PAMAM Dendrimer-Based Multifunctional Conjugate for Cancer Therapy: Synthesis, Characterization, and Functionality

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Poly(amidoamine) (PAMAM) dendrimer-based multifunctional cancer therapeutic conjugates have been designed and synthesized. The primary amino groups on the surface of the generation 5 (G5) PAMAM dendrimer were neutralized through partial acetylation, providing enhanced solubility of the dendrimer (in conjugation of FITC (fluorescein isothiocyanate)) and preventing nonspecific targeting interactions (in vitro and in vivo) during delivery. The functional molecules fluorescein isothiocyanate (FITC, an imaging agent), folic acid (FA, targets overexpressed folate receptors on specific cancer cells), and paclitaxel (taxol, a chemotherapeutic drug) were conjugated to the remaining nonacetylated primary amino groups. The appropriate control dendrimer conjugates have been synthesized as well. Characterization of the G5 PAMAM dendrimer and its nanosize conjugates, including the molecular weight and number of primary amine groups, has been determined by multiple analytical methods such as gel permeation chromatography (GPC), nuclear magnetic resonance spectroscopy (NMR), potentiometric titration, high-performance liquid chromatography **(**HPLC), and UV spectroscopy. These multifunctional dendrimer conjugates have been tested in vitro for targeted delivery of chemotherapeutic and imaging agents to specific cancer cells. We present here the synthesis, characterization, and functionality of these dendrimer conjugates.

Introduction

Paclitaxel (Taxol) is a novel anticancer drug which induces apoptosis by binding to microtubules within a dividing cell during mitosis, causing kinetic stabilization and thereby preventing cell division through mitotic arrest.[1,2] Taxol inhibits microtubule depolymerization, thus encouraging tubulin assembly into stable aggregate structures.[2] So far, microtubule damage has been the only recognized means of cellular activity by Taxol currently identified, and this is not specific for cancer cells. It has been indicated by other researchers, however, that there may be other methods of Taxol-induced apoptosis.[1] A variety of conjugates have been synthesized with the goal of increasing the specificity of Taxol to cancer cells. Multifunctional polymer−Taxol drug delivery systems are also being widely evaluated, as their ability to target overexpressed genes or receptors found on some cancerous cells helps greatly lessen or eliminate undesirable side effects to the rest of the body.[2−5] A bifunctional therapeutic dendrimer conjugate has been synthesized containing chemotherapeutic Taxol and herceptin (trastuzumab), which works by utilizing herceptin (HER) to target the overexpressed HER2 gene found at abnormally high levels in 25−30% of breast cancers, to stop cell growth.[6−8] Covalent attachment of the cytotoxic drug to a macromolecular polymeric carrier creates a prodrug, which is inactive until cleavage of the drug from the carrier or solvation of the carrier within the cellular matrix of a malignant cell, which in turn frees (thereby activating) the drug.[2] Macromolecular polymeric carriers such as dendrimers have been found to be an effective approach to drug delivery.[9−16] We propose to conjugate Taxol to dendrimers for drug delivery. The chemical structure of Taxol (Figure 1) shows the active 2′ position −OH group (arrows) where attachment to the dendrimer is proposed to occur.

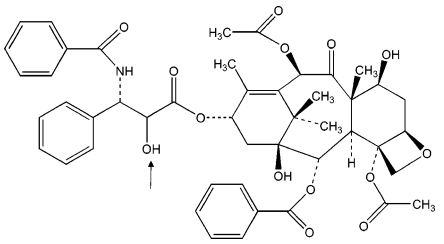

Figure 1. Chemical structure of Taxol, displaying the 2′ active −OH group used for attachment to a dendrimer conjugate.

Described within this paper is the design, synthesis, and characterization of a polymer-based, multifunctional engineered conjugate for targeted cancer therapy utilizing Taxol as the chemotherapeutic drug. In vitro studies are described as the second part of this research.

Experimental Section

Materials and Methods. The G5 (generation 5) PAMAM (poly(amidoamine))dendrimer was synthesized and characterized at the Center for Biologic Nanotechnology, University of Michigan, under cGMP-compatible conditions.

MeOH (HPLC grade), acetic anhydride (99%), triethylamine (99.5%), DMSO (99.9%), fluorescein isothiocyanate (98%), glycidol (racemic form, 96%), DMF (99.8%), 1-[3-(dimethylamino)-propyl-3-ethylcarbodiimide HCl (EDC) (98%), citric acid (99.5%), sodium azide (99.99%), D_2O, NaCl, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Aldrich Co. and used as received. The folic acid (98%) was from Sigma. Spectra/Por dialysis membrane (MWCO 3500), Millipor Centricon ultrafiltration membrane

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YM-10, and phosphate buffer saline (PBS, pH 7.4) were from Fisher. Succinic anhydride, *N*-hydroxysuccinimide, pyridine, CH_2Cl_2, silica gel, EtOAc, diphenylphosphoryl chloride, Et_2O, $MgSO_4$, acetonitrile, and Taxol were used as purchased from Sigma Aldrich.

Potentiometric Titration. Titration was carried out manually using a Mettler Toledo MP230 pH Meter and MicroComb pH electrode at room temperature, 23 ± 1 °C. A volume of 10 mL of a solution of 0.1 M NaCl was added to a precisely weighed 100 mg sample of PAMAM dendrimer to shield amine group interactions. Titration was performed with 0.1028 N HCl, and 0.1009 N NaOH was used for back-titration. The numbers of primary and tertiary amines were determined from back-titration data.

Gel Permeation Chromatography. Gel permeation chromatography experiments were performed on an Alliance Waters 2690 Separation Module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt Dawn DSP laser photometer, an Optilab DSP interferometric refractometer (Wyatt Technology Corporation), and with TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 μm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 μm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 μm), and G 4000 PW (300 mm × 7.5 mm, 17 μm) columns. Column temperature was maintained at 25 ± 0.1 °C by a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025% sodium azide, pH 2.74, at a flow rate of 1 mL/min. Sample concentration was 10 mg/5 mL with an injection volume of 100 μL. Molecular weight, molecular weight distribution, and rms radius of the PAMAM dendrimers and its conjugates were determined using Astra 4.7 software (Wyatt Technology Corporation).

Nuclear Magnetic Resonance Spectroscopy. ^1H and ^{13}C NMR spectra were taken in D_2O and were used to provide integration values for structural analysis by means of a Bruker AVANCE DRX 500 instrument.

UV Spectrophotometry. UV spectra were recorded (in PBS) using a Perkin-Elmer UV−vis spectrometer Lambda 20 and Lambda 20 software.

Cell Culture and Treatment. The KB cell line (ATCC, Manassas, VA) is a human epidermoid carcinoma that overexpresses folate receptors, especially when grown in low folic acid medium.[17] The KB cells were grown continuously as a monolayer at 37 °C and 5% CO_2 in folic acid-deficient RPMI 1640 medium. This medium was supplemented with penicillin (100 units/mL), streptomycin (100 μL/mL), and 10% heat-inactivated FBS, yielding a final folic acid concentration approximately that of normal human serum. Approximately 2×10^4 cells per well were seeded the day before experiments in 12-well plates, either with complete medium (KB folate receptor down-regulated cells) or folic acid-deficient medium (KB folate receptor up-regulated cells). An hour before each experiment, the cells were washed with their respective media, then 500 μL of either the complete medium or folic acid-deficient medium was put in each well. An hour later, the cells were treated with either G5−Ac^3−FITC−FA−OH−Taxol^e, the control dendrimer conjugate G5−Ac^3−FITC−FA, or free Taxol (FITC, fluorescein isothiocyanate; FA, folic acid; Ac, acylated; Taxol^e, Taxol conjugated through an ester (^{e}) bond). After 1 h of treatment, the cells were washed with PBS and fresh medium was added to each well. After incubation for an additional 72 h, the cells were harvested and washed with PBS containing 0.1% bovine serum albumin (BSA) before analysis by flow cytometry. In some experiments, the KB cells were treated for 72 h and then analyzed.

Flow Cytometric Analysis. To estimate the cell death, the KB cells were incubated with propidium iodide (1.25 μg/mL) for 5 min at room temperature. The dead cells are not able to exclude propidium iodide dye, and thereby dye binds to cellular nucleic acids generating red fluorescence in the cells; on contrary, living cells exclude propidium iodide and remain nonfluorescent. After incubation with propidium iodide cells were acquired on a Beckman-Coulter EPICS-XL MCL flow cytometer, and data were analyzed using Expo32 software (Beckman-Coulter, Miami, FL).

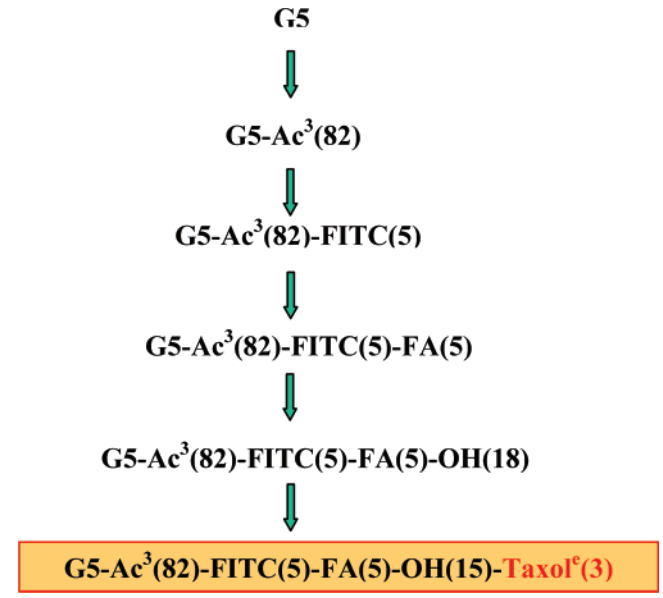

Figure 2. Synthetic strategy for production of functional dendrimer conjugates with imaging (FITC, fluorescein isothiocyanate), targeting (FA, folic acid), and cytotoxic drug (Taxol, paclitaxel) units (G5, poly(amidoamine) dendrimer generation 5; Ac, acetamide; OH, hydroxyl).

Cytotoxicity Assay. For cytotoxicity experiments, KB cells (2×10^3 per well) were seeded in 96-well microtiter plates in complete or folic acid-deficient media for 24 h before the treatment. An hour before each experiment, the cells were washed with the respective media, then 100 μL of either complete medium or folic acid-deficient medium was put in each well. One hour later, the cells were treated with either G5−Ac^3−FITC−FA−OH−Taxol^e, the control dendrimer conjugate G5−Ac^3−FITC−FA, or free Taxol for an additional 60 min. After incubation, the cells were washed to remove the treatment and further incubated in their respective media for an additional 120 h. An XTT assay was performed using a Cell Proliferation kit II from Roche Diagnostics Corporation (Indianapolis, IN) to assess cell viability following the manufacturer's assay procedure. The XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) assay is utilized to quantify cell viability based on the enzymatic conversion of XTT to formazan by active mitochondria of live cells. After incubation with an XTT labeling mixture, microtiter plates were read on an ELISA reader (Spectra Max 340, Molecular Devices, Sunnyvale, CA) at 492 nm with the reference wavelength at 650 nm.

Synthesis. The synthetic strategy for production of dendrimer conjugates presented in Figure 2 details the method used for production of the multifunctional engineered dendrimer conjugate utilizing Taxol as the chemotherapeutic drug. UV, NMR, and gel permeation chromatography have been utilized in order to fully characterize the intermediates and final dendrimer conjugate.

1. G5 Carrier. The G5 PAMAM dendrimer was synthesized and characterized at the Center for Biologic Nanotechnology, University of Michigan. PAMAM dendrimers are highly branched macromolecules composed of an ethylenediamine (EDA) core with four dendron arms branching radially from it. Exhaustive Michael addition of methyl acrylate (MA) and condensation (amidation) of the resulting ester with large excesses of EDA produces each successive generation.[13,14] Each consecutive reaction sequence theoretically doubles the number of surface amino groups, to which functional molecules can be attached.[14] The molecular weight of the G5 PAMAM dendrimer as determined by gel permeation chromatography is 26 530 g/mol, and the average number of primary amino groups was determined by potentiometric titration to be 110. This means that this dendrimer structure differs from the theoretical one.

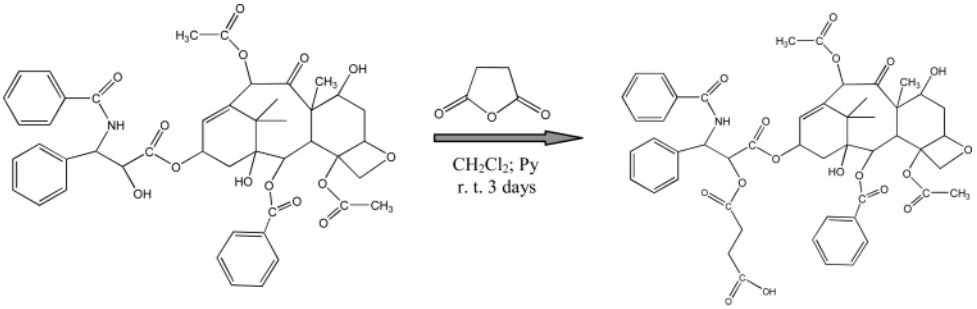

Figure 3. Synthesis of Taxol-2′-hemisuccinate.

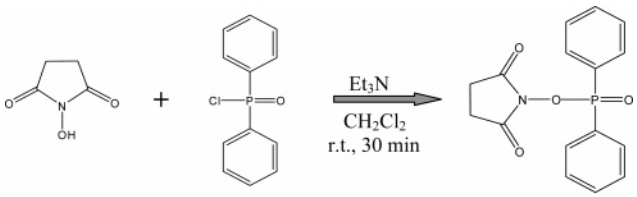

Figure 4. *N*-Hydroxysuccinimido diphenyl phosphate formation.

2. G5−Ac^3(82). An amount of 2.38696 g (8.997×10^{-5} mol) of G5 PAMAM dendrimer in 160 mL of absolute MeOH was allowed to react with 679.1 μL (7.198×10^{-3} mol) of acetic anhydride in the presence of 1.254 mL (8.997×10^{-3} mol, 25% molar excess) of triethylamine. After dialysis, membrane filtration, and lyophilization, 2.51147 g (93.4%) of G5−Ac^3(82) product was yielded. The average number of acetyl groups (82) has been determined based on ^{1}H NMR calibration.[19]

3. G5−Ac^3(82)−FITC. An amount of 1.16106 g (3.884×10^{-5} mol) of G5−Ac^3(82) partially acetylated PAMAM (MW = 29 880 g/mol by gel permeation chromatography) in 110 mL of absolute DMSO was allowed to react with 0.08394 g (90% pure) (1.94×10^{-4} mol) of FITC under nitrogen overnight. After dialysis, and lyophilization, 1.10781 g (89.58%) of G5−Ac^3(82)−FITC product was yielded. Further purification was taken by membrane filtration using PBS and DI water, followed by lyophilization.

4. G5−Ac^3(82)−FITC−FA. An amount of 0.03756 g (8.51×10^{-5} mol) of FA (MW = 441.4 g/mol) was allowed to react with 0.23394 g (1.22×10^{-3} mol) of EDC (1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide HCl; MW = 191.71 g/mol) in a mixture of 27 mL of dry DMF and 9 mL of dry DMSO under nitrogen atmosphere for 1 h. Then this organic reaction mixture was added dropwise to the DI water solution (100 mL) of 0.49597 g (1.55×10^{-5} mol; MW = 32 150 g/mol) G5−Ac^3(82)−FITC. The reaction mixture was vigorously stirred for 2 days. After dialysis, and lyophilization, 0.5202 g (98.1%) of G5−Ac^3(82)−FITC−FA was obtained. Further purification was taken by membrane filtration using PBS and DI water, followed by lyophilization.

5. G5−Ac^3(82)−FITC−FA−OH. An amount of 0.29597 g (8.63×10^{-6} mol) of G5−Ac^3(82)−FITC−FA partially acetylated PAMAM dendrimer conjugate (MW = 34 710 g/mol by gel permeation chromatography) in 200 mL of DI water was allowed to react with 25.8 μL (3.9×10^{-4} mol, 25% molar excess) of glycidol (MW = 74.08 g/mol) for 3 h. After dialysis, lyophilization, and repeated membrane filtration, 0.27787 g (90.35%) of fully glycidylated G5−Ac^3(82)−FITC−FA−OH product was yielded.

6. Taxol-2′-hemisuccinate.[20] To a stirred solution of 25 mg of Taxol and 3.6 mg (1.23 equiv) of succinic anhydride in 1.5 mL of CH_2Cl_2 at room temperature, 3.4 μL (~10-fold molar excess) of dry pyridine was added. The reaction mixture was stirred for 3 days at room temperature and then concentrated in vacuum. The residue was dissolved in 1 mL of CH_2Cl_2, and the Taxol-2′-hemisuccinate was purified on silica gel (and washed with hexane, followed by elution with ethyl acetate) to give 27.2 mg of product (97.5%). Figure 3 presents the synthesis of Taxol-2′-hemisuccinate.

7. *N*-Hydroxysuccinimido Diphenyl Phosphate. This was synthesized as described in ref 21 (see Figure 4).

8. Taxol−NHS Ester. The procedure for synthesis of Taxol−NHS ester was followed as described in ref 20. A volume of 15 μL (4.3 equiv) of triethylamine was added to a solution of 27.2 mg of Taxol−hemisuccinate and 1.5 mol equiv of SDPP in 1.5 mL of acetonitrile. The reaction was stirred for 6 h at room temperature, than concentrated under vacuum. The residue was dissolved in 2.5 mL of ethyl acetate and 1 mL of hexane and purified on silica gel. SDPP gave superior yields in less time and under milder conditions than did any carbodiimide-coupling reagent. The purified Taxol−NHS ester was dried for 24 h in vacuum at room temperature to give 29.37 mg (98.0%). Figure 5 presents the synthesis of the Taxol−NHS ester.

9. Synthesis of G5−Ac^3−FITC−FA−OH−Taxol^e. In 9 mL of DMF and 3 mL of DMSO solvent mixture, 0.00609 g (5.79×10^{-6} mol) of Taxol−NHS and 0.01554 g (8.1×10^{-5} mol) of 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC) were dissolved, kept at room temperature, and stirred for 2 h under nitrogen. This solution was added dropwise to a DI water solution of 0.02001 g (5.67×10^{-7} mol) of the bifunctional dendritic carrier G5−Ac^3−FITC−FA−OH. This reaction mixture was stirred at room temperature for 3 days. After membrane filtration using PBS and DI water, and lyophilization, 0.01984 g (75%) of the trifunctional nanodevice was yielded and analyzed by H^1 NMR and gel permeation chromatography.

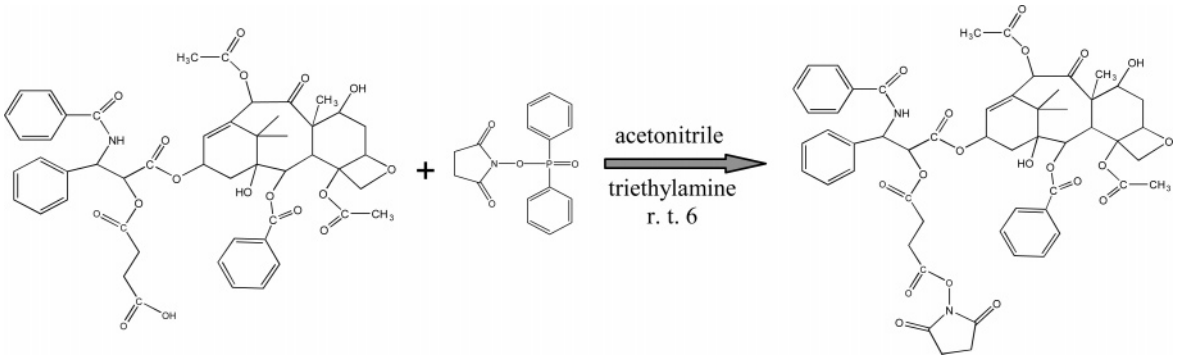

Figure 5. Synthesis of the Taxol-hemisuccinate-*N*−succinimidyl ester.

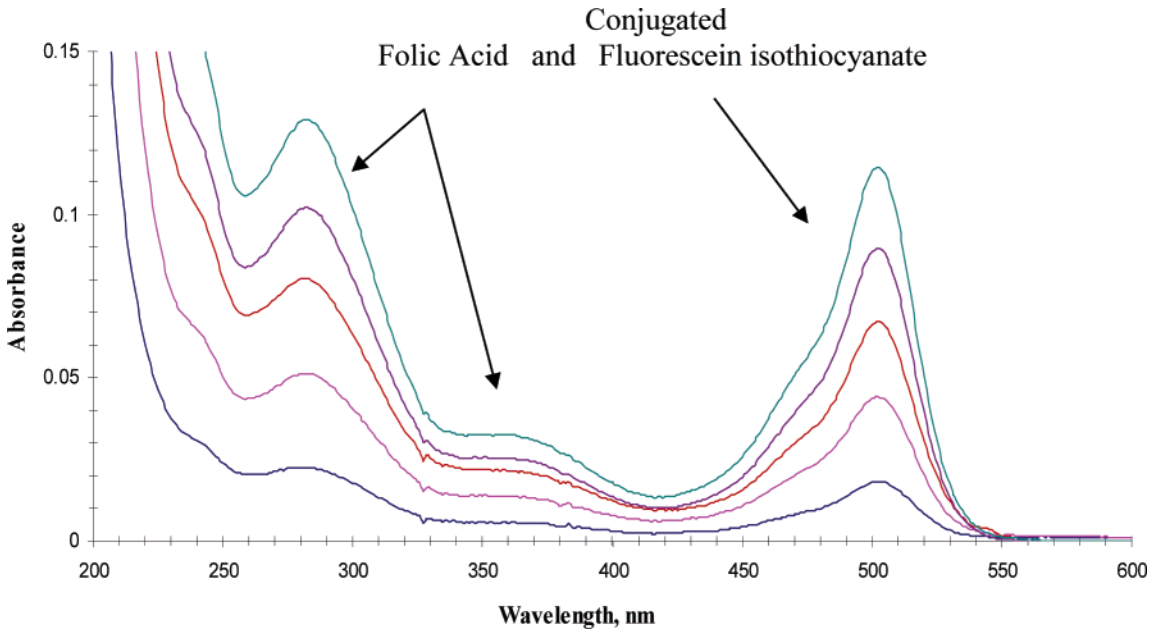

Figure 6. Absorbance vs wavelength relationship for G5−Ac^{3}−FITC−FA−OH in PBS at different concentrations.

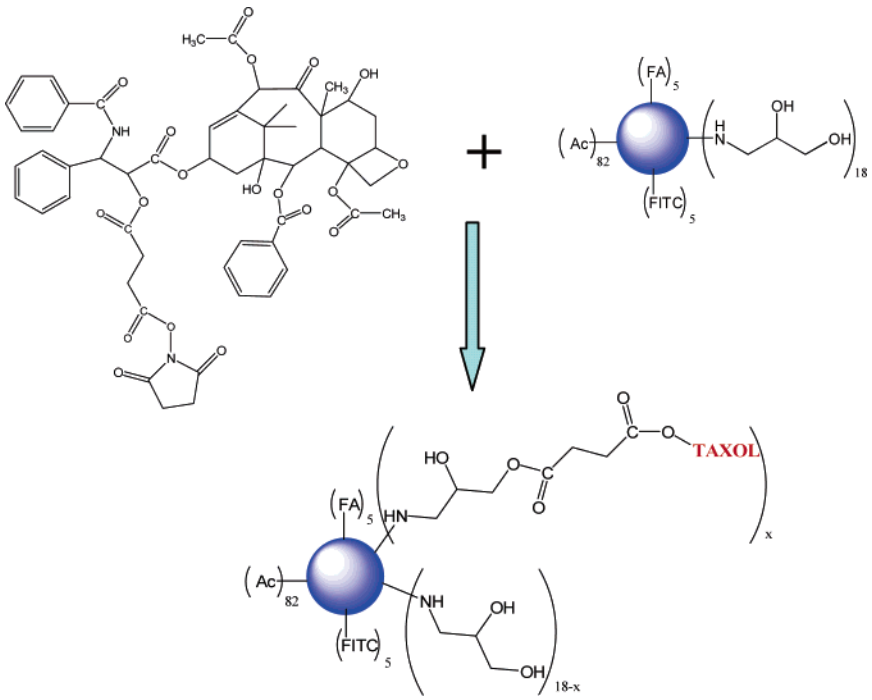

Figure 7. Conjugation of Taxol to the carrier G5−Ac^{3}−FITC−FA−OH, forming the trifunctional dendrimer conjugates G5−Ac^{3}−FITC−FA−OH−Taxol^{e}.

Results and Discussion

Our goal was to produce a working, proof-of-principle example of a single macromolecule-based therapeutic agent with three functions. We synthesized and performed in vitro testing on the trifunctional dendrimer conjugate G5−Ac^{3}−FITC−FA−OH−Taxol^{e}, which employs FITC as an imaging unit, folic acid as a folate receptor targeting agent, and Taxol as a therapeutic drug.

The G5 PAMAM dendrimer was synthesized and characterized at the Center for Biologic Nanotechnology, University of Michigan, under cGMP conditions. Precise characterization of the dendrimer allowed for the proper stoichiometry of the ensuing reactions to be determined. Potentiometric titration was used to determine the number of primary surface amino groups (110) available for conjugation to various functional molecules. The G5 dendrimer was partially acetylated before attachment of FITC (forming G5−Ac^{3}) to prevent solubility difficulties after FITC conjugation. The average number of acetyl groups (82) was determined based on H^{1} NMR calibration.[19] FITC was attached through a thiourea bond to the carrier. The number of attached FITC (5) was determined by examination of H^{1} NMR, UV, and GPC. Folic acid was conjugated to the partially acetylated monofunctional dendrimer conjugate via condensation between the γ- carboxyl group of folic acid and the primary amino groups of the dendrimer, forming G5−Ac^{3}−FITC−FA. NMR, UV, and GPC were used to confirm the number of folic acid molecules attached to the dendrimer. The broadening of the aromatic proton peaks in the G5−Ac^{3}(82)−FITC−FA NMR spectrum (not shown, but similar to Figure 4 in the Supporting

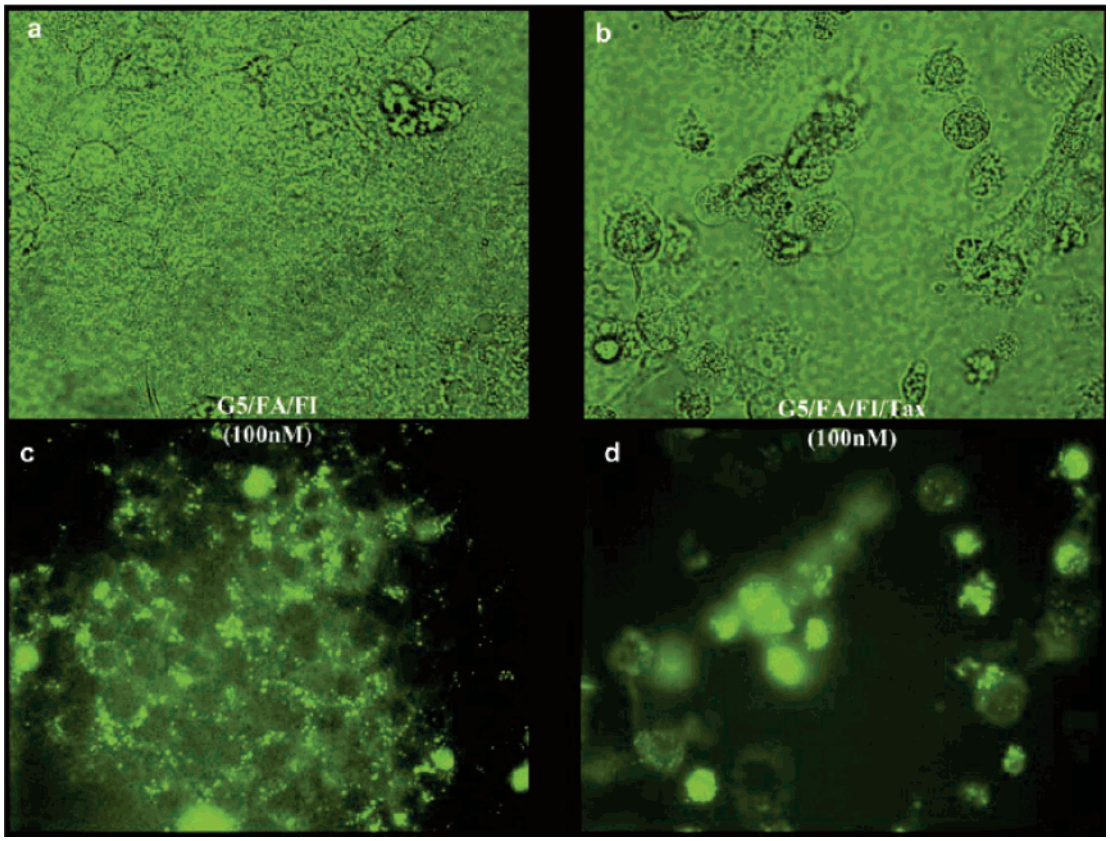

Figure 8. KB (FR+) cells treated with either 100 nM G5−Ac^{3-}FITC−FA (a and c) or 100 nM G5−Ac^{3-}FITC−FA−OH−Taxol^{e} (b and d). Cells were treated with dendrimer conjugates for 60 min, then washed and incubated for 72 h before microscopic examination. Targeted KB (FR+) cells examined after treatment by fluorescent microscopy. Panels a and b show the visible light image of panels c and d, respectively. The cells treated with the drug-free G5−Ac^{3-}FITC−FA bind to the KB cells but are nontoxic (panel c). The cells treated with the drug containing G5−Ac^{3-}FITC−FA−OH−Taxol^{e} bind to the KB cells and kill them (panel d). They are rounded and detached from the bottom of the plate.

Table 1. Molecular Weights and Molecular Weight Distribution for the PAMAM Dendrimer Carrier and Its Mono-, Bi-, and Trifunctional Dendrimer Conjugates

	$\bar{M}_n$ (g/mol)	$\bar{M}_w$ (g/mol)	$\bar{M}_w/\bar{M}_n$
G5	26530	26890	1.020
G5−Ac^3	29880	30760	1.030
G5−Ac^{3-}FITC	32150	32460	1.100
G5−Ac^{3-}FITC−FA	34710	35050	1.010
G5−Ac^{3-}FITC−FA−OH	36820	37390	1.016
G5−Ac^{3-}FITC−FA−OH−Taxol^{e}	41180	43110	1.047

Information) indicates the presence of a covalent bond between the folic acid and the dendrimer. If folic acid or other small molecules are not covalently attached, due to sample impurities, sharp aromatic peaks appear. On the basis of the integration values of the methyl protons in the acetamide groups and the aromatic protons in the folic acid, the average number of attached folic acid molecules was calculated to be 4.5. UV spectroscopy, utilizing the free folic acid concentration calibration curve, determined the number of folic acid molecules to be 4.8. G5−Ac^{3-}FITC−FA was fully glycidylated, yielding the attachment of 18 OH. Attachment of glycidol was a necessary precursor step to the attachment of Taxol through an ester linkage. Conversion of the remaining primary amino groups to OH prevents nonspecific targeting during delivery.[16] The average number of attached OH was calculated using gel permeation chromatography data. The absorbance versus wavelength relationship for the bifunctional dendritic carrier G5−Ac^{3-}FITC−FA−OH (in concentrations of 10, 20, 30, 40, and 50 μL of PBS) is presented in Figure 6 as an illustrative UV sample. Comparison of UV data of the bifunctionally conjugated dendrimer with UV data of the G5 dendrimer, G5−Ac^3 dendrimer, and of the free folic acid, FITC, and Taxol (not shown here) has allowed us to determine which peaks can be attributed to which dendrimer conjugate. G5−Ac^3 exhibits no characteristic peaks over 300 nm. The peak attributed to FITC is present at 502 nm, and the peaks characteristic of folic acid appear at approximately 262 nm and 358 nm. The peak for maximum absorbance for Taxol lies around 226 nm; therefore, utilization of the UV spectrum of the dendrimer conjugate after the attachment of Taxol to quantify the number of Taxol molecules attached is impossible. We have used GPC to determine number of attached Taxol in this study (another useful method is mass spectrometry).

Conjugation of the Taxol−NHS ester to the bifunctional dendritic carrier via an ester linkage formed G5−Ac^{3-}FITC−FA−OH−Taxol^{e}. Conjugation via an ester link was utilized due to the reactivity of the ester bond, which is characterized by ease of cleavage. Cleavage most probably occurs through enzymatic hydrolysis. The average number of Taxol molecules attached (3) was determined by calculation using gel permeation chromatography. NMR data was not useful for this calculation because the aliphatic proton peaks from folic acid and Taxol overlap with the proton peaks belonging to the dendrimer, making it virtually impossible to distinguish which protons belong to which molecule. Figure 7 presents a graphic representation of the conjugation of Taxol to the bifunctional dendritic carrier G5−Ac^{3-}FITC−FA−OH, forming the trifunctional dendrimer conjugate G5−Ac^{3-}FITC−FA−OH−Taxol^{e}.

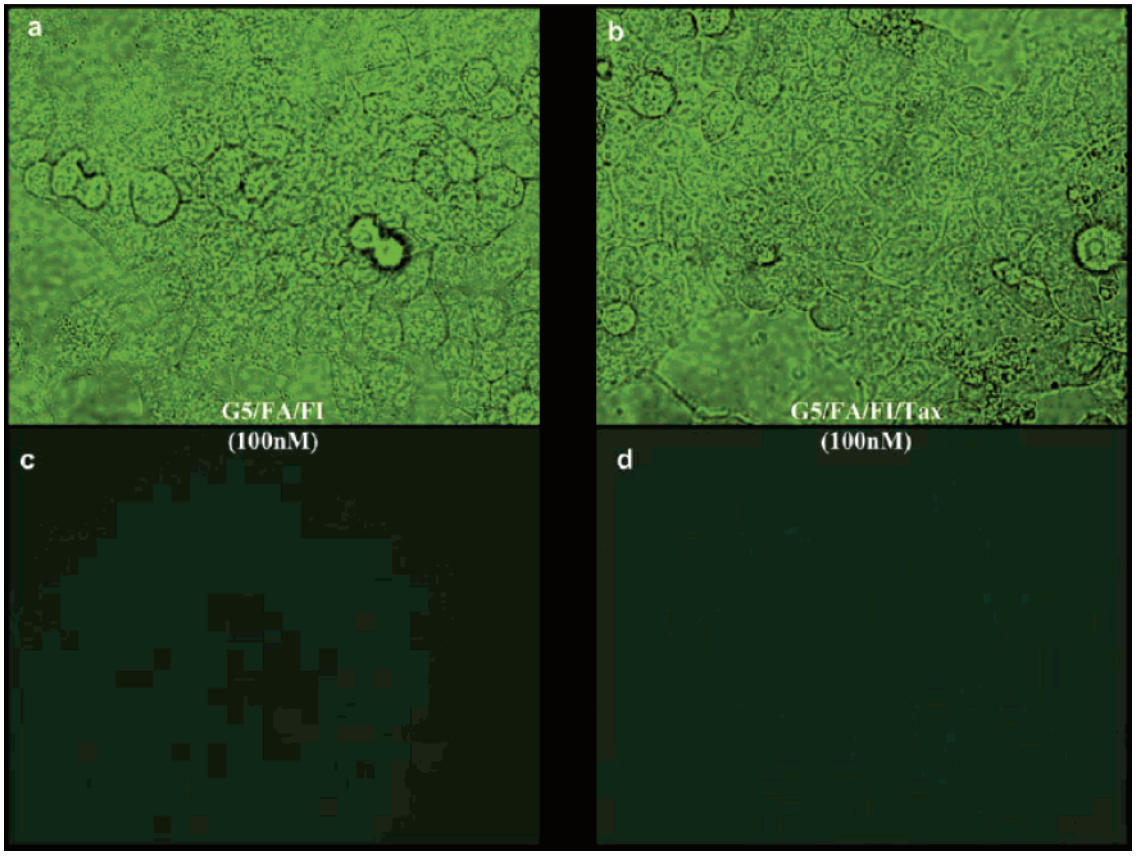

Figure 9. KB (FR−) cells treated with either 100 nM G5−Ac^{3}−FITC−FA (a and c) or 100 nM G5−Ac^{3}−FITC−FA−OH−Taxol^{e} (b and d). Cells were treated with dendrimer conjugates for 60 min, then washed and incubated for 72 h before microscopic examination. Nontargeted KB (FR−) cells were examined after treatment by fluorescent microscopy. Panels a and b show the visible light image, while panels c and d show the fluorescent image. Panels a and c show the same view, as do panels b and d. Neither the G5−Ac^{3}−FITC−FA nor G5−Ac^{3}−FITC−FA−OH−Taxol^{e} binds to the KB cells or shows any toxic effects.

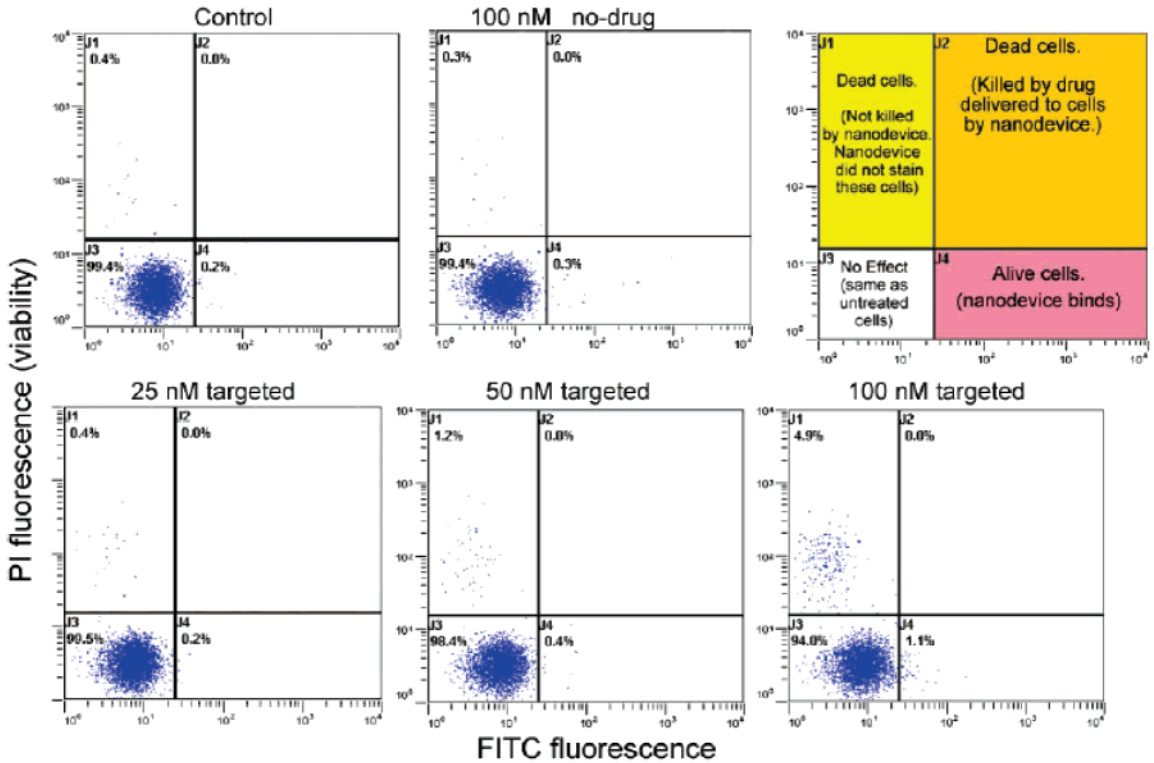

Figure 10. Nontargeted KB cells—folic acid receptor-negative—examined after treatment by flow cytometry. No significant cytotoxicity was observed. KB (FR−) cells were treated as indicated on the scattergram for 60 min, then washed and incubated for 72 h. Before flow cytometry analysis, the cells were stained with propidium iodide to identify dead or dying cells.

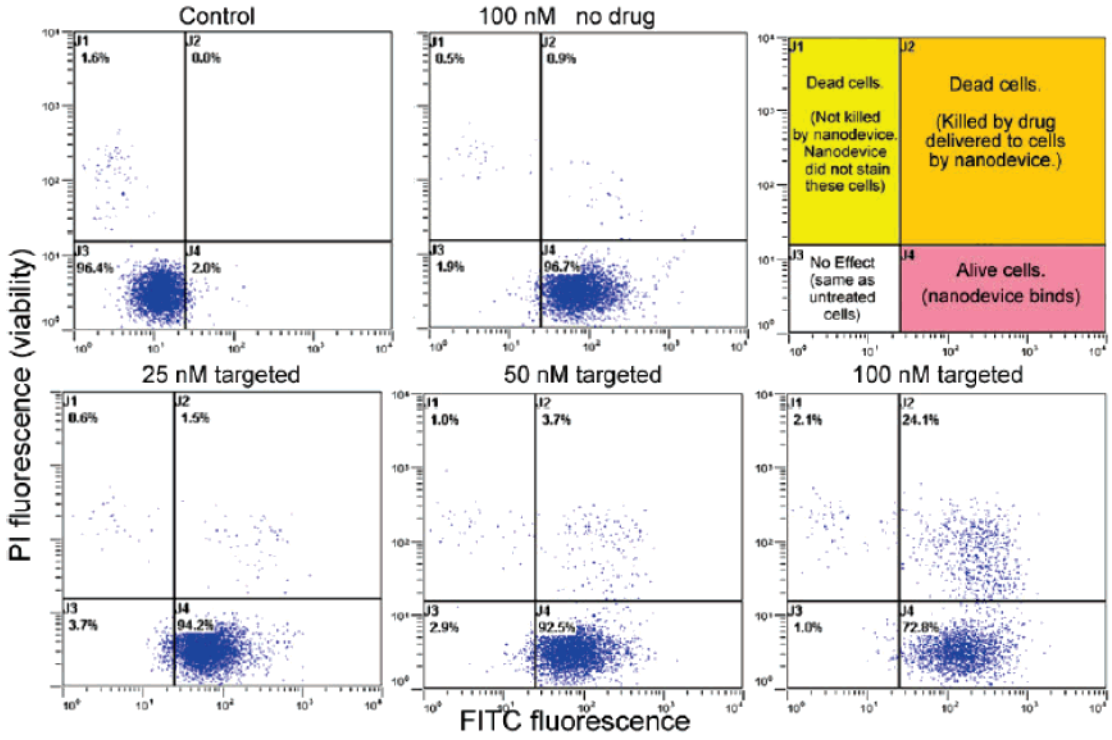

Figure 11. Targeted KB cells—folic acid receptor-positive—examined after treatment by flow cytometry. No significant cytotoxicity was observed. More than 24% of the cells died due to treatment with 100 nM of the targeting G5−Ac^{3}−FITC−FA−OH−Taxol^{e} dendrimer conjugate. KB (FR+) cells were treated as indicated on the scattergram for 60 min, then washed and incubated for 72 h. Before flow cytometry analysis, cells were stained with propidium iodide to identified dead or dying cells.

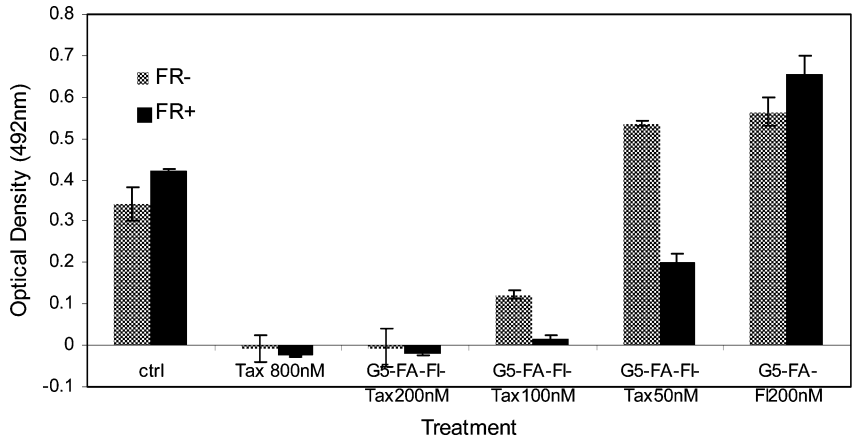

Figure 12. KB folate receptor and folate receptor + cells were treated as indicated for 60 min, then washed and incubated for 120 h before XTT assay was performed. A 200 nM concentration of G5−Ac^{3}−FITC−FA−OH−Taxol^{e} is equivalent to an 800 nM concentration of free Taxol.

Molecular weights and molecular weight distribution for the PAMAM dendrimer carrier and its mono-, bi-, and trifunctional conjugates (Table 1) were calculated using gel permeation chromatography equipped with multiangle laser light scattering and an RI detector as a concentration detector. The values in Table 1 were calculated utilizing gel permeation chromatography data for each dendrimer conjugate (Figures 1 and 2; Supporting Information) to derive the precise number of each functional group attached to the carrier.

The concentration and time needed for this dendrimer conjugate to induce cytotoxic effects in KB cells have been determined. KB cells with up- and down-regulated folic acid receptors were treated with the trifunctional G5−Ac^{3}−FITC−FA−OH−Taxol^{e} dendrimer conjugate or control dendrimer conjugate (G5−Ac^{3}−FITC−FA) at concentrations of 100, 50, and 25 nM for 1 h, then the treatment was washed out and cells were incubated in fresh medium for an additional 72 h before examination under a fluorescent microscope. Dendrimer conjugates at concentrations higher than 100 nM were cytotoxic to both types of KB cells (data not shown). Only KB cells with up-regulated folate receptor and treated either with the trifunctional dendrimer conjugate or the control dendrimer conjugate showed green fluorescence, documenting uptake of the dendrimer conjugate (Figure 8). The folate receptor up-regulated KB cells treated with the targeted dendrimer conjugate (Figure 8, parts b and d) became rounded and detached from the bottom of the plate, indicating the cytotoxic effects of the Taxol. Additionally, we tested folate-starved cells treated with Taxol and compared them with KB cells grown on complete medium. We did not observe any difference in Taxol toxicity between these two subgroups of KB cells (data not shown).

The folate receptor down-regulated KB cells did not show any green fluorescence with either the targeted or nontargeted dendrimer conjugates, indicating that they did not internalize

either dendrimer conjugate (Figure 9). The visible light image shows that after either treatment these cells remained viable and attached to the surface. This demonstrates that the toxicity seen in the targeted cells is due to the specific delivery of the Taxol and is not due to its presence in the media alone. The fluorescent images of folate receptor down-regulated KB cells show no fluorescence, indicating that the folic acid is in fact targeting the dendrimer conjugate to the cells. In the absence of folic acid receptor, there is no association between the cells and the dendrimer conjugate.

The cytotoxic effect was observed at the highest experimental concentration of dendrimer conjugate (100 nM). The folate down-regulated KB cells that were treated with the trifunctional and control dendrimer conjugate (Figure 10) did not show any increase in green fluorescence (uptake of dendrimer conjugate), and the number of dead cells (red fluorescence) was comparable to that of the untreated cells.

Flow cytometry analysis showed that the KB cells with the up-regulated folate receptor were sensitive to the cytotoxic effect of the dendrimer conjugate at a concentration of 100 nM (increase in red fluorescence) as shown in Figure 11. FCM analysis also documented that folate up-regulated KB cells also internalized the dendrimer conjugate (increase in green fluorescence).

The cytotoxic effect of the dendrimer conjugate on KB folate receptor + cells was further confirmed by XTT proliferation assay (Figure 12). The dendrimer conjugate at a concentration of 200 nM was cytotoxic to KB folate receptor− and folate receptor + cells showing unspecific binding of the dendrimer conjugate and killing folate receptor cells. The dendrimer conjugate at a 4 times lower concentration (50 nM) showed selective cytotoxicity only toward KB folate receptor + cells, leaving KB folate receptor cells unaffected (Figure 12). The control dendrimer conjugate without a drug was completely nontoxic to the cells at the concentration of 200 nM.

Although, obtained in vitro data is a very promising, at this point we do not know whether targeting nanodevice can be equally efficient in vivo. We will address this issue in the next in vivo study.

Conclusion

Novel multifunctional conjugates with PAMAM dendrimer as a carrier have been designed, synthesized, characterized, and tested in vitro. The syntheses have been carried out step by step, and each intermediate has been characterized. This strategy allowed us to build up engineered molecular structures. Biological in vitro testing has shown uptake and cytotoxic effect of this multifunctional dendrimer conjugate in comparison to the appropriate control dendrimer conjugate.

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Supporting Information Available. Supporting GPC and ^{1}H NMR data for characterization of the dendrimer conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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