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Targeted Dendrimeric Anticancer Prodrug: A Methotrexate-Folic Acid-Poly(amidoamine) Conjugate and a Novel, Rapid, “One pot” Synthetic Approach

Yuehua Zhang, Thommey P. Thomas, Ankur Desai, Hong Zong, Pascale R. Leroueil, Istvan J. Majoros, and James R. Baker Jr.

Michigan Nanotechnology Institute for Medicine and Biological Sciences, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA

Abstract

A targeted dendrimeric anticancer prodrug, a conjugate of Generation 5 (G5) polyamidoamine (PAMAM) dendrimer, folic acid (FA), and methotrexate (MTX), has been successfully synthesized by using a novel “one pot” approach which is simple, reproducible and feasible for large-scale synthesis. All dendrimer products have been characterized by ^1H NMR, MALDI-TOF, GPC, and HPLC. With this new method, the ratio of FA versus MTX attached to the dendrimer can be easily tuned to achieve the desired therapeutic effect. A new analytical approach for calculating the numbers of FA and MTX attached to the dendrimer has been established. *In vitro* studies performed on FA receptor-expressing KB cells show that the new conjugate has a similar affinity and cytotoxic potency to G5-FA-MTX synthesized using the traditional multiple-step approach.

Introduction

PAMAM dendrimers are highly branched, narrowly dispersed synthetic macromolecules with well-defined chemical structures. PAMAM dendrimers can be easily modified and conjugated with multiple functionalities such as targeting molecules, imaging agents, and drugs.(1) They are water-soluble, biocompatible, and cleared from the blood through the kidneys,(2) which eliminates the need for biodegradability. Because of these desirable properties, PAMAM dendrimers have been widely investigated for drug delivery,(3–7) gene therapy,(8–10) and imaging applications.(11)

Folic acid belongs to the vitamin B family. It is important in cell division because it participates in the biosynthesis of nucleotide bases. There are two membrane-bound folic acid receptors (FR), FR- α and FR- β . Both of the 38 kDa FR isoforms bind folic acid with a high affinity ($K_D < 1$ nM).(12) The expression of FR in normal tissues is low and restricted to various epithelial cells, such as those of the placenta, choroid plexus, lungs, thyroid, and kidneys.(13, 14) However, the folate receptors are overexpressed in many epithelial cancer cells, including breast, ovary, endometrium, kidney, lung, head and neck, brain, and myeloid cancers,(14–17) and is internalized into cells after ligand binding.(18) Although FAR is expressed in some normal tissues, as the normal epithelial cells but not the tumor cells are

Supporting Information Available: ^1H NMR spectrum of folic acid (FA), methotrexate (MTX), G5 PAMAM dendrimer (G5-NH₂), hydroxyl-terminated G5 dendrimer (G5-Gly-OH), and conjugates (G5-FA-MTX) in DMSO-d₆; MALDI-TOF mass spectrum and GPC chromatogram of G5-NH₂, G5-Gly-OH, and G5-FA-MTX; HPLC chromatograms of G5-FA-MTX under UV 210 nm and 280 nm; and three batches of parallel synthesis of G5-FA-MTX and characterization. This material is available free of charge via the internet at <http://pubs.acs.org>.

highly polarized and as the FR has been shown to be exclusively localized in the apical side of the cells (e.g., the urine side in kidney), an FR-targeted drug delivery has been shown to cause only minimum non-specific cytotoxicity when administered systemically.(19, 20) Tumor-selective targeting has been achieved by an FA-targeted PEG-gemcitabine prodrug, (21) FA-conjugated multi-arm-block copolymer-doxorubicin,(22) FA-conjugated liposomes encapsulating an antineoplastic drug(23) or antisense oligonucleotides,(24) FA-conjugated protein toxin,(25) and FA-derivatized antibodies or their Fab/scFv fragments binding to the T-cell receptor.(26) *In vivo* studies from our own group showed that the administration of multivalent, folate-targeted dendrimer-methotrexate conjugates resulted in significantly lower toxicity and a ten-fold enhancement in efficacy compared to free methotrexate at an equal cumulative dose.(5, 27).

In our previous studies, FA and MTX were conjugated to PAMAM dendrimers through amide and ester linkages, respectively, using a multiple-step synthetic route.(5, 6, 28) The synthetic steps involved partial acetylation of the dendrimer, conjugation of FA using EDC chemistry through amide bonds, glycidation of the remaining amino groups, and finally conjugation of MTX through ester linkage through some of the glycidol moieties. The variability in efficiency of each of these synthetic steps resulted in batch-to-batch reproducibility problems which limited the application of this technology. In order to address this issue, we have developed a simplified approach for synthesizing multifunctional dendrimers that is amenable to large-scale synthesis. In this approach, the dendrimer is initially fully glycidated and the FA and MTX are simultaneously conjugated to the dendrimer through ester linkages in a “one pot” reaction.

Experimental Procedures

Materials

Amine-terminated G5-PAMAM dendrimer (G5-NH₂) was purchased from Dendritech, Inc. (Midland, MI, USA) and characterized at the Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan. All chemicals were purchased from Sigma-Aldrich and used as received, unless otherwise specified. Water used in all experiments was purified by a Mili-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ cm. The Spectra/Por® dialysis membranes (MWCO 1,000 and MWCO 10,000) and phosphate buffer saline were acquired from Fisher.

Nuclear Magnetic Resonance Spectrometry (NMR)

¹H NMR spectra were recorded on a 400 MHz Varian Inova 400 nuclear magnetic spectrometer in dimethyl sulfoxide (DMSO-d₆).

Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry

MALDI-TOF mass spectra were recorded on a Waters ToFSpec-2E spectrometer (Beverly, MA, USA), running in linear mode with the high-mass PAD detector, and 2,5-dihydroxybenzoic acid (DHB) in acetonitrile/water (50:50, v/v) was used as the matrix. The instrument was calibrated with bovine serum albumin (BSA, M_w = 66.43 × 10³) in sinapic acid. The data were acquired and processed by MassLynx 3.5 software.

High-Performance Liquid Chromatography (HPLC)

A reverse-phase HPLC instrument consisting of a System GOLD 126 solvent module, a model 507 auto sampler equipped with a 100 μL loop and a model 166 UV detector (Beckman Coulter, Fullerton, CA USA), and a Phenomenex (Torrance, CA, USA) Jupiter

C5 silica-based HPLC column (250 × 4.6 mm, 300 Å) were used for analysis of the products in this work. Two Phenomenex safety guards were installed upstream of the HPLC column. The mobile phase for elution of the PAMAM dendrimer products was a linear gradient beginning with 100:0 water/acetonitrile (ACN) (both containing 0.14 wt% TFA) at a flow rate of 1 mL/min., reaching 20:80 (or 50:50) within 35 minutes. Trifluoroacetic acid (TFA) (0.14 wt% in both water and ACN) was used as a counter-ion to make the dendrimer-conjugate surface hydrophobic. All samples were dissolved in the aqueous mobile phase (water containing 0.14% TFA). The injection volume in each case was 35 µL with a sample concentration of 1 mg/mL, and the detection wavelength was 210 or 280 nm. The analysis was performed using Beckman's System GOLD Nouveau software.

Gel Permeation Chromatography (GPC)

GPC was used to evaluate the molecular weights and molecular weight distribution of the G5-PAMAM dendrimer, dendrimer derivatives, and conjugates. GPC experiments were performed on an Alliance Waters 2690 separation module (Waters Corp., Milford, MA) equipped with a Waters 2487 dual wavelength UV absorbance detector (Waters Corp.), a Wyatt Dawn HELEOS light scattering detector (Wyatt Technology Corp., Santa Barbara, CA), an Optilab rEX differential refractometer (Wyatt Technology Corp.), and TosoHaas TSK-Gel Guard PHW 06762 (75 × 7.5 mm, 12 µm), G 2000 PW 05761 (300 × 7.5 mm, 10 µm), G 3000 PW 05762 (300 × 7.5 mm, 10 µm), and G 4000 PW (300 × 7.5 mm, 17 µm) columns. Column temperature was maintained at 25 ± 0.2°C with a Waters temperature control module. Citric acid buffer (0.1 M) with 0.025% sodium azide in water, pH 2.74, was used as a mobile phase at a flow rate of 1 mL/min. The sample concentration was 2 mg/mL, with an injection volume of 100 µL. The number average molecular weight, M_n , weight average molecular weight M_w and polydispersity index (PDI) were calculated using ASTRA 5.34.14 software (Wyatt Technology Corp.)

Synthesis of hydroxyl-terminated G5 PAMAM dendrimer (G5-Gly-OH 2)

G5-GLY-OH was synthesized using methods similar to that which has been previously reported. (28–30) G5-NH₂ (500 mg, 0.0174 mmol) was dissolved in methanol (10 mL) in a 50-mL flask. To the solution was added glycidol (442 mg, 4.77 mmol). The mixture was stirred at room temperature under nitrogen overnight. The mixture was dialyzed against water (4 × 4L) with a cellulose dialysis membrane (MWCO = 1,000) for 48 hours and was dried by lyophilization (3 days) to yield G5-Gly-OH as a white solid (677 mg, 85%).

Synthesis of a conjugate of G5 PAMAM dendrimer, folic acid and methotrexate (G5-FA-MTX) 3

A solution of G5-Gly-OH 2 (200 mg, 5.06×10^{-3} mmol), MTX (34.1 mg, 0.075 mmol), and FA (17.7 mg, 0.040 mmol) in dimethyl sulfoxide (15 mL) was stirred at room temperature under nitrogen. To the solution was added 2-chloro-1-methylpyridinium iodide (35.3 mg, 0.138 mmol) and 4-(dimethylamino)pyridine (33.7 mg, 0.276 mmol). The mixture was further stirred overnight at room temperature and then poured into water (75 mL). The product was purified by dialysis against isotonic phosphate buffered saline (PBS) buffer (3 × 4L) and then water (3 × 4L) with a cellulose dialysis membrane (MWCO = 10,000) over 48 hours. The final product was dried by lyophilization (3 days) to yield G5-FA-MTX as a yellow solid (231 mg, 98%).

Measurement of cellular binding and cytotoxicity

KB cells, a sub-line of the cervical carcinoma HeLa cells (ATCC, Manassas, VA, USA), were grown as a monolayer cell culture at 37°C and 5% CO₂ in FA-deficient RPMI 1640

medium supplemented with 10% fetal bovine serum (FBS). The 10% FBS provided an FA concentration equivalent to that present in the human serum (~20 nM).

For assessment of the cellular binding of the synthesized conjugate, we have utilized the competition of the conjugate with another previously synthesized dendrimer conjugate ("G5-FI-FA-MTX") that contained 4 molecules of the fluorescent dye fluorescein isothiocyanate (FI), 5 molecules of FA, and 7 molecules of MTX.(7) The KB cells, plated in 24-well plates, were treated with a mixture of 100 nM of the G5-FI-FA-MTX and varying concentrations of the newly synthesized G5-FA-MTX, added as a mixture at the same time. The cells were incubated at 37°C for 1 hour, and the FL1 fluorescence of 10,000 cells was determined by flow cytometry, as described previously. (7).

For the cytotoxicity experiments, the cells were seeded in 96-well microtiter plates (3000 cells/well) in medium containing dialyzed serum. Two days after plating, the cells were treated with the dendrimer conjugates in tissue culture medium under the indicated conditions. A colorimetric "XTT" (sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Molecular Biochemicals, Indianapolis, IN) 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.(7)

Results and Discussion

Synthesis

In this study, G5-NH₂ **1** was used as the starting material to synthesize the targeted dendrimeric anticancer drug G5-FA-MTX **3**, Figure 1. The number of available primary amino groups on the surface of G5-NH₂ is 110.(31) Starting with G5-NH₂ **1**, the amino groups of G5-NH₂ react with glycidol to yield G5-Gly-OH **2**. In this reaction, excess glycidol was used (glycidol:NH₂ = 3:1) to fully cap the free amino groups on the surface of **1**. Each amino group can theoretically react with two molecules of glycidol to form a tertiary amine. However, calculations based upon the MALDI indicated that the hydroxylation reaction was incomplete, as previous reported by us (30). It is hypothesized that steric hindrance prevents some of the terminal amines residing inside the dendrimer from being available for hydroxylation, resulting in an incomplete reaction.(32) The G5-Gly-OH **2** was purified by dialysis with a cellulose dialysis membrane against water. MTX and FA were attached to the hydroxyl groups of the G5-Gly-OH **2** through ester bonds in a one-pot reaction, using 2-chloro-1-methylpyridinium iodide and 4-(dimethylamino)pyridine as the coupling reagents (Mukaiyama reagent), which is efficient for preparation of esters from equimolar amounts of free carboxylic acid and alcohol. The reaction was conducted in DMSO under nitrogen at room temperature for 24 hours. The final product, conjugate **3**, was purified by dialysis with a cellulose dialysis membrane against PBS buffer and then water. The G5-FA-MTX is highly water-soluble, even with 5 FAs and 15 MTXs attached. It was also observed that the numbers of FA and MTX attached on the surface of the PAMAM dendrimer can be easily and reproducibly adjusted by changing the feeding ratio of FA and MTX in the feed. The numbers of FA and MTX attached on the PAMAM dendrimer were calculated using results from MALDI-TOF, GPC, and ¹H NMR. Despite the fact that we have used dialysis as a purification step in these procedures, the application of ultrafiltration would make the synthetic procedure feasible for scale-up synthesis.

¹H NMR spectroscopy

¹H NMR was used to confirm the surface capping of the terminal amines of the dendrimer with glycidol and the attachment of the FA and MTX to the hydroxyl group of the G5-Gly-

OH (Figure 2). The ^1H NMR of the G5 PAMAM dendrimer shows 6 broad peaks which correspond to the protons of the amide bond (CONH) at 7.95 ppm, the amino groups (NH_2), the internal methylene groups (CH_2), and those CH_2 next to the amino groups at 3.05, 2.64, 2.58, 2.42, and 2.20 ppm. The ^1H NMR spectrum of the G5-Gly-OH clearly shows three new peaks, compared with that of the G5- NH_2 . The peak at 3.348 ppm (peak 3) is the resonance of protons of the CH_2 at position 3 of the 2,3-dihydroxypropyl group. The peak at 3.542 ppm (peak 2) belongs to the CH at position 2 of the 2,3-dihydroxypropyl group. The broad peak at 3.961 ppm is attributed to two hydroxyl groups of the 2,3-dihydroxypropyl group. The peak at about 2.47 ppm (30) belongs to the protons of the CH_2 at position 1 of the 2,3-dihydroxypropyl group, which overlaps with peaks associated with the internal protons of the G5- NH_2 . Compared with the spectrum of the G5-Gly-OH, the spectrum of the G5-FA-MTX shows additional peaks. Some of the peaks overlap with the peaks of the G5-Gly-OH. The H-7 peaks of the conjugated FA and MTX are present at 8.617 ppm and 8.561 ppm, respectively. They are sharp and well separated single peaks. The integration ratio of these two peaks represents the molar ratio of MTX and FA because both MTX and FA contain only one H-7 (Figure 3). Significantly, it is also found that the integration ratio of these two protons is proportional to the feed ratio of MTX and FA in the “one-pot” reaction (Table 1). Therefore, the actual numbers of FA and MTX conjugated to the surface of the dendrimer can be calculated using this integration ratio and molecular weight gain from G5-Gly-OH **2** to G5-FA-MTX **3**, as measured by MALDI-TOF and GPC.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry has been proven to be an important technique for characterization of dendrimers and dendrimer derivatives. It not only provides the average molecular weight but also gives information about the success of each conjugation reaction and the number of molecules that have been conjugated. Figure 4 shows the MALDI-TOF mass spectra of the G5- NH_2 , G5-Gly-OH, and G5-FA-MTX. The molecular weight increase for each species along the synthetic pathway is clearly seen and demonstrates that the hydroxylation (first step) and conjugation (second step: the “one pot” reaction) have occurred. The molecular weight gain of the G5-Gly-OH from the G5- NH_2 is due to the attachment of the 2,3-dihydroxypropyl group. The average number of 2,3-dihydroxypropyl groups attached to each amino group may be calculated by the difference divided by the molecular mass of the 2,3-dihydroxypropyl group and the number of the amino groups on the surface of the dendrimer.

The molecular weight increase from the G5-Gly-OH to the G5-FA-MTX is due to the addition of both FA and MTX. The numbers of FAs and MTXs attached to the dendrimer can be calculated using formula (1) and (2), which are based on the molecular weight gain from the G5-Gly-OH to the G5-FA-MTX, as measured by MALDI-TOF or GPC, and the integration ratio of H-7 of the conjugated FA and MTX. Calculation results have been listed in Table 1.

$$N_{\text{FA}} = \frac{\Delta\text{MW} \times F_{\text{FA}/(\text{MTX}+\text{FA})}}{\text{MW}_{\text{FA}} - 18} \quad (1)$$

$$N_{\text{MTX}} = \frac{\Delta\text{MW} \times F_{\text{MTX}/(\text{MTX}+\text{FA})}}{\text{MW}_{\text{MTX}} - 18} \quad (2)$$

Note: N_{FA} : molecules of folic acid attached to each PAMAM dendrimer molecule; N_{MTX} : molecules of MTX attached to each PAMAM dendrimer molecule; ΔMW : molecular weight gain of the conjugate from hydroxyl-terminated PAMAM dendrimer; F_{LA} : ^1H NMR

integration fraction of H-7 (FA over FA and MTX); F_{MTX} : ^1H NMR integration fraction of H-7 (MTX over FA and MTX); MW_{FA} : molecular weight of FA; MW_{MTX} : molecular weight of MTX.

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a widely accepted method for separation and purification of small molecules and macromolecules and is commonly used in chemical, pharmaceutical, and biotech laboratories. Recently, HPLC has been used as a vital tool to analyze PAMAM dendrimers of various generations and terminal groups, as well as their conjugates.(33–35) In this study, we have used HPLC to evaluate the purity and molecular weight distributions of the conjugates. Small molecule impurities such as unreacted FA, MTX, coupling reagent, and by-products are very easily differentiated from the dendrimers (G5-NH₂, G5-Gly-OH, and G5-FA-MTX) by HPLC due to the significant difference in retention time. Fig. 5 is a representative chromatogram of the conjugate (G5-FA-MTX) under UV 280 nm, which shows a single symmetric peak of the conjugate and a few small impurity peaks attributed to the presence of less than 0.1% free FA, MTX and other impurities. The result also indicates that the FA and MTX have been successfully attached to the G5-Gly-OH, resulting in a very narrow molecular weight distribution, which is in agreement with the GPC results shown below.

Gel permeation chromatography (GPC)

A gel permeation chromatography (GPC) instrument equipped with two detectors, a multiangle laser light scattering (MALLS) and a differential refractive index, was used to evaluate the molar mass of the starting PAMAM dendrimer, the hydroxyl-terminated PAMAM dendrimer, and the conjugates. Fig. 6 shows that differential mass fraction profile of the G5-NH₂, G5-Gly-OH, and G5-FA-MTX respectively. All dendrimer samples exhibit a single peak. The number average molecular weights M_n and polydispersity index (PDI: M_w/M_n) of the G5-NH₂, G5-Gly-OH, and G5-FA-MTX are 26,060 (1.025), 39,110 (1.042), and 52,800 (1.044), respectively. The ratios of weight- to number-average molecular weights of the G5-NH₂, G5-Gly-OH, and G5-FA-MTX are very close to 1. This indicates that the distribution mass of the individual components is narrow and that the conjugation process at each step is nearly uniform.

The GPC results are somewhat different from those measured by MALDI-TOF, as shown by Fig. 4 and Fig. 6. This difference is due to the broadening of the MALDI-TOF spectra, as compared to the GPC spectra. Generally, however, GPC is thought to reflect the real average molar mass.

In vitro studies

The binding of the newly synthesized G5-FA-MTX onto FA receptor-expressing KB cells was examined. For this, we have utilized a previously synthesized G5-FI-FA-MTX conjugate in which the FA and MTX were conjugated to the dendrimer through the classic synthetic pathway.(28) In addition, a fluorescent dye FITC (FI) was conjugated to the surface of the dendrimer. As shown in Figure 7, at a fixed concentration of G5-FI-FA-MTX (100 nM; concentrations shown are for the intact conjugates), the G5-FA-MTX competed with the former for binding, with a 50% inhibition of binding occurring at ~60 nM. This shows that the G5-FA-MTX binds through the FA receptor with an affinity similar to or slightly better than the G5-FA-MTX synthesized through the classic synthetic pathway.(28)

The cytotoxicity of the newly synthesized conjugate was determined by the XTT assay, as described in Methods. We have compared the cytotoxicity with another batch of G5-FA-MTX synthesized by Cambrex Inc., using the classic synthetic pathway in which the FA and

MTX were conjugated through amide and ester linkages, respectively. Our previous studies have shown that this batch of Cambrex conjugates was cytotoxic *in vitro*, and tumoricidal *in vivo*, with a potency similar to that of our previously published compound.(5) As shown in Figure 8, the newly synthesized conjugate was as cytotoxic as the Cambrex batch.

Conclusion

A targeted dendrimeric anticancer prodrug, a conjugate of PAMAM dendrimer, FA, and MTX, has been successfully synthesized using an improved and simple “one pot” approach. Using this method, FA and MTX can be attached to the dendrimer in any desired ratios in a simple one-step reaction. A new analytical approach for calculating the molecules of FA and MTX attached to each dendrimer molecule has been established. The *in vitro* studies performed on FA receptor-expressing KB cells show that the new conjugates have similar affinity and cytotoxic potency as the G5-FA-MTX synthesized using the traditional multiple step approach. The results of parallel synthesis have demonstrated that this method is reproducible. The new method will allow the easy conjugation of multiple functionalities such as a targeting agent, a drug, and even an imaging agent by utilizing the simple “one-pot” synthetic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of a G5-FA-MTX conjugate using folic acid (FA), methotrexate (MTX), and G5 polyamidoamine (PAMAM) dendrimer. Reagents and conditions: (a) glycidol, methanol, room temperature, 24 hours; (b) FA, MTX, 2-chloro-1-methylpyridinium iodide, 4-(dimethylamino)pyridine, dimethyl sulfoxide, room temperature, 24 hours.

Figure 2.

Comparison of the ^1H NMR spectra of the G5 PAMAM dendrimer, the hydroxyl-terminated G5 PAMAM dendrimer, and the conjugate of G5 PAMAM dendrimer, FA, and MTX.

Figure 3.
Enlarged scale of the ^1H NMR spectrum of proton-7 of conjugated FA and MTX.

Figure 4. MALDI-TOF mass spectra of the G5 PAMAM dendrimer, the hydroxyl-terminated G5 PAMAM dendrimer, and the conjugate of G5 PAMAM dendrimer, folic acid, and methotrexate.

Figure 5.
HPLC chromatogram of the conjugate (G5-FA-MTX) under 280 nm.

Figure 6.

Graph showing the differential molar mass fractions for the G5 PAMAM dendrimer, the hydroxyl-terminated G5 PAMAM dendrimer, and the conjugate of G5 PAMAM dendrimer, FA and MTX.

Figure 7.

Competition of G5-FA-MTX synthesized using the “one-pot” approach with G5-FI-FA-MTX. The KB cells were treated with the two conjugates simultaneously, as described in Methods, and the mean FL1 fluorescence of 10,000 cells was determined by flow cytometry. The data is expressed as the percent fluorescence obtained for the binding of 100 nM G5-FI-FA-MTX in the absence of G5-FA-MTX. As indicated by the arrow, the concentration of G5-FA-MTX that is required for 50% reduction in binding of the G5-FI-FA-MTX is ~60 nM.

Figure 8.

In vitro cytotoxicity of the newly synthesized G5-FA-MTX. The KB cells were treated with different concentrations of the newly synthesized G5-FA-MTX (▲), and free MTX (●). The data also show the cytotoxicity of G5-FA-MTX synthesized through the classic synthetic pathway in which the FA and MTX were conjugated through amide and ester linkages, respectively (□). The cells were treated with the drugs for 5 days, with one change of fresh medium/drug after 3 days, and the live cells were quantified by the XTT assay as described in Methods. The data represents the Mean $\pm$ SE of 5 replicate cell samples in a representative experiment, with identical data obtained in 3 independent experiments.

Table 1

Reaction feed molar ratio of MTX/FA/G5-Gly-OH, ¹H NMR integration ratio of H-7 of MTX/FA, and Numbers of MTX and FA on the conjugate (G5-FA-MTX)

Reaction feed molar ratio of MTX/FA/ G5-Gly-OH	¹ H NMR Integration Ratio of H-7s (MTX/FA)	Average number of MTX and FA attached on PAMAM
10:7:1	1.38:1.00	6.79 and 5.20
15:8:1	1.86:1.00	9.44 and 5.22
23:8:1	2.83:1.00	14.90 and 5.42
15:10:1 ^a	1.33:1.00	9.26 (±0.28 ^b) and 7.18 (± 0.1 ^c)

^a) Three batches of parallel synthesis under the same condition: G5-Gly-OH (50 mg), MTX (8.54 mg), FA (5.52 mg), 2-chloro-1-methylpyridinium iodide (8.00 mg), 4-(dimethylamino)pyridine (7.65 mg), DMSO (2.5 mL), N₂, 24 hours, cellulose membrane dialysis (6 times 4L over 48 hours), and lyophilization (72 hours).

^b) Standard deviation of average number of MTX.

^c) Standard deviation of average number of FA.