidoamine (PAMAM) G5 (Dendritech, Inc., Midland, MI; 0.5 g) in MeOH (100 mL) was added triethylamine (0.73 mL, 5.24 mmol) and glutaric anhydride (0.547 g, 4.79 mmol). The mixture was stirred at rt for 48 h, and concentrated to ~5 mL in volume *in vacuo* at rt. After mixed with phosphate-buffered saline solution (without Ca^{2+} and Mg^{2+}; 10 mL), the solution was added into a membrane dialysis tubing (Spectrum® Labs, Inc.; MWCO 10 kDa), and dialyzed extensively against phosphate-buffered saline solution (1×2 L), and deionized water (3×4 L) over 3 days. The solution in the tubing was collected and lyophilized, yielding **6** (PAMAM G5-glutaric acid) as colorless foam (0.576 g). The molecular weight of **6** was characterized by measuring MALDI-TOF and size exclusion chromatography. MS (MALDI-TOF): *m/z* = 40200; gel permeation chromatography: Mn = 40,850, Mw = 42,729, polydispersity index = 1.046. Average number of glutaric acids attached on the surface of PAMAM G5 dendrimer was calculated to be 100: (increase in molecular weight relative to PAMAM G5) ÷ (MW of glutaric anhydride) = [(40200 − 28800) ÷ 114] =

100. ^{1}H NMR (400 MHz, D_2O): δ = 3.28-3.2 (br), 3.14 (br s), 2.9-2.76 (br), 2.74-2.6 (br), 2.42-2.3 (br), 2.09-2.07 (br t), 2.03-1.99 (br t), 1.66-1.62 (br t) ppm.

7 (PAMAM G5-FA-doxorubicin): To a suspension of **6** (PAMAM G5-glutaric acid; 40 mg) in anhydrous DMF (10 mL) was added 4-dimethylaminopyridine (12 mg, 98.3 μmol), *N*-hydroxysuccinimide (12 mg, 104 μmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (20 mg, 104 μmol). The mixture was stirred at rt for 36 h, and followed by the addition of **1** (folic acid-linker; 9.9 mg, 20.0 μmol) and **5** (freshly prepared from 25 mg of **4**, 25.8 μmol). Then triethylamine (54 μL, 387 μmol) was added to the reaction mixture to adjust the solution to be slightly basic (pH~9). The mixture was stirred at rt for 36 h in the dark prior to the addition of water (2 mL). After stirring for 4 h at rt, the mixture was concentrated to a volume of ~2 mL, and diluted with water (15 mL). The solution was added into a into a membrane dialysis tubing (MWCO 10 kDa), and dialyzed extensively against deionized water (4 L), phosphate-buffered saline solution (1×2 L), and deionized water (3×4 L) over 2 days. The solution in the tubing was collected and lyophilized, yielding **7** (PAMAM G5-FA-doxorubicin) as brick red fluffy solid (33 mg). The molecular weight of **7** was characterized by measuring MALDI-TOF: *m/z* = 48000. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.46 (br), 7.9 (br), 7.65-7.55 (br m), 7.4 (br), 7.40-7.15 (br m), 6.70 (br), 5.25 (br), 4.7 (br), 4.51 (br), 3.90-3.80 (br m), 3.2-2.8 (br s), 2.6 (br s), 2.4 (br s), 2.35 (br), 2.2-2.0 (br m), 1.8 (br s), 1.1-0.95 (br) ppm. Average number of folate ligand and doxorubicin attached to the surface of PAMAM G5-glutaric acid was estimated to be ~8 (**1**) and ~4 (**5**) respectively from the analysis of the relative ratio between **1** and **5** in ^{1}H NMR spectral data in combination with their contribution to the increase in molecular weight of **7** relative to **6** (Δ = 48000 − 40200 = 7800).

8 (PAMAM G5-FA-doxorubicin-FITC): The conjugate was prepared following the procedure as described in the synthesis of **7** but further conjugated with a fluorescent molecule, fluoresceinisothiocyanate (FITC)-NH$(CH_2)_4$-NH_2 (prepared as described elsewhere: Gapski, G. R.; Whiteley, J. M.; Rader, J. I.; Cramer, P. L.; Hendersen, G. B.; Neef, V.; Huennekens, F. M. Synthesis of a fluorescent derivative of amethopterin. *Journal of Medicinal Chemistry* **2002,** 18, 526-528). To a suspension of **6** (PAMAM G5-glutaric acid; 50 mg) in anhydrous DMF (15 mL) was added 4-dimethylaminopyridine (15 mg, 123 μmol), *N*-hydroxysuccinimide (15 mg, 130 μmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (24 mg, 125 μmol). The mixture was stirred at rt for 36 h, and followed by the addition of **5** (prepared from 13.2 mg of **4**, 13.6 μmol) and FITC-NH$(CH_2)_4$-NH_2 (3.6 mg, 7.5 μmol). Then triethylamine (9 μL, 65 μmol) was added to the reaction mixture to adjust the solution to be slightly basic (pH~9). The mixture was stirred at rt for 24 h in the dark, and then **1** (6 mg, 12.1 μmol) dissolved in DMF (2 mL) was added. After stirring for 24 h, water

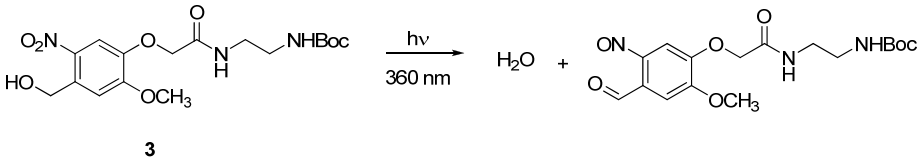
(2 mL) was added to quench the coupling reaction. After stirring for 4 h, the mixture was concentrated to a volume of ~2 mL, and diluted with water (15 mL). The solution was added into a into a membrane dialysis tubing (MWCO 10 kDa), and dialyzed extensively against deionized water (4 L), phosphate-buffered saline solution (1×2 L), and deionized water (3×4 L) over 2 days. The solution in the tubing was collected and lyophilized, yielding **8** as brick red solid (31 mg). The molecular weight of **8** was characterized by measuring MALDI-TOF: *m/z* = 48800. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.55 (br s), 8.15-7.7 (br m), 7.6-7.5 (br m), 7.2-7.15 (m), 7.1 (m), 6.8 (br), 6.6-6.5 (br m), 5.3-5.2 (br), 4.5 (br s), 4.4 (br m), 4.0-3.4 (br), 3.2-2.8 (br), 2.6-2.5 (br m), 2.4-2.3 (br), 2.2-1.9 (br m), 1.8 (br m) ppm. UV-vis spectroscopy (PBS, pH 7.2): 498, 354, 283 nm. Mean value of folate ligand, doxorubicin, and FITC attached to the surface of PAMAM G5-glutaric acid was estimated to be 6.4 (**1**), 5.9 (**5**), and 1.2 (FITC) respectively from the analysis of UV-vis, and MALDI-TOF mass spectroscopic data.

9 (PAMAM G5-doxorubicin-FITC): The conjugate was prepared according to the procedure as described in the synthesis of **8** under identical conditions, only different that **1** was not added. Starting with 50 mg of **6** (PAMAM G5-glutaric acid) afforded **9** as brick red solid (27 mg). MALDI-TOF: *m/z* = 45800. ^{1}H NMR (400 MHz, D_2O): δ = 7.7-7.2 (br m), 7.1-6.6 (br), 6.5-6.2 (br m), 5.2-5.0 (br), 4.5-4.2 (br m), 4.0 (br), 3.8-3.5 (br m), 3.4-2.0 (br m), 1.8-1.6 (br m), 1.4 (br), 1.0 (br) ppm. UV-vis spectroscopy (PBS, pH 7.2): 498, 354, 283 nm. Mean value of doxorubicin, and FITC attached to the surface of PAMAM G5-glutaric acid was estimated to be 5.9 (**5**), and 1.2 (FITC) respectively from the analysis of UV-vis, and MALDI-TOF mass spectroscopic data.

5. Photolysis experiments of **3** and **7**

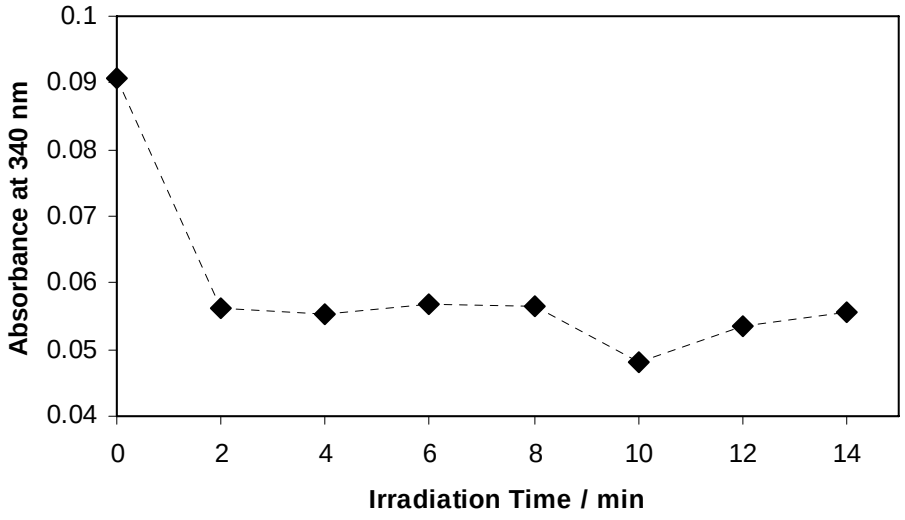
Photolysis experiments were carried out using Spectroline® UV bench lamps (XX-15A) at long wavelength (320-400 nm) with a maximum intensity at 365 nm. Linker **3** or PAMAM G5 nanoconjugate **7** was dissolved in phosphate-based saline solution (PBS, pH 7.2) unless specified otherwise. Each of the solution contained in a Petri dish was placed without any cover under UV lamps at the distance of ~ 5 cm from lamps, and irradiated over up to 14 min. Progress of the photolysis was monitored by analyzing an aliquot (700 μL) from each irradiated solution at specific time by UV-vis spectrometry and analytical HPLC.

5a. UV-vis absorption spectra of photolinker **3** before and after UV exposure. An aqueous solution of **3** (33 μM in 0.5% MeOH/H_2O) was exposed to long wavelength UV over specified time (t = 0 to 12 min) and measured for UV-vis spectra.



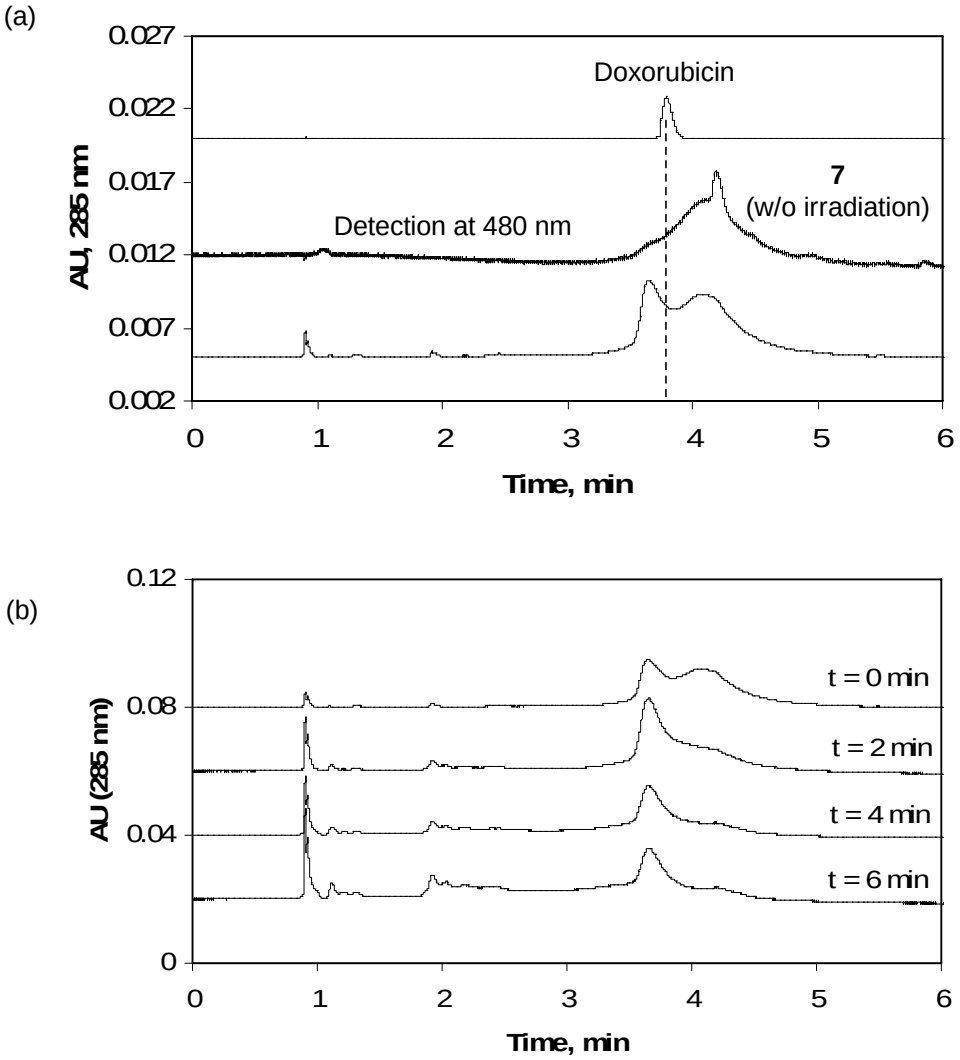
3

5b. Time course of photolysis of **3**. A plot of absorbance at λ_{max} (340 nm) against irradiation time.



5c. Photolysis of PAMAM G5-FA-doxorubicin (**7**). An aqueous solution of **7** (3.88 μM in PBS, pH 7.2) was exposed to long wavelength UV over variable time and the progress of photolysis was analyzed by analytical HPLC. (a) HPLC chromatograms for doxorubicin, and conjugate **7** before irradiation (t = 0 min). The chromatogram monitored at 480 nm, the wavelength specific for doxorubicin, is shown in the

middle, indicating an uneven distribution of PAMAM doxorubicin dendrimers; (b) HPLC chromatograms after UV irradiation (t = 2 to 6 min). Please note that the current HPLC method allowed to monitor the progress of the photolysis over time, but was unable to separate doxorubicin from a distribution of dendrimer conjugates, both assigned to a broad peak.



6. *In vitro* cell based assays

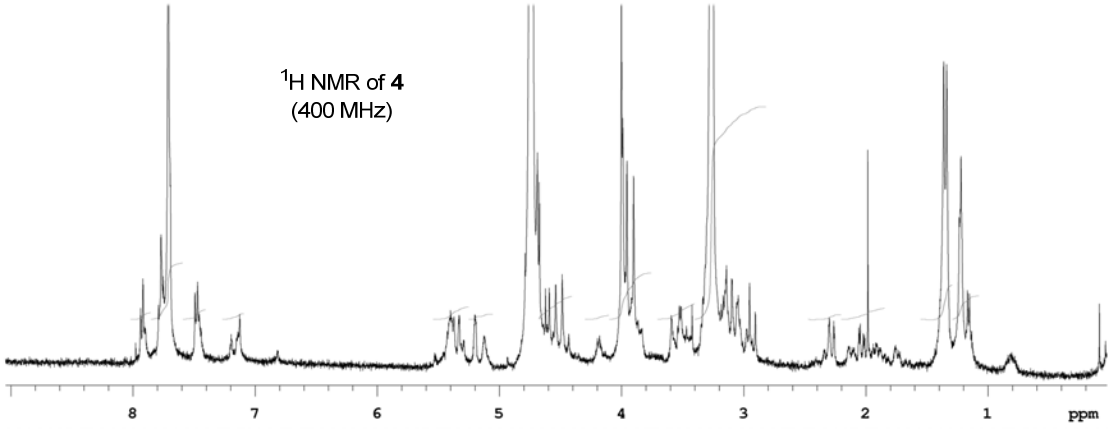
In vitro cytotoxicity assay. It was measured in KB cells, a sub-line of the cervical carcinoma HeLa cells (ATCC, Manassas, VA, USA). The cells were grown as a monolayer cell culture at 37 °C and 5% CO_2 in folic acid-deficient RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The 10% FBS provided folic acid concentration equivalent to that is present in the human serum (~20 nM). Cells were seeded in 96-well microtiter plates (3000 cells/well) and two days after plating, they were rinsed with phosphate buffered saline containing 0.1% bovine serum albumin (PBSB). The cells were then covered with 50 μL of each PBSB in the presence or absence of 3 μM each of **7**

(PAMAM G5-FA-Dox) or doxorubicin. The plates were exposed to UV light (Spectroline® UV bench lamp XX-15A: 320-400 nm, power = 1.1 mW/cm^2) kept ~ 1 cm above from the bottom of the plate for different time periods. The control (zero time) plate was incubated under normal light in parallel. Following the exposure to light, the cells were supplemented with 150 µL each of RPMI medium containing 10% serum and incubated for 24 h, changed to fresh medium and allowed to grow for another 4 days. For cytotoxicity measurement, a colorimetric XTT (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; Roche Molecular Biochemicals, Indianapolis, IN) assay was performed following the vendor's protocol. After incubation with the XTT labeling mixture, the microtiter plates were read on an ELISA reader (Synergy HT, BioTek) at 492 nm with the reference wavelength at 690 nm.

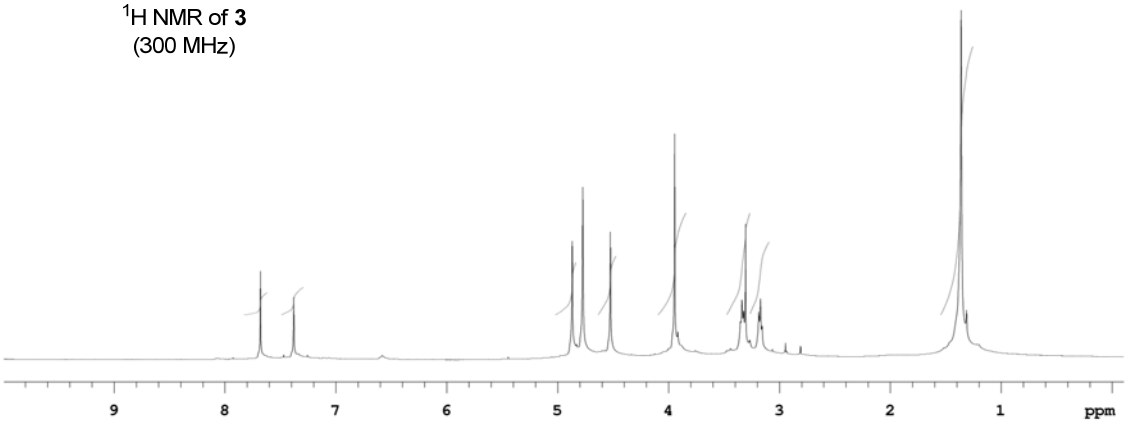
Flow cytometry. KB Cells were incubated in the presence of different concentrations of the dendrimer conjugate for 2 h and the mean FL1 fluorescence of 10,000 cells were taken by flow cytometry. Some cells were pre-incubated with 50 uM of free FA for 15 min prior to adding the conjugates as described in the Figure 3 legends.

Confocal fluorescence microscopy. KB Cells were incubated in the presence of 300 nM of the indicated dendrimer conjugates for 2 h and the cells were fixed and stained for DAPI. The DAPI (blue) and FITC (green) fluorescence were measured using a confocal microscope. Some cells were pre-incubated with 50 uM of free FA for 15 min prior to adding the dendrimer conjugates.

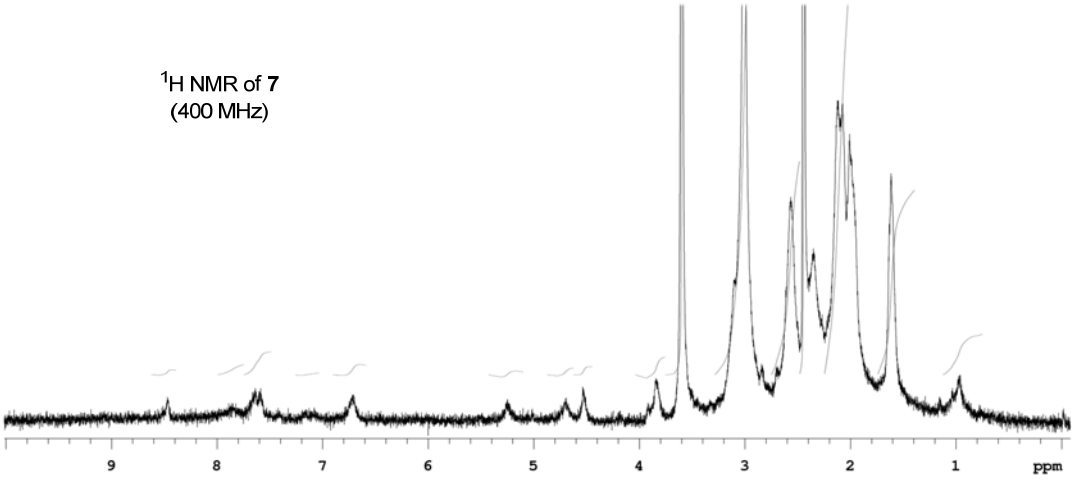
7a. ^{1}H NMR spectra of **4**, **3**, and **7**, and HRMS of **5**.



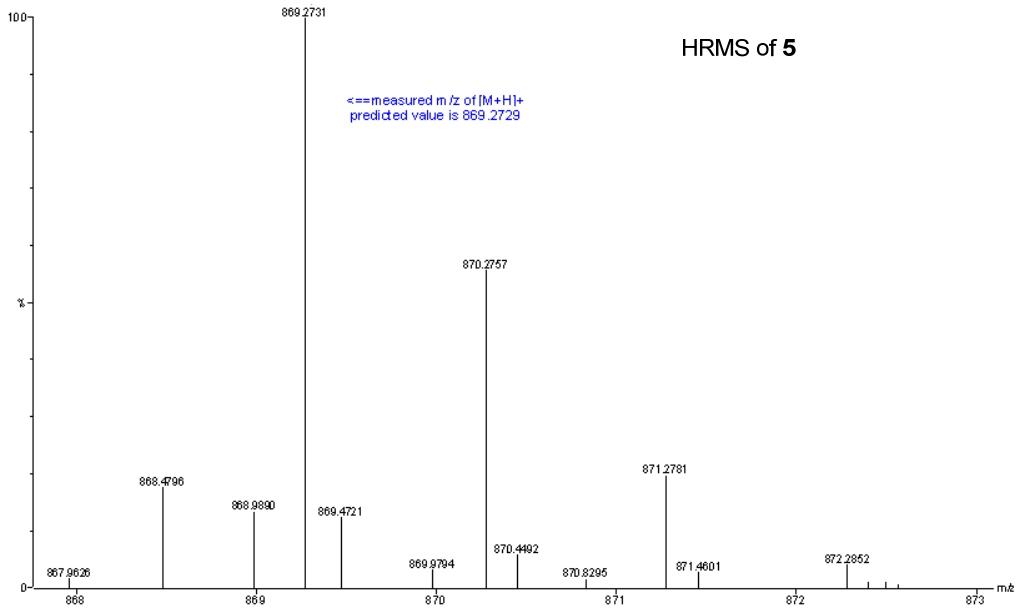
^{1}H NMR of **3**
(300 MHz)



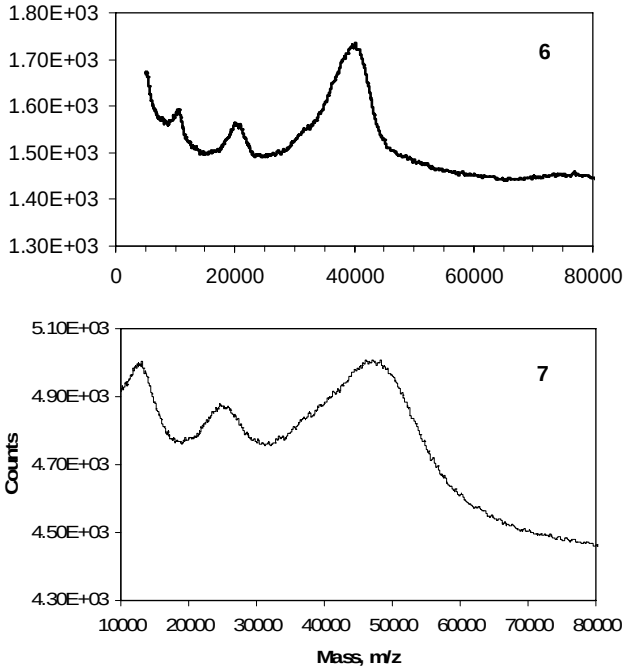
^{1}H NMR of **7**
(400 MHz)

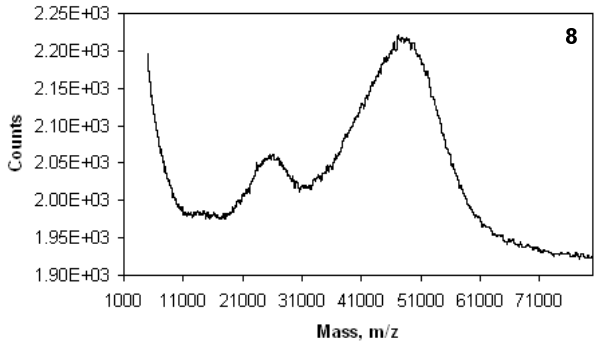


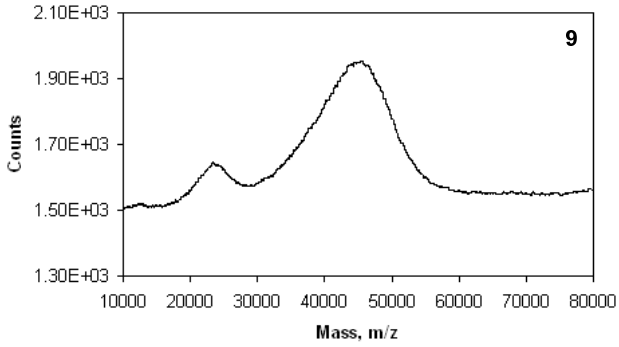
HRMS of **5**



7b. MALDI-TOF mass spectra of PAMAM G5-glutaric acid **6** and its doxorubicin conjugates **7** to **9**







7c. GPC trace of PAMAM G5-glutaric acid **6**: detection by light scattering (red) and refractive index (blue).