Enhancing the Efficacy of Ara-C through Conjugation with PAMAM Dendrimer and Linear PEG: A Comparative Study

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ABSTRACT: 1β-D-Arabinofuranosylcytosine (Cytarabine, Ara-C) is a key drug in the treatment of acute myeloid leukemia. Ara-C has a number of limitations such as a rapid deactivation by cytidine deaminase leading to the formation of a biologically inactive metabolite, Ara-U (1β-d-arabinofuranosyluracil), a low lipophilicity, and fast clearance from the body. To address these problems, we developed a conjugate in which hydroxyl-terminated PAMAM dendrimer, G4-OH ["D"] and PEG were used as carriers for the drug (Ara-C). The conjugates were synthesized using an efficient multistep protection/deprotection method resulting in the formation of a covalent bond between the primary hydroxyl group of Ara-C and dendrimer/PEG. The structure, physicochemical properties, and drug release kinetics were characterized extensively. 1H NMR and MALDI-TOF mass spectrometry suggested covalent attachment of 10 Ara-C molecules to the dendrimer. The release profile of Ara-C in human plasma and in PBS buffer (pH 7.4) showed that the conjugates released the drug over 14 days in PBS, with the release sped up in plasma. In PBS, while most of the drug is released from PEG-Ara-C, the dendrimer continues to release the drug in a sustained fashion. The results also suggested that the formation of the inactive form of Ara-C (Ara-U) was delayed upon conjugation of Ara-C to the polymers. The inhibition of cancer growth by the dendrimer-Ara-C and PEG-Ara-C conjugates was evaluated in A549 human adenocarcinoma epithelial cells. Both dendrimer- and PEG-Ara-C conjugates were 4-fold more effective in inhibition of A549 cells compared to free Ara-C after 72 h of treatment.

1. INTRODUCTION

Cytarabine (Ara-C) is a widely used anticancer drug for the treatment of various forms of cancer such as acute myelogenous leukemia, colon, breast, and ovary carcinoma. As in other pyrimidine analogue nucleosides, the potency of Ara-C is limited by its low stability after intravenous administration and the narrow therapeutic index. Consequently, the minimum effective dose is high and has to be increased regularly after the initial treatment. In this context, toxicity becomes a key limiting factor for treatment. Its rapid clearance from the body is due to the enzymatic conversion to the inactive and more soluble form of the drug (Ara-U) by cytidine deaminase, mainly in liver and kidney. Many efforts have been made to address the problem, including continuous intravenous infusion or frequent and high-dose schedules. To improve its in vivo stability, a combination therapy with tetrahydrouridine (a cytidine deaminase inhibitor) was used, which did not significantly improve the therapeutic efficacy of Ara-C. Moreover, the hydrophilic character of Ara-C strongly limits its intracellular uptake because of the low membrane permeability of the molecule. These issues raise the need for improved formulations, stability, and intracellular delivery, and provide new impetus for the present study. A variety of Ara-C conjugates have been synthesized to increase the stability and bioactivity. Macromolecular derivatives and prodrugs obtained by acylation at the N-4 position of the nucleoside have been synthesized to prevent deamination and improve pharmacokinetics. In fact, conjugation of antitumor drugs to high molecular weight polymers, antibodies and polypeptides, are now representing new and promising approaches in chemotherapy. These conjugates may act as classical prodrugs while taking advantage of longer circulation time and the enhanced permeability and retention (EPR) effect, resulting in improved accumulation in the tumor microvasculature and cellular internalization by endocytosis to reach their intracellular targets. It was also reported that Ara-C conjugated with polyethylene glycol (PEG) through a norleucine linker is less cytotoxic in human HeLa cells. This work builds on these efforts by using PAMAM dendrimer-based delivery of Ara-C.

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Poly(amideamine) dendrimers (PAMAM) are promising drug delivery vehicles because of their multivalency, well-defined and globular structure, low polydispersity, and amenability to post-synthetic manipulation that are extensively investigated for various biomedical applications.16–19 PAMAM dendrimers are the most widely studied polymers used as vehicles for drug delivery,20–22 antiviral agents,23,24 MRI contrast agents,25 and selective tumor targeting.26–30 Conjugation to a PAMAM dendrimer significantly improved the efficacy of methotrexate26 and paclitaxel.31 Khandare et al. have shown that PAMAM dendrimer—Paclitaxel conjugate was more efficacious than a PEG—Paclitaxel conjugate in A2780 human ovarian carcinoma cells.31 Folic acid-conjugated conjugate was more efficacious than other end functionalities, and its kidney-based targeting overexpressed folate receptors on mouth epidermal cell line improved efficacy, and significantly improved the efficacy of methotrexate and paclitaxel.31 When an anti-inflammation agent N-acetyl cysteine conjugated to the PAMAM dendrimer because of its improved cytotoxicity profile compared to other end functionalities, and its kidney-based targeting overexpressed folate receptors on mouth epidermal carcinoma (KB) cells.32 In this study, we used the hydroxyl-functionalized generation-4 PAMAM dendrimer because of its improved cytotoxicity profile compared to other end functionalities, and its kidney-based clearance.35,36 We have previously reported extensively on the improved efficacies, based on this delivery platform. Dendrimer-antibiotic conjugates were prepared for the treatment of Staphylococcus aureus bacteria in periprosthetic infection and Chlamydia trachomatis infection in reactive arthritis.33,34 Conjugation of poorly soluble drugs to dendrimer can increase the bioavailability, and decrease the dose frequency.35–37 The most common covalent linkage of the drugs to the surface functional groups of dendrimers can be achieved through the ester or amide bond, which can be cleaved hydrolytically or enzymatically.38 When a conjugate, appropriately designed to improve cell uptake and drug release profile, is further combined with a targeting ligand, significant improvements in efficacy can be obtained.39 Interestingly, neutral PAMAM dendrimers with no targeting ligands have been shown to have an intrinsic ability to localize in cells associated with neuro-inflammation in a rabbit model of cerebral palsy and a rat model of retinal degeneration.40,41 When an anti-inflammatory/antioxidant agent N-acetyl cysteine conjugated to the PAMAM dendrimer and administered intravenously to newborn rabbit kits with cerebral palsy (after birth), the dendrimer delivered the drug to activated microglia and astrocytes, resulting in significant improvements in motor function, myelinization, and neuronal injury by day 5 of life.42 The aim of this study is to design and synthesize PAMAM dendrimer-Ara-C and polyethylene glycol-Ara-C (PEG-Ara-C) conjugates using appropriate protection/deprotection chemistry, to improve the drug stability, release profile and intracellular delivery. We report the synthesis, structural characterization, drug release, and the efficacy of the conjugates in an AS49 human epithelial carcinoma cell line.

2. MATERIALS AND METHODS

2.1. Materials. Generation-four hydroxyl-terminated PAMAM dendrimer (G4-OH, “D”) in methanol was purchased from Dendritech, Inc. (Midland, Michigan). mPEG5000 (PEG) was purchased from Fluka Chemicals. Benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 4-(dimethylamino)pyridine (DMAP), N,N'-carbonyldiimidazole (Cy4), tert-butyl(dimethyl)chloride (TBDMS-Cl), di-tert-butyl dicarbonate, succinimide anhydride, triethylamine (TEA), diisopropylethylamine (DIEA), acetonitrile (ACN), and trfluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, Missouri). The anhydrous dimethylsulfoxide (DMSO), dimethylformamide (DMF), 1,4-dioxane, tetrahydrofuran (THF), and dichloromethane (DCM) solvents were purchased from Acros Organics (Morris Plains, New Jersey). Deuterated dimethylsulfoxide (DMSO-d6) and deuterated chloroform (CDCl3) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts). All other solvents and chemicals were purchased from Fisher Scientific and were used without further purification. Regenerated cellulose (RC) dialysis membrane with molecular weight cutoff of 2000 Da was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, California).

The reactions were carried out under nitrogen. Thin-layer chromatography (TLC) was performed on silica gel GF254 plates (Whatman, Piscataway, New Jersey), and the spots were visualized with UV light. NMR spectra were recorded on a Varian INOVA 400 spectrometer, using commercially available deuterated solvents. Proton chemical shifts are reported in ppm (δ) and tetramethylsilane (TMS) used for internal standard. Coupling constants (J) are reported in hertz (Hz).

2.2. Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF) Mass Spectrometry. Electrospray ionization (ESI) mass spectra were recorded on a Waters Micromass QZ spectrometer (Waters Corporation, Milford, Massachusetts). MALDI-TOF mass spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm; Bruker Daltonics Inc., Milford, Massachusetts), operating in positive ion reflector mode, using 19 kV acceleration voltage. 2,5-Dihydroxybenzoic acid was used as matrix and Cytochrome c (MW 12361 g/mol) was used as external standard. The matrix solution was prepared by dissolving 20 mg of matrix in 1 mL of deionized water/ACN (0.1% TFA; 1:1). Analytical samples were prepared by mixing 10 μL of dendrimer solution (2 mg/mL in methanol) with 100 μL of matrix solution, followed by deposition of 1 μL of sample mixture onto the MALDI plate. This mixture was allowed to air-dry at room temperature (22–25 °C) and used for analysis.

2.3. High-Performance Liquid Chromatography (HPLC) Analysis. The conjugates were analyzed by Waters HPLC instrument (Waters Corporation, Milford, Massachusetts) equipped with binary pump, dual UV detector, and autosampler interfaced with breeze software. The HPLC chromatogram was monitored at 210 and 272 nm simultaneously using dual UV absorbance detector. The water/acetoni-trile (0.14% w/w TFA) was freshly prepared, filtered, degassed, and used as a mobile phase. The flow rate was 0.8 mL/min. Elution was monitored at 210 nm (for dendrimer detection), 272 nm (for Ara-C detection) and refractive index without further purification. The anhydrous dimethylsulfoxide (DMSO), dimethylformamide (DMF), 1,4-dioxane, tetrahydrofuran (THF), and dichloromethane (DCM) solvents were purchased from Acros Organics (Morris Plains, New Jersey). Deuterated dimethylsulfoxide (DMSO-d6) and deuterated chloroform (CDCl3) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts). All other solvents and chemicals were purchased from Fisher Scientific and were used without further purification. Regenerated cellulose (RC) dialysis membrane with molecular weight cutoff of 2000 Da was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, California).

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deionized water (18.2 Ω) to make the solution with the final concentration of 0.1 mg/mL. The solution was filtered through a cellulose acetate membrane (0.45 μm, PALL Life Science) and DLS measurements were performed at 25 °C with a scattering detector angle of 173°. Zeta potentials were calculated using the Smoluchowski model and measurements were performed in triplicate.

2.7. Synthesis of Compounds 1–3. The intermediates 1, 2a, 2b, 3a, and 3b were synthesized using previously published procedures.43 The detailed experimental procedure and 1H and 13C NMR spectra are described in the Supporting Information.

2.8. Synthesis of Boc-Protected Ara-C Succinic Acid Linker (4a/4b). Synthesis of Mono Boc-Protected Ara-C Succinic Acid Linker (4a). To a stirred solution of 3a (170 mg, 0.264 mmol) in DCM (10 mL), succinic anhydride (43 mg, 0.428 mmol), and DMAP (32 mg, 0.264 mmol) were added, 1H and 13C NMR spectra were determined at room temperature for 6 h. The reaction mixture was concentrated on a rotary evaporator and the crude product was purified on a column packed with silica gel using chloroform/MeOH (10:1) as eluent to yield 4a as a white powder (150 mg, 71.4%). 1H NMR (CDCl3) δ 7.85 (d, 1H, H-6, J = 6.4 Hz), 7.21 (d, 1H, H-5, J = 6.8 Hz), 6.28 (d, 1H, H-1′′, J = 4.0 Hz), 5.26 (m, 1H, H-2′′), 4.87 (m, 1H, H-3′), 4.27–4.59 (m, 3H, H-4′, H-5′ and H-5′′), 2.64–2.71 (m, 1H), 1.34–1.51 (m, 27H, −C(CH3)3). ESI-MS Calcd for C38H32N2O6: 625.210; measured, 624.2509.

Synthesis of Boc-Protected Ara-C Succinic Acid Liner Intermediate (4b). The compound 4b (80%) was synthesized by the same procedure as above for compound 4a. 1H NMR (DMSO-d6) δ 7.99 (d, 1H, H-6, J = 7.6 Hz), 6.92 (d, 1H, H-5, J = 7.6 Hz), 6.21 (d, 1H, H-1′′, J = 3.6 Hz), 5.22 (m, 1H, H-1′′), 5.10 (m, 1H, H-3′), 4.28–4.43 (m, 3H, H-4′, H-5′ and H-5′′), 2.49–2.57 (m, 4H), 1.47 (s, 18H, −C(CH3)3), 1.44 (s, 9H, −C(CH3)3) and 1.28 (s, 9H, −C(CH3)3). ESI-MS Calcd for C44H44N2O6: 744.3191; measured, 744.3180.

2.9. Synthesis of Dendrimer-Ara-C Conjugate (6). To a stirred solution of PAMAM G4-OH (50 mg, 0.0035 mmol) in anhydrous DCM (5 mL), PyBOP (72.8 mg, 0.14 mmol) was added under nitrogen. A solution of PAMAM G4-OH (50 mg, 0.0035 mmol) in anhydrous DCM (10 mL) was added and stirred for 3 h at room temperature. The solvent of the crude product was precipitated in anhydrous diethyl ether. The solid was collected by filtration and dried over high vacuum to get PEG-linker, 7 (0.75 g, 86.5%). 1H NMR (CDCl3) δ 4.22 (t, 2H, −OCH2CH2OH, J = 4.4 Hz), 3.42–3.80 (m, backbone CH2), 3.35 (s, 3H, −OCH3), 2.53–2.63 (m, 1H, linker CH2).

Synthesis of Boc-Protected Ara-C-PEG Intermediate (8). To a solution of 7 (100 mg, 0.02 mmol) in DCM (10 mL), EDC (7.5 mg, 0.039 mmol), and DMAP (4.8 mg, 0.039 mmol) were added and stirred for 30 min under nitrogen. The Boc-protected Ara-C (3b, 25.24 mg, 0.039 mmol) was added to the reaction mixture and stirred for 16 h at room temperature. The solvent was evaporated to get the oily crude product. The crude product was dissolved in DMF (7 mL) and purified by dialysis with DMF (membrane MW cutoff 1000 Da). The resulting dialyzed solution was evaporated and dissolved in 2 mL of DCM and added dropwise to anhydrous diethyl ether to get white solid (90 mg, 80.3%) and dried over vacuum. 1H NMR (CDCl3) δ 7.81 (d, 1H, Ar, J = 7.6 Hz), 7.07 (d, 1H, Ar, J = 7.6 Hz), 6.29 (d, 1H, H-1′, J = 3.2 Hz), 5.30 (d, 1H, H-2′, J = 3.2 Hz), 4.97 (d, 1H, H-3′, J = 2.4 Hz), 4.39–4.53 (m, 3H, H-4′ and H-5′), 4.22–4.26 (m, 2H, −OCH2CH2OH), 3.43–3.80 (m, mPEG-backbone H), 3.35 (s, 3H, −OCH3), 2.63–2.69 (m, 4H), 1.53 (s, 18H, N-Boc), 1.47 (s, 9H, O-Boc), 1.34 (s, 9H, O-Boc).

PEG-Ara-C Conjugate (9). The B-protected PEG-Ara-C conjugate 8 (70 mg, 0.012 mmol) was dissolved in anhydrous DCM (3 mL), and triluorooxacetic acid (1 mL) was added dropwise to it at 0 °C and the solution was stirred for 3 h. The solvent was evaporated in vacuum and the residue was dissolved in DMF (10 mL) and purified through dialysis with DMF (membrane MW cut off 1000 Da). The resulting dialyzed solution was evaporated in vacuum and the obtained oily mass was precipitated in anhydrous diethyl ether to produce a white solid powder (55 mg, 84%). 1H NMR (CDCl3) δ 7.79 (d, 1H, Ar, J = 7.6 Hz), 6.19 (d, 1H, H-1′, J = 3.2 Hz), 6.07 (d, 1H, Ar, J = 7.6 Hz), 4.55 (m, 1H, H-2′), 4.34 (s, 1H, H-3′), 4.14–4.24 (m, 4H, H-4′ and −OCH2CH2OH), 4.08 (s, 1H, H-4′), 3.42–3.80 (m, mPEG-backbone H), 3.35 (s, 3H, −OCH3), 2.60–2.70 (m, 4H).

2.11. Drug Release Study in PBS. Release of Ara-C from D-Ara-C and PEG-Ara-C conjugates was characterized in 0.1 M phosphate buffer (pH 7.4) at 37 °C. A concentration of 1 mg/mL of the conjugate was maintained in a water bath with constant mixing, and at appropriate time points 300 μL of the sample was withdrawn from the incubation mixture and directly analyzed by GPC, without further treatment. PBS solution (0.1M, pH 7.4) with 0.025% sodium azide was used as mobile phase and the release of Ara-C was monitored at 210 and 272 nm for both dendrimer and Ara-C respectively. Percentage of Ara-C and SA-Ara-C (Ara-C linker) released from the conjugates were quantified by respective calibration curves.

2.12. Drug Release Study in Plasma. D-Ara-C (2 mg/mL) and PEG-Ara-C (3 mg/mL) were incubated in human pooled plasma diluted to 80% with 0.1 M PBS with constant mixing in water bath (Dual-action shaker; Polyscience, Niles, Illinois) at 37 °C. At periodic intervals, plasma samples (200 μL) were withdrawn. The precipitation of protein was done by addition of 2 M trichloroacetic acid, followed by addition of ice cold acetoneitrile (200 μL) and centrifuged at 10000 rpm for 5 min at 4 °C. The clear supernatant was then filtered through 0.2 μm filter and stored at −80 °C until GPC analysis. The samples were analyzed by GPC to evaluate the stability and release of Ara-C from the conjugates.

2.13. Cell Culture. Human lung adenocarcinoma epithelial cell line A549 was obtained from ATCC (Manassas, VA, U.S.A.) and was cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% antibiotics (penicillin/streptomycin) in a humidified incubator at 37 °C with 5% CO2.

2.14. MITT Assay of Cell Inhibition. Human lung adenocarcinoma A549 epithelial cells (5 × 104) were plated and grown for 24 h in 200 μL of growth medium in 96-well microtiter plates (Costar, Cambridge, MA) and then the culture medium was replaced by fresh phenol red-free DMEM medium containing different concentrations (0.005–2.0 μM) of Ara-C, D-Ara-C, and PEG-Ara-C at increasing concentrations. The control cells were treated with medium containing fresh phenol-red-free DMEM. The cells are incubated in standard conditions (explained in section 2.13) for 72 h. After a 72 h incubation...
3. RESULTS AND DISCUSSION

3.1. Preparation of the Dendrimer-Ara-C Conjugate (D-Ara-C, 6). The synthetic strategies were aimed at synthesizing a Boc-protected Ara-C linker that would possess reactive functional groups suitable for efficient conjugation to hydroxyl-terminated PAMAM dendrimer and PEG. The Ara-C contains heterofunctional reactive hydroxyl and amine groups. Our objective was to introduce a linker at C-5 position of Ara-C that could finally be used for subsequent reaction with dendrimer or PEG. Our previous studies suggested that linker plays a major role in releasing the drug from the dendrimer conjugates. Since the functional groups on the dendrimer used for conjugation on the surface are "sterically crowded", enzymatic release can be challenging compared to linear PEG. Here, we have used succinic acid as a spacer to conjugate Ara-C to the dendrimer surface to provide steric relief. The synthesis of Boc-protected Ara-C with succinic acid linker intermediates (4a, 4b) are depicted in Scheme 1. The intermediates 3a and 3b were synthesized by modification of the previously published procedure. In brief, Ara-C was reacted with TBDMS-Cl in pyridine in the presence of a catalytic amount of imidazole to get a white solid product 1 in good yield. The intermediate 1 was then converted to Boc-protected Ara-C (2a) by using excess Boc anhydride in dioxane, and when we changed the solvent to dioxane-chloroform (DCM) mixture, it resulted in completely Boc-protected hydroxyl groups intermediate (2b). A free hydroxyl group was obtained by deprotecting the TBDMS group at C-5 position of 2a and 2b with triethylamine trihydrofluoride in THF to get 3a and 3b, respectively. Finally, the carboxylic acid derivatives, 4a and 4b, were obtained by reacting succinic anhydride with 3a and 3b in the presence of a catalytic amount of DMAP in DCM. The above intermediates were purified on silica gel by gradient elution, and the structures of 1–4 were confirmed by 1H and 13C NMR and high-resolution mass spectroscopy.

The synthesis of the desired dendrimer–drug conjugate, D-Ara-C (6), is shown in Scheme 1. The Boc-protected dendrimer–drug conjugates (5a and 5b) were prepared using a coupling reaction between G4-OH and 4a and 4b in the presence of PyBOP in DMF, and the crude products were purified by dialysis (membrane MW cutoff 2000 Da). The intermediate 5a was characterized by 1H NMR, which suggested that 10 drug molecules were attached to the dendrimer based on the proton integration method. The final dendrimer–drug conjugate, D-Ara-C (6), was obtained by elimination of the Boc protecting groups of 5a and 5b with trifluoroacetic acid (TFA) in DCM and subsequent purification by dialysis. The structure of D-Ara-C conjugate was established by 1H NMR; loading of the drug molecules was calculated by MALDI-TOF mass spectroscopy; purity was determined by reverse-phase HPLC and size exclusion chromatography (SEC); and the number of amine groups originating from the Ara-C moieties and internal tertiary amines of the dendrimer were calculated by potentiometric analysis. In 1H NMR, the presence of two doublets corresponding to the aromatic protons of Ara-C at 7.76 and 8.20 ppm, to the aromatic protons of the drug conjugate, D-Ara-C (6), shows the drug conjugate is linear.
Ara-C were conjugated to the dendrimer. A peak at 16971 m/z in the MALDI-TOF mass spectrum indicated the presence of 10 drug molecules on the dendrimer, based on the molecular weight of the dendrimer (13672 Da) and linker (118 Da), which is in agreement with the 1H NMR results (Figure 2A). The HPLC and SEC chromatograms of Ara-C, Ara-C linker (SA-Ara-C), and D-Ara-C indicate that there were no low molecular weight impurities present in the conjugate (Figure 3). Appearance of a new peak of D-Ara-C at 18.78 min in the HPLC chromatogram at 272 nm, which is different from Ara-C (3.2 min) and SA-Ara-C (10.0 min), confirms the formation of the conjugate (Figure 3A). Additionally, in the SEC chromatogram a peak at 29.4 min for D-Ara-C indicates the formation of the product, whereas Ara-C and SA-Ara-C elute at 44.4 and

Figure 1. Proton NMR spectra of (A) dendrimer-Ara-C conjugate (D-Ara-C, 6) in DMSO-d6 and (B) polyethylene glycol-Ara-C conjugate (PEG-Ara-C, 9) in CDCl3.
38.6 min, respectively (Figure 3B). The difference in retention times of Boc-protected Ara-C-dendrimer and dendrimer-Ara-C conjugate in HPLC and SEC also confirms the formation of the product (data not shown). Finally, potentiometric titration measurements suggested that 10 ± 2 Ara-C molecules (corresponding to 17% of the end OH groups being consumed in conjugation) were attached to the dendrimer, based on the titration curves of the G4-OH dendrimer as the reference. This is in good agreement with 1H NMR and MALDI-TOF mass data (Figure 4). The number of tertiary amines present in the dendrimer does not change upon conjugation of Ara-C to the dendrimer but, at the same time, there will be additional primary amine groups that are present in Ara-C to the conjugates. Using a theoretical molecular weight of G4-OH (MW = 14277), the number of tertiary amines in the dendrimer was calculated to be 62 (Figure 4A), which is the same as that of the D-Ara-C conjugate (Figure 4B). In addition, appearance of a shoulder peak in the titration curve of the D-Ara-C conjugate indicates the presence of primary amines originating from Ara-C molecules, and the number was calculated to be 10 ± 2.

3.2. Preparation of the PEG-Ara-C Conjugate (PEG-Ara-C, 9). The synthesis of PEG-Ara-C conjugate is summarized in Scheme 2. In this case, we attached a linker to the PEG molecules (PEG-linker, 7) instead of the Ara-C (as before for synthesis of D-Ara-C conjugate). The desired PEG-Ara-C (9) was obtained by using EDC·HCl instead of PyBOP as the coupling agent followed by Boc deprotection. The purity of the final conjugate was confirmed by the chromatogram in HPLC and SEC (Figure 3), where no small amounts of starting material and other components were seen as impurities. The characterization of all intermediates and the final compound were done by 1H NMR (Figure 1B and supplement) and finally confirmed by the evident changes of molecular weight in MALDI-TOF MS spectroscopy (Figure 2B).

3.3. Particle Size and Zeta Potential. The surface properties and size of the dendrimer conjugates/nanoparticles

Figure 2. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra of (A) D-Ara-C (6) shows the relative increase of molecular weight upon Ara-C conjugation. The difference in the molecular weights of main fraction of G4-OH (13672 Da) and D-Ara-C (16971 Da) indicates 10 Ara-C molecules are attached. (B) MALDI-TOF mass spectra of PEG-linker (PEG-SA; MW 5117 Da) and PEG-Ara-C (MW 5338 Da) conjugates show one molecule is attached to the PEG.

Figure 3. (A) Elution profiles in HPLC chromatograms of Ara-C (3.29 min), SA-Ara-C (10.03 min), D-Ara-C conjugate (18.78 min), and PEG-Ara-C conjugate (35.81 min) are different from each other monitored at 272 nm. Unmodified G4-OH (13.8 min) and PEG (30.49 min) monitored at 210 nm are different from their respective conjugates (monitored at 272 nm). There is no measurable amount of free Ara-C, dendrimer, or PEG observed, indicating the purity of the desired products; (B) SEC chromatograms of conjugates with different retention time; D-Ara-C (29.49 min), PEG-Ara-C conjugate (29.55 min), unmodified Ara-C (44.44 min), and SA-Ara-C (38.68 min). Both G4-OH and PEG exhibited very low intensity at 28.76 and 29.89 min, respectively, monitored at 272 nm (for clarity, data not shown).
play a crucial role in determining the potential of the drug carrier and its interaction with biological systems. We measured the particle size and zeta potential of G4-OH (D), D-Ara-C, PEG, and PEG-Ara-C (Table 1). It appears that Ara-C conjugation to the dendrimer did not change the size appreciably. This indicates that the drug (with ketone and amine groups) may have folded back into the “hydrophobic” interior of the dendrimer (amide and amine groups). It may also be due to the small size of the dendrimer and the conjugates, which are at the low end of the instrument sensitivity. G4-OH has a small positive zeta potential (+4.5 mV), which is due to the presence of tertiary amines in its core. The zeta potential of D-Ara-C increased to +15.0 mV due to the presence of amine functionality in the Ara-C molecule. There are 10 Ara-C molecules attached to the dendrimer. Since PEG is a linear molecule, the hydrodynamic diameter of PEG and its conjugates are in between 4.5 to 7.0 nm. Linear PEG and PEG-SA have negative zeta potential (Table 1). After conjugation with Ara-C, the zeta potential of PEG-Ara-C increases to +6.9 mV due to the presence of an amine group in the Ara-C.

3.4. Release Study. In vitro release characteristics of the prepared conjugates were studied to investigate their stability in physiologically relevant solutions such as PBS (pH 7.4) and 80% human plasma. Human plasma is used to simulate the biological condition of intravenous injection where degradation may occur by hydroxyl ions and enzymes.15 Free Ara-C released from the conjugates binds to plasma proteins and gets deaminated converting to Ara-U, an inactive form of Ara-C. Hence, the peak corresponding to Ara-U was also taken into account for total drug release. We found that both of the conjugates release the drug in as, (i) free Ara-C, (ii) SA-Ara-C, and (iii) Ara-U, in both plasma and PBS.

Release in PBS. In PBS, both of the conjugates released the drug “relatively slowly” and had different release kinetics. The released Ara-C (elution time of 44.4 min SEC in Figure SC) and conjugated Ara-C (elution time of ~30 min for both PEG-Ara-C and D-Ara-C in SEC Figure SC) could be identified based on their different elution times. For PEG-Ara-C in PBS, the release is nearly zero order (20% in 100 h), with no initial burst (Figure SA). The release from D-Ara-C shows an initial burst (~10% in 4 h), followed by a sustained release (nearly zero order, 20% over the next 350 h). This estimation based on free Ara-C is consistent with the estimation based on the conjugated Ara-C (Figure SB). Figure SC illustrates this through the increase in the SEC peak associated with free Ara-C and the

Table 1. Particle Size and Zeta Potential of Dendrimer and PEG Conjugates

<table>
<thead>
<tr>
<th>compound</th>
<th>diameter (nm)</th>
<th>zeta potential (mV)</th>
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<tr>
<td>G4-OH (D)</td>
<td>4.28 ± 0.24</td>
<td>+4.54 ± 0.10</td>
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<tr>
<td>D-Ara-C (6)</td>
<td>3.39 ± 0.57</td>
<td>+15.03 ± 1.62</td>
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<tr>
<td>PEG</td>
<td>4.68 ± 0.69</td>
<td>−6.97 ± 0.76</td>
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<tr>
<td>PEG-SA</td>
<td>6.89 ± 1.01</td>
<td>−13.50 ± 2.14</td>
</tr>
<tr>
<td>PEG-Ara-C (9)</td>
<td>5.89 ± 1.78</td>
<td>+6.89 ± 1.10</td>
</tr>
</tbody>
</table>

Figure 4. Potentiometric titration curves of dendrimer (G4-OH) and dendrimer-Ara-C (D-Ara-C) conjugate. D-Ara-C conjugate shows an increase in the molecular weight upon conjugation with Ara-C. Number of tertiary amines (3’-N) in the dendrimer was found to be 62, based on the theoretical molecular weight (MW = 14277). The number of 3’-N and primary amine (1’-N) in the D-Ara-C conjugate was calculated considering the relative increase of the molecular weight (MW = 17527) and number of moles of titrant used for titration. The number of 1’-N is found to be 10 and the number of 3’-N did not change upon Ara-C conjugation with dendrimer.

Scheme 2. Synthesis of Polyethylene Glycol-Succinic Acid Linker (PEG-Linker, 7); Boc-Protected Ara-C-PEG Conjugate (8); and PEG-Ara-C (9) Conjugate Using Boc-Protection/Deprotection Chemistry
decrease of signal for conjugated Ara-C. Faster release was observed from PEG conjugate, over 14 days, approximately 79% (56% as free Ara-C and 23% as SA-Ara-C), whereas D-Ara-C released only 42% (35 and 6.6% of free Ara-C and SA-Ara-C, respectively), suggesting that ester bonds in linear PEG conjugate were more susceptible to hydrolysis due to their increased size in water and the fact that the drug is attached to one end of the linear PEG. In the dendrimer conjugate, there are 10 Ara-C molecules attached to the dendrimer, and the steric crowding on the dendrimer surface may hinder enzymatic cleavage of the ester bond (Figure 5A,B). In the dendrimer conjugate, the ester linkage is surrounded by electron-rich hydroxyl groups, which makes the ester bond less electron-deficient and less accessible to nucleophile-like hydroxyl ions or water molecules. So, the rate of hydrolysis slows down in the case of the dendrimer conjugate, whereas PEG being a linear molecule has no such effect. Since the formation of the inactive form of Ara-U requires the deaminase, there is no Ara-U formation in PBS.

Release in Plasma. In human plasma, the extent of release was relatively higher for both PEG and dendrimer conjugates compared to PBS at the same time point. This is expected because of the presence of enzymes (e.g., esterases), which can specifically cleave the ester bond between the linker and the drug. One of the limitations of Ara-C as a drug is its low stability in plasma and rapid conversion into a more inactive and soluble form, Ara-U. Such conversion is mediated by deaminase enzyme in plasma. PEG conjugate showed higher release rates (approximately 73% of the drug in 5 h in the form of Ara-U, Ara-C, and SA-Ara-C, and almost all the drug molecules were released within 4 days (Figure 5D). The Ara-C and SA-Ara-C released from the PEG conjugate were converted to Ara-U in plasma, which was confirmed by the decrease of Ara-C peak and appearance of Ara-U peak in the GPC chromatogram. From 5 h to 5 days, there appears to be a conversation of ∼35% of the released Ara-C and SA-Ara-C into the inactive Ara-U form, suggestive of instability of Ara-C in plasma. This suggests that the expected increased circulation time of PEG-Ara-C may not be able to directly address the conversion of Ara-C to Ara-U.

The release from D-Ara-C conjugate was comparatively slower, releasing approximately 70% of its payload in 5 days in the form of free Ara-C (30%), Ara-U (35%), and SA-Ara-C (5%; Figure 5E). Interestingly, at the end of 5 days, 30% of the released Ara-C from the dendrimer is still in the active form, whereas only 10% of that released from the PEG conjugate was in the active form. This could be attributed to the initial rapid release of Ara-C from PEG compared to the dendrimer. From 5 days to the end of 10 days, there is a decrease in the free Ara-C associated with an increase in Ara-U. At the end of

Figure 5. Controlled release profile of the Ara-C from D-Ara-C and PEG-Ara-C conjugates, monitored by size exclusion chromatography (SEC) at 272 nm. (A) Release profiles of Ara-C and SA-Ara-C from D-Ara-C and PEG-Ara-C conjugates in PBS buffer (pH 7.4) and (B) the decrease of the concentration of both conjugates with time; (C) The representative chromatograms of the D-Ara-C conjugate incubated in phosphate buffer (pH 7.4) at a concentration of 1 mg/mL for 0, 1, 4, and 48 h, showing an increase in the signals related to Ara-C and SA-Ara-C and a decrease in the peak associated with D-Ara-C conjugate; (D) Graph showing the relative percentage of Ara-C, SA-Ara-C, and the inactive form of Ara-C (Ara-U) from PEG conjugate in 80% human plasma; (E) Graph showing the relative percentage of Ara-C, SA-Ara-C, and the inactive form of Ara-C (Ara-U) release from D-Ara-C in 80% human plasma; (F) Decrease of D-Ara-C concentration in percentage with time in 80% human plasma.
Table 2. IC50 Values of Ara-C and Ara-C Conjugates

<table>
<thead>
<tr>
<th>compounds</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>Ara-C</td>
<td>0.66 ± 0.11</td>
</tr>
<tr>
<td>D-Ara-C (6)</td>
<td>0.17 ± 0.05a</td>
</tr>
<tr>
<td>Dendrimer + Ara-C</td>
<td>0.46 ± 0.13</td>
</tr>
<tr>
<td>PEG-Ara-C (9)</td>
<td>0.14 ± 0.04a</td>
</tr>
<tr>
<td>PEG + Ara-C</td>
<td>0.42 ± 0.08</td>
</tr>
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</table>

“IC50 values of Ara-C conjugates in human lung adenocarcinoma epithelial cells after 72 h treatment (MTT assay). Values represent the average from three independent experiments. Values are mean ± SE.

10 days, ~20% of the Ara-C is still conjugated to the dendrimer (Figure SF). In both plasma and PBS, the release of Ara-C from dendrimer conjugate was comparatively slower than that of PEG conjugate, suggesting a steric hindrance at the dendrimer surface affecting the enzymatic action on the linker between the dendrimer and the drug. On the other hand, PEG, being a linear polymer, is more susceptible to the enzymatic cleavage, because the drug is attached to only one end of the monofunctional-PEG.

It was also noticed that, in plasma, SA-Ara-C was released at a much slower rate from dendrimer conjugates compared to that of PEG conjugates. The half-life (t1/2) of Ara-C in plasma is reported to be 2 min and, hence, the conversion of Ara-C to Ara-U is unavoidable.15 We have also seen that the free Ara-C was completely converted to Ara-U after an incubation of 15 min in human plasma. In the release study of Ara-C from dendrimer conjugate, no Ara-U was detected for the first 2 h, and a negligible amount was observed after 5 h of incubation. In the case of the PEG conjugate, a considerable amount of Ara-U was detected within the first 2 h. These studies suggest that the advantage of using dendrimers as drug carriers since resulting conjugates are stable for a longer period of time and can reach the target site before a considerable amount of drug is released from them.

3.5. In Vitro Drug Efficacy. The efficacy of prepared conjugates in inhibiting the growth of cancer cells was evaluated in A549 human lung adenocarcinoma epithelial cells after 72 h of incubation (Figure 6). The cells were treated with dendrimer/PEG conjugates based on equivalent Ara-C concentrations. Our results suggested that both D-Ara-C and PEG-Ara-C conjugates were 4-fold more efficacious in inhibiting cell growth, based on IC50 values (Table 2), suggesting superior intracellular transport and release from the conjugates compared to cell uptake of free drug. The free dendrimer and PEG at concentrations equivalent to those used in the conjugates did not have any effect on the A549 cells. After 24 and 48 h incubations, the dendrimer and PEG conjugates showed lower inhibition activity compared to that of free Ara-C. This lower efficacy is probably due to the low amount of free Ara-C hydrolytically released from the conjugates inside cells at short times (up to 48 h), rather than due to slower uptake of the dendrimer conjugates.15 We have previously shown that neutral PAMAM dendrimers are taken up by A549 cells by nonspecific interactions relatively rapidly.45 Moreover, these dendrimers are mostly localized in the lysosomes of the cytosol,21 where the ester bonds are susceptible to cleavage by the action of lysosomal enzymes in acidic pH.

The delayed efficacy of the conjugates can be correlated with the stability of dendrimer conjugates in plasma where ~50% of the incubated conjugates (Figure SF) were stable up to 50 h, releasing ~60% of its payload in the form of Ara-C and SA-Ara-C, whereas PEG conjugates were comparatively less stable in plasma, releasing more that 70% of the drug within 5 h.

4. CONCLUSIONS

We have developed conjugates of Ara-C with a hydroxyl-terminated PAMAM dendrimer, and a linear polymer (PEG), using an ester linkage, and evaluated them in human adenocarcinoma epithelial cells. Our results suggest a significant increase in the inhibition of growth of A549 cells in both the conjugates compared to free drug. Release studies of Ara-C from the conjugates in PBS and in human plasma indicated their stability against hydrolytic cleavage and susceptibility in plasma. Release of the drug from PEG-Ara-C conjugate in plasma is much faster compared to D-Ara-C conjugate. Results also indicate the formation of the inactive form of Ara-C (Ara-U) is delayed upon polymeric conjugate compared to its free form. In MTT assay, both D-Ara-C and PEG-Ara-C conjugates were found to be 4-fold more efficacious than Ara-C in inhibiting the growth of A549 cells (based on IC50), suggesting a superior intracellular transport and release compared to free drug. Hence, it could be expected that both of the Ara-C conjugates would have much better stability and longer circulation time than free Ara-C in vivo. It is evident from the study that dendrimer plays a crucial role in increasing the stability as well as the efficacy of the drug, addressing a key need for treatment with Ara-C related to poor blood stability. Conjugation with dendrimer, in addition to further ligand targeting to the target site, may improve the effectiveness of Ara-C in vivo, enabling better delivery of this important drug in the fight against cancer.

ASSOCIATED CONTENT

Supporting Information

Preparation of compounds 3a and 3b is described in the method section. The 1H and 13C NMR spectra of the intermediates are presented in Figures S1–S13. This material is available free of charge via the Internet at http://pubs.acs.org.
The authors wish to thank the Ralph Wilson Foundation for Biomedical Engineering and the Wayne State University Nanotechnology Initiative (Nano Incubator) for financial support; and Admira Bosnjakovic for MALDI-TOF mass spectroscopy.

REFERENCES