Synergistic Induction of Apoptosis in Brain Cancer Cells by Targeted Codelivery of siRNA and Anticancer Drugs

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S *Supporting Information*

ABSTRACT: Multiple dysregulated pathways in tumors necessitate targeting multiple oncogenic elements by combining orthogonal therapeutic moieties like short-interfering RNAs (siRNA) and drug molecules in order to achieve a synergistic therapeutic effect. In this manuscript, we describe the synthesis of cyclodextrin-modified dendritic polyamines (DexAMs) and their application as a multicomponent delivery vehicle for translocating siRNA and anticancer drugs. The presence of β-cyclodextrins in our DexAMs facilitated complexation and intracellular uptake of hydrophobic anticancer drugs, suberoy-lanilide hydroxamic acid (SAHA) and erlotinib, whereas the cationic polyamine backbone allowed for electrostatic interaction with the negatively charged siRNA. The DexAM complexes were found to have minimal cytotoxicity over a wide range of concentrations and were found to efficiently deliver siRNA, thereby silencing the expression of targeted genes. As a proof of concept, we demonstrated that upon appropriate modification with targeting ligands, we were able to simultaneously deliver multiple payloads —siRNA against oncogenic receptor, EGFRvIII and anticancer drugs (SAHA or erlotinib)—efficiently and selectively to glioblastoma cells. Codelivery of siRNA-EGFRvIII and SAHA/erlotinib in glioblastoma cells was found to significantly inhibit cell proliferation and induce apoptosis, as compared to the individual treatments.

KEYWORDS: RNA interference, codelivery, cyclodextrins, SAHA, brain tumor cells, targeted delivery
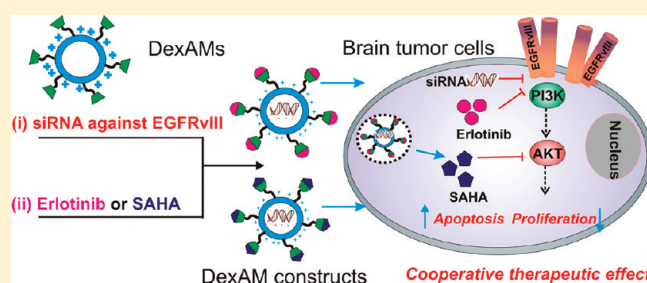
■ INTRODUCTION

Advances in the field of chemical genetics and molecular cell biology have triggered a surge in development of genetic manipulation based therapies for cancer.[1,2] Such genetic manipulation methods typically rely on either the traditional small-molecule/protein modalities[3] or the newly discovered RNA interference (RNAi) based modalities,[4] each having their own advantages and disadvantages. For example, RNAi therapeutics can provide attractive solutions to the major shortcomings of the conventional therapeutics, including difficulty in lead identification and complex synthesis of small organic molecules and proteins, and potentially can be applicable to all molecular targets for cancer therapy.[5] However, RNAi-based therapeutics, such as small interfering RNA (siRNA) and micro RNA (miRNA), are inherently antagonistic and their downstream effects (i.e., gene-silencing) are delayed, compared to those of conventional small-molecule/protein-based therapeutics.[6] Additionally, owing to their short serum half-life and poor cellular uptake, successful clinical application of siRNA requires appropriate chemical modifications and better delivery vehicles to overcome the numerous cellular barriers.[4] On the other hand, small organic molecules can act as both antagonists and agonists for molecular targets and their drug effects can be much faster than siRNA with minimal problems during their intracellular uptake.[5] Hence, from a biological perspective, it would be beneficial to combine the

advantages of these therapeutic modalities to potentially enhance their individual efficacy. For example, it was recently demonstrated that simultaneous delivery of siRNA against multidrug resistance genes in cancer cells led to the enhanced efficacy of the codelivered anticancer drugs.[7,8] These studies show that it would be desirable to target multiple oncogenic signaling elements using different therapeutic modalities for cooperative effect, especially considering the molecular heterogeneity of tumors. However, to achieve this goal, the primary requirement is to develop noncytotoxic codelivery platforms capable of efficient translocation of siRNA and small molecules with specificity as well as identify the right combination of siRNA and small molecules for a cooperative therapeutic effect.

To address the aforementioned need for cooperative chemotherapeutics, herein we describe the synthesis of a multifunctional delivery platform consisting of a dendritic polyamine backbone conjugated with β-cyclodextrin (β-CD) moieties [henceforth referred to as DexAMs] and its application for target-specific codelivery of two orthogonal chemotherapeutic molecules (siRNA and anticancer drug). We hypothesize that
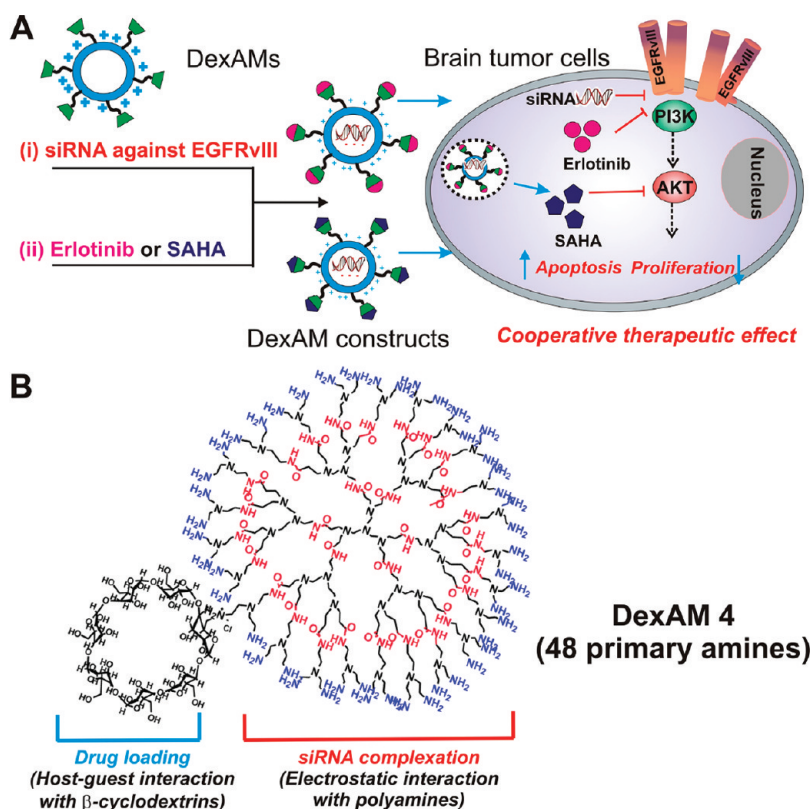
Scheme 1. (A) General Scheme Showing Codelivery of Small Molecules Like Anticancer Drugs and siRNA to Cancer Cells Using Cyclodextrin Modified Polyamines (DeXAMs). (B) Chemical Structure of the Delivery Vehicle[a]

[a] See Supporting Information for other DexAM generations.

codelivery of siRNA and anticancer drugs will have a cooperative therapeutic effect against the target oncogenic signaling pathway (EGFRvIII-PI3K/AKT), resulting in the selective induction of apoptosis in brain tumor cells (Scheme 1). Additionally, conjugation of targeting ligands against receptors overexpressed in brain cancer cells (EGFR) would allow for selective uptake of our complexes into glioblastoma cells, thereby minimizing toxic side effects on normal cells.

Additionally, our delivery platform and synthetic methods have several advantages, as compared to conventional carrier molecules (e.g., polyethyleneimine (PEI) and polyamidoamine (PAMAM)). These include (i) minimal cytotoxicity and high transfection efficiency of siRNA/drug—DexAM constructs, (ii) significantly higher yields and purity of DexAMs and increased aqueous solubility of DexAM constructs, and (iii) capability of simultaneously delivering nucleic acids, small organic molecules and proteins, thereby achieving cooperative therapeutic effects.

■ MATERIALS AND METHODS

Starting materials, reagents, and solvents were purchased from commercial suppliers (Sigma-Aldrich, Acros, and Fisher) and used as received unless otherwise noted. All reactions were conducted in flame-dried glassware with magnetic stirring under an atmosphere of dry nitrogen. Reaction progress was monitored by analytical thin layer chromatography (TLC) using 250 μm silica gel plates (Dynamic Absorbents F-254). Visualization was accomplished with UV light and potassium permanganate stain, followed by heating. Proton nuclear magnetic resonance (^{1}H NMR)

spectra were recorded on either a Varian-300 instrument (300 MHz), a Varian-400 instrument (400 MHz) or a Varian-500 instrument (500 MHz). Chemical shifts of the compounds are reported in ppm relative to tetramethylsilane (TMS) as the internal standard. Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), and coupling constants (Hz).

Quantification of siRNA Loading Efficiency. The complexes were prepared at various charge ratios by mixing equal volumes of DexAM with siRNA in PBS. Charge ratios (N/P) were calculated as a ratio of the number of primary amines in the polymer, determined from ^{1}H NMR spectra, to the number of anionic phosphate groups in the siRNA. The samples were then incubated at room temperature for 30 min to ensure complex formation. The complexes were prepared at a final siRNA concentration of 0.2 μg of siRNA/100 μL of solution. 100 μL of each complex was transferred to a 96-well (black-walled, clear-bottom, nonadsorbing) plate (Corning, NY, USA). A total of 100 μL of diluted Pico-Green dye (1:200 dilution in Tris- EDTA (TE) buffer) was added to each sample. Fluorescence measurements were made after 10 min of incubation at room temperature using a M200 Pro Multimode Detector (Tecan USA Inc., Durham, NC, USA), at excitation and emission wavelengths of 485 and 535 nm, respectively. All measurements were corrected for background fluorescence from a solution containing only buffer and PicoGreen dye.

Particle Size and Zeta Potential Analysis. Dynamic light scattering (DLS) and zeta potential analyses were performed using a Malvern Instruments Zetasizer Nano ZS-90 instrument (Southboro, MA) with reproducibility being verified by collection

and comparison of sequential measurements. Polymer/siRNA complexes (siRNA concentration = 330 nM) at different polymer concentrations were prepared using purified water (resistivity = 18.5 MΩ-cm). DLS measurements were performed at a 90° scattering angle at 25 °C. Z-average sizes of three sequential measurements were collected and analyzed. Zeta potential measurements were collected at 25 °C, and the Z-average potentials following three sequential measurements were collected and analyzed.

Cell Culture. Cells were cultured in the following growth media: DMEM (Dulbecco's modified Eagle's medium) with high glucose (Invitrogen), 10% fetal bovine serum (FBS), 1% streptomycin—penicillin, 1% glutamax (Invitrogen), and selection markers, G418 (100 μg/mL) and hygromycin B (30 μg/mL) for U87-EGFP and U87-EGFRvIII respectively. PC-12 cells were cultured in DMEM with 10% horse serum, 5% FBS and 1% streptomycin—penicillin. For the knockdown experiment and targeted delivery, passaged cells were prepared to 40—60% confluency in 24-well plates. For the knockdown experiment, targeted delivery and cell viability assay, medium was exchanged with serum-free basal medium (500 μL) and siRNA—DexAM solution (50 μL) was added after 20—30 min. After incubation for 12 h, medium was exchanged with normal medium. Fluorescence measurement and cellular assays were performed after 48—96 h from the starting point.

Cytotoxicity Assays. The percentage of viable cells was determined by MTS assay following standard protocols described by the manufacturer. All experiments were conducted in triplicate and averaged. The quantification of polymer-mediated toxicity was done using MTS assay after incubating the glioblastoma cells in the presence of varying concentrations of only polymer vehicle for 48—96 h. The data is represented as formazan absorbance at 490 nm, considering the control (untreated) cells as 100% viable.

Quantification of Knockdown of EGFP Expression (ImageJ). Following siRNA treatment, cells were washed with DPBS and fixed with 2—4% paraformaldehyde solution prior to imaging. For the fluorescence, DIC and phase contrast images were obtained using the Zeiss Axio observer inverted epifluorescence microscope. Each image was captured with different channels and focus. Images were processed and overlapped using Image-Pro (Media Cybernetics) and ImageJ (NIH).

Targeted Delivery. Highly tumorigenic U87-EGFP cells and low-tumorigenic PC-12 cells were cultured in 24-well plates, at a density of 5×10^4 cells per well. For PC-12 cells, the normal growth medium was DMEM (with high glucose, Invitrogen), 5% horse serum, 10% FBS, 1% Glutamax, and 1% penicillin—streptomycin. For the delivery of EGFR-Ab conjugated DexAM polyplexes, medium was exchanged with serum free DMEM medium. The cells were incubated in the Ab-conjugated polyplex medium for 6—8 h. Fluorescence images were taken after replacing the serum-free medium with regular medium.

Apoptosis Assay. Cells were harvested by trypsinization and stained using an Annexin V FITC Apoptosis Detection kit (Roche, Cambridge, MA) according to the manufacturer's protocol. The stained cells were immediately analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lake, NJ). Early apoptotic cells with exposed phosphatidylserine but intact cell membranes bound to Annexin V—FITC but excluded propidium iodide. Cells in necrotic or late apoptotic stages were labeled with both Annexin V—FITC and propidium iodide.

RESULTS AND DISCUSSION

Using multistep solution-phase and solid-phase synthesis, we generated a series of highly water soluble dendritic polyamine compounds conjugated to one or more β-cyclodextrin (β-CD) molecules, referred to as DexAMs, with higher yield and purity as compared to reported syntheses (Scheme 2). The first step for synthesizing DexAM involved generating a dendritic polyamine backbone by Michael addition of tris(2-aminoethyl)amine and methyl acrylate, followed by amidation of the amino esters generated after Michael addition. The use of tris(2-aminoethyl)amine as the core initiator yielded higher surface amine groups and hence more compact dendrimers as compared to the reported synthetic methods (for, e.g., ethylenediamine, ammonia) for PAMAM dendrimers.[9] The conjugation of β-cyclodextrin to the polyamine backbone involved tosylation of β-cyclodextrin, followed by nucleophilic addition with amine group. Compared to the previously reported protocol,[10] where tosyl chloride was used for regioselective tosylation of β-cyclodextrin resulting in very low yields, we improved the synthetic yield (~50%) and purity by using tosylimidazole, instead of tosyl chloride, under reflux conditions to generate 6-monotosylated β-cyclodextrin (see Supporting Information). In the final step, polyamine backbone was conjugated to tosylated CD via nucleophilic addition to generate cyclodextrin conjugated polyamines, resulting in a 25-fold increase in the aqueous solubility of CD (>50 g/100 mL) as compared to that of CD alone (<1.8 g/100 mL), owing to generation of an aminium salt (see Supporting Information for the detailed synthesis).

The first component of our delivery vehicle—β-CD—has been extensively used in pharmaceutical applications to improve solubility of hydrophobic moieties, such as anticancer drugs.[11] Many anticancer drugs are known to have poor aqueous solubility, thereby necessitating the use of toxic organic solvents like dimethyl sulfoxide (DMSO), which can be detrimental in biological applications.[12] The presence of β-CD in our DexAM moiety and the optimized drug loading would not only prevent the use of such toxic solvents but also improve the water solubility of CD—drug complex for the optimal cellular uptake and drug efficacy. In our study, two hydrophobic anticancer drugs [erlotinib and suberoylanilide hydroxamic acid (SAHA)] were synthesized and loaded into the β-CD cavity by using our optimized protocols (Figure 2b).[13,14] For instance, by utilizing the pH-dependent solubility of erlotinib, we could load drug up to almost 50% of the molar ratio of β-CD, resulting in a significant increase in its aqueous solubility (178 mg/100 mL).[15,16] Similarly, we complexed SAHA with β-CD under reflux conditions to obtain highly water soluble SAHA—CD complexes (solubility: 175 mg/100 mL) (see Supporting Information for the more detailed synthesis and experimental protocols).[17] The second component of our DexAMs—dendritic polyamine backbone—provides a positive surface charge which can interact electrostatically with the negatively charged nucleic acids, condensing them into cationic complexes (known as *polyplexes*), thus facilitating their intracellular uptake and endosomal escape.[18—20] However, these primary/tertiary amines are also responsible for cytotoxicity by interacting with the cellular components and interfering in the cellular processes.[21] Hence, there is a clear need to develop synthetic chemistry to control the ratio of electrostatic properties and the size of polymer structures. Our synthetic methods enabled us to precisely control the number of primary amine head groups from 4 to 48 leading to four different generations

Scheme 2. (A) Schematic Representation of Synthesis of DexAMs. (B) Conjugation of Drugs and Antibodies to DexAMs
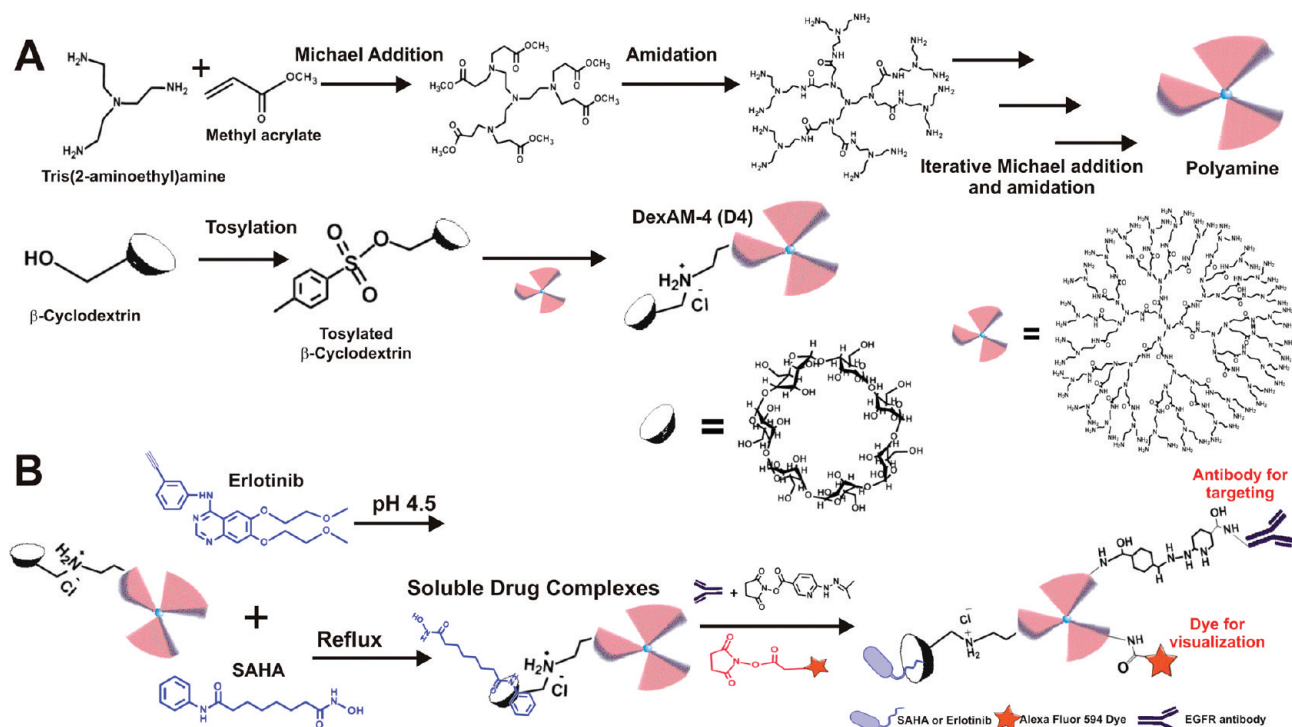
of DexAMs molecules (D1−D4), thereby allowing us to achieve an optimal balance between cytotoxicity and complexation ability.

We assessed the capability of our four different generations of DexAMs (D1−D4) to spontaneously form complexes with the negatively charged siRNA using a well-established dye exclusion assay. As the number of amine groups increased from DexAM-1 (D1, 4 primary amines) to DexAM-4 (D4, 48 primary amines), the amount of free/unbound siRNA decreased correspondingly at a given DexAM concentration (see Figure S1 in the Supporting Information). Since we found that the complexation ability of DexAM-4 is higher than that of the other generations with minimal cytotoxicity, we proceeded with using DexAM-4 for the subsequent experiments. Additionally, the hydrodynamic diameters of the resultant polyplexes could be controlled from 250 to 400 nm with polydispersity index of 0.8−1.0 by increasing the polymer concentration (see Figure S2a in the Supporting Information). The zeta potentials of the resulting polyplexes were in the range of 8−10 mV at pH 7.4 (see Figure S2b in the Supporting Information), demonstrating the cationic nature of the polyplexes. Cytotoxicity of the DexAM molecules was assessed using MTS assay. First we confirmed the effect of the β-CD moiety on the cytotoxicity of DexAMs by comparing the cytotoxicity of the DexAM (containing CD) to that of the DexAM without CD. Our cytotoxicity assay data clearly shows that the DexAM constructs with CD show significantly less cytotoxicity as compared to those without CD (Figure 1). We believe this is due to the presence of CDs on a polycationic backbone in DexAM, which can potentially reduce nonspecific binding of the DexAM constructs with proteins or cellular structures.[20−23] We also compared the cytotoxicity of our DexAMs with the commercially available transfection agents, polyethyleneimine (PEI), Lipofectamine 2000 (LF) and X-tremeGENE (Xgene)

at the recommended concentrations for transfection, and found that those agents were significantly more cytotoxic at those concentrations as compared to DexAMs (Figure 1b).

The optimization of gene silencing with our siRNA−DexAM constructs and assessment of knockdown efficiency were performed by measuring the suppression of enhanced green fluorescent protein (EGFP) in glioblastoma cell lines (U87-EGFP), which were genetically modified to constitutively express EGFP. The decrease of green fluorescence intensity due to siRNA-mediated EGFP silencing was monitored over a time period of 48−96 h to quantify the knockdown efficiency of our DexAM/siRNA constructs (see Figure S4 in the Supporting Information). Approximately 70% of the U87-EGFP cells showed no EGFP signal after 96 h of siRNA treatment as compared to the control cells at a polymer concentration of 100 μM (Figure 1c) with negligible cytotoxicity (∼95% cell viability). In parallel, we compared the transfection efficiency and the corresponding cytotoxicity of our delivery platform with that of the commercially available transfection agent (X-tremeGENE) under the same condition, in which X-tremeGENE-based transfection demonstrated similar levels of EGFP knockdown (∼70% knockdown efficiency), albeit with significant toxicity (∼30% cell viability) (see Figure S5 in the Supporting Information).

In addition to efficient translocation of siRNA across the cell membrane with minimal cytotoxicity, successful therapeutic application of siRNA also requires the siRNA to interact with the RNAi machinery within a target cell, thereby minimizing off-target effects.[24] Brain tumor cells, particularly glioblastoma cells, are known to present high levels of epidermal growth factor receptors (EGFRs) on their cell surface, thus making it a specific biomarker for cell-specific delivery toward brain tumor cells.[25] For targeted delivery to glioblastoma cells, we modified our DexAM-4 with appropriate ratios of EGFR antibodies (DexAM-4:EGFR-Ab = 1:5)
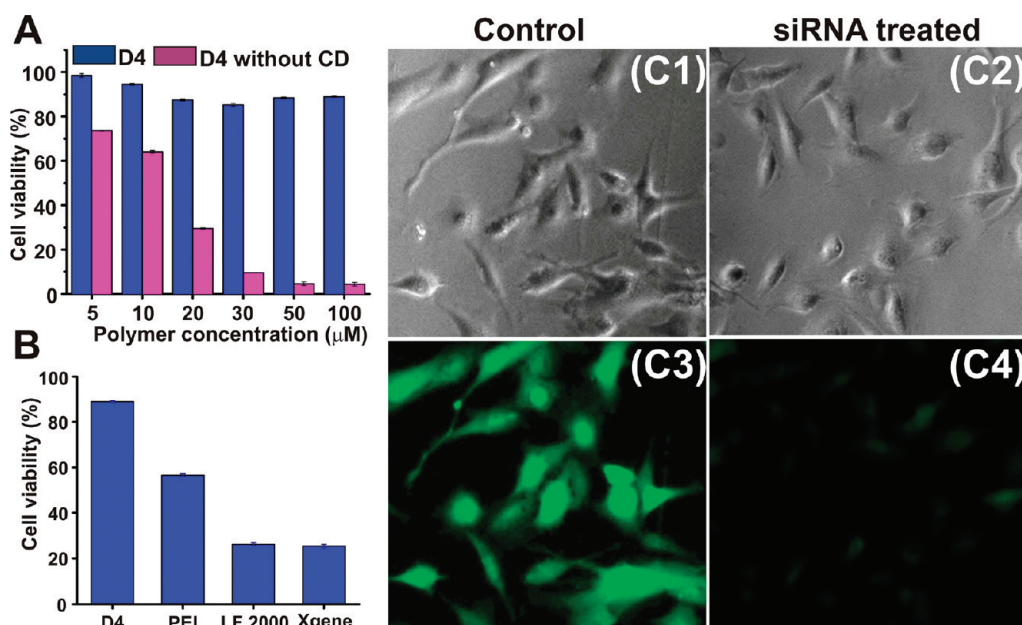
Figure 1. Cytotoxicity and transfection efficiency of DexAM-4 (D4). (A) Effect of cyclodextrin grafting on polymer-mediated toxicity. (B) Comparison of toxicities of DexAM-4 with commercially used transfection agents at optimized concentrations of delivery agent and siRNA. (LF 2000, Lipofectamine 2000; and Xgene, X-tremeGENE). (C) Phase contrast (C1, C2) and fluorescent (C3, C4) images showing siRNA-mediated decrease in green fluorescence in treated and control (untreated) U87-EGFP cells.

and incubated them in U87 (glioblastoma cell line, target cells) and other less-tumorigenic PC-12 cells (control cells) which tend to have low levels of expression of EGFRs. The DexAM-4 constructs were also labeled with a fluorescent dye (Alexa Fluor 594) to monitor their intracellular uptake using fluorescence microscopy. From our data we could see that EGFR-antibody modified DexAM-4 were selectively translocated into U87 (target glioblastoma cells) with high efficiency as compared to the PC-12 (control cells) (Figure 2a).

Having demonstrated the target-specific delivery and efficient gene silencing capability of the siRNA–DexAM constructs, we then focused on our main goal of codelivering siRNA and anticancer drugs for targeting key oncogenic signaling pathways (e.g., EGFRvIII-(phoshphatidylinositol-3-kinase)PI3K/AKT) to achieve a cooperative chemotherapeutic effect. Tumors harbor multiple dysregulated signaling pathways, thus limiting the clinical utility of single target agents.[26] Hence, combining approaches targeting multiple oncogenic elements, using a single delivery platform, can not only increase the likelihood of blocking tumor survival and metastasis, as compared to individual treatments, but also simplify clinical applications. For this purpose, we focused on developing a combinatory therapeutic approach based on siRNA and anticancer cancer drugs targeting oncogenic pathways in glioblastoma multiforme (GBM), an extremely aggressive and difficult-to-treat form of primary brain tumor. We aimed at downregulating the EGFRvIII-PI3K/AKT pathway, implicated in the proliferation and apoptosis of brain tumor cells, by delivering siRNA against epidermal growth factor receptor variant III (EGFRvIII), which is known to enhance the tumorigenicity of GBM.[27−29] However, due to tumor molecular heterogeneity, only siRNA-based downregulation of a single oncogenic target (EGFRvIII) may not be efficacious. Histone deacetylase (HDAC) inhibitors like suberoylanilide hydroxamic acid (SAHA) and EGFR tyrosine kinase inhibitors like erlotinib have been reported to enhance the efficacy of other EGFR

antagonists.[26,30] To this end, we used either SAHA or erlotinib for codelivery with siRNA against EGFRvIII oncogene to deactivate the target signaling pathway in a selective and efficient manner. These drugs have already shown some promising results for GBM therapy, but have met with limited success since they require higher doses and longer exposures, which may lead to increased toxic side effects.[31]

Our hypothesis is that combination of anticancer drugs against complementary therapeutic targets with siRNA therapeutics against EGFRvIII would have a cooperative effect on induction of apoptosis in brain tumor cells. To test this hypothesis, we initially compared the antiproliferative capability of anticancer drugs (SAHA and erlotinib) and siRNA against EGFRvIII in glioblastoma cells, either individually or in combination by using cell viability assay (Figure 2b). From the data, we could clearly observe a cooperative inhibition of glioblastoma cell proliferation when SAHA (5 μM) was codelivered with the siRNA (200 nM; polymer concentration 100 μM), as compared to treating the cells with only SAHA at the same concentration (5 μM). This can be attributed to the fact that SAHA is known to significantly enhance the efficacy of agents targeting EGFR signaling pathway by modulating several indirect downstream targets, which in turn are key regulators of EGFR pathways. Similarly, codelivery of erlotinib (30 μM) and siRNA (200 nM) also inhibited tumor cell proliferation to a higher extent (Figure 2b). Additionally, we also monitored the effect of codelivery of both siRNA and anticancer drugs on inducing cell death in glioblastoma cells using the apoptosis assay (Annexin-V/propidium iodide assay). A significantly higher proportion of cell population treated with both siRNA and SAHA was Annexin-V−FITC-positive as compared to the individual treatments as well as untreated cells. These results indicate greater induction of apoptosis in cells treated with both siRNA and SAHA, as compared to those with only SAHA and only siRNA treatment (Figure 2c). A similar trend in the cooperative induction of apoptosis was seen in the case of
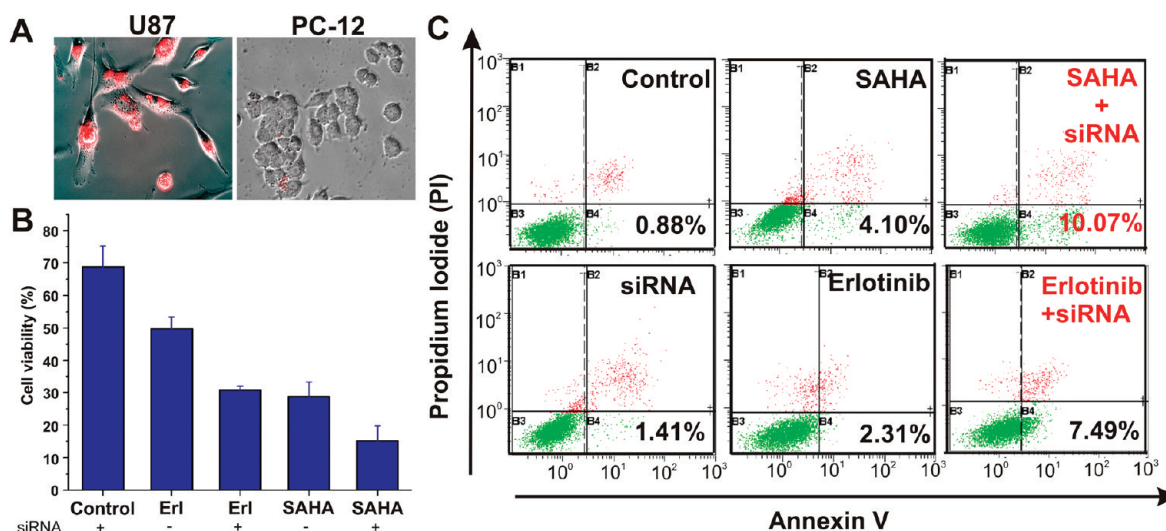
Figure 2. Targeted delivery of DexAMs and cooperative effect of anticancer drugs and siRNA on glioblastoma cells. (A) Targeted delivery of DexAMs modified with EGFR antibodies in highly tumorigenic U87-EGFP cells and less-tumorigenic PC-12 cells. (B) Viability of glioblastoma cells following individual treatments and codelivery of drugs and siRNA, based upon MTS assay. (C) Flow cytometry based Annexin-V/PI assay demonstrating the apoptotic effect of combined and individual siRNA and drug treatments. Percentages represent Annexin-V-positive (apoptotic cells). For all experiments, the polymer concentration was kept constant (100 μM), whereas the concentrations of SAHA, erlotinib and siRNA were 5 μM, 30 μM and 200 nM respectively.

combined erlotinib/siRNA treatment (Figure 2c). We also found that complexation of SAHA and erlotinib within the CD cavity improved their aqueous solubility and hence increased their potency, measured as IC_{50} values, by approximately 2-fold as compared to its DMSO solution (see Figure S6 in the Supporting Information). Thus, these results show the cooperative effect on selectively inducing the apoptosis of brain tumor cells by the right combination of siRNA and anticancer drugs and the capability of our delivery molecules (DexAMs) for target-specific delivery and improved chemotherapeutic efficacy.

In conclusion, we synthesized a multimodal delivery platform to simultaneously deliver two orthogonal therapeutic modalities having cooperative therapeutic efficacy in an efficient and selective manner to the target brain tumor cells. As a proof-of-concept experiment, we demonstrated that target-specific codelivery of siRNA and anticancer drugs having complementary therapeutic results would be a novel method to enhance the apoptotic signaling pathways and inhibit the proliferation signaling pathways in brain tumor cells. Potentially, our approach and methodology can be beneficial for introducing exogenous siRNA combined with small molecules into other mammalian cells, which can represent a powerful approach for the optimal manipulating signal transduction. Our synthetic techniques afforded facile manipulation of the polymer structure to achieve efficient transfection with minimal polymer-mediated cytotoxicity. The strategy of codelivering anticancer drug with therapeutic siRNA is particularly advantageous for *in vivo* applications, so that both the moieties are delivered to the target cells using a single delivery platform. Our versatile delivery platform can also be used to codeliver different kinds of small molecules and nucleic acids to regulate cancer cell fate such as proliferation, migration and apoptosis by targeting multiple signaling pathways. Collectively, our DexAM-based codelivery strategy has significant potential for cancer therapy as well as regulating cell fate by modulating key signaling cascades.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. Detailed synthesis of DexAMs; NMR characterization of synthesized compounds; conjugation of targeted moieties to DexAMs, complexation of drugs, particle size and zeta potentials of DexAM polyplexes, siRNA loading efficiency. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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