Published in final edited form as:

Bioconj Chem. 2015 August 19; 26(8): 1651–1661. doi:10.1021/acs.bioconjchem.5b00254.

Combination of nanoparticle-delivered siRNA for Astrocyte elevated gene-1 (AEG-1) and all-*trans* retinoic acid (ATRA): an effective therapeutic strategy for hepatocellular carcinoma (HCC)

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Abstract

Hepatocellular carcinoma (HCC) is a fatal cancer with no effective therapy. Astrocyte elevated gene-1 (AEG-1) plays a pivotal role in hepatocarcinogenesis and inhibits retinoic acid-induced gene expression and cell death. Combination of a lentivirus expressing AEG-1 shRNA and all-*trans* retinoic acid (ATRA) profoundly and synergistically inhibited subcutaneous human HCC xenografts in nude mice. We now have developed liver-targeted nanoplexes by conjugating poly(amidoamine) (PAMAM) dendrimers with polyethylene glycol (PEG) and lactobionic acid (Gal) (PAMAM-PEG-Gal) which were complexed with AEG-1 siRNA (PAMAM-AEG-1si). The polymer conjugate was characterized by ¹H-NMR, MALDI and mass spectrometry, and optimal nanoplex formulations were characterized for surface charge, size and morphology. Orthotopic xenografts of human HCC cell QGY-7703 expressing luciferase (QGY-luc) were established in the livers of athymic nude mice and tumor development was monitored by bioluminescence imaging (BLI). Tumor-bearing mice were treated with PAMAM-siCon, PAMAM-siCon+ATRA, PAMAM-AEG-1si and PAMAM-AEG-1si+ATRA. In the control group the tumor developed aggressively. ATRA showed little effect due to high AEG-1 levels in QGY-luc cells. PAMAM-AEG-1si showed significant reduction in tumor growth and the combination of PAMAM-AEG-1si +ATRA showed profound and synergistic inhibition so that the tumors were almost undetectable

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by BLI. A marked decrease in AEG-1 level was observed in tumor samples treated with PAMAM-AEG-1si. The group treated with PAMAM-AEG-1si+ATRA nanoplexes showed increased necrosis, inhibition of proliferation and increased apoptosis when compared to other groups. Liver is an ideal organ for RNAi therapy and ATRA is an approved anti-cancer agent. Our exciting observations suggest that the combinatorial approach might be an effective way to combat HCC.

Keywords

Astrocyte elevated gene-1; all-*trans* retinoic acid; nanoparticle; hepatocellular carcinoma; combinatorial therapy; orthotopic xenograft

Introduction

Hepatocellular carcinoma (HCC) is a major global health problem. The incidence of HCC is increasing in the West, and it is the third highest cause of cancer-related death globally [1]. In the US, the estimated new cases of HCC for 2014 were 33,190 out of which 23,000 were expected to die [2]. HCC, diagnosed at early stages, is amenable to potentially curative treatments, such as surgical therapies (resection and liver transplantation) and loco-regional procedures (radiofrequency ablation) [3, 4]. Five-year survival rates of up to 60 to 70% can be achieved in well-selected patients. However, disease that is diagnosed at an advanced stage or with progression after loco-regional therapy has a dismal prognosis, owing to the underlying liver disease and lack of effective treatment options. Sorafenib is the only FDA-approved drug for advanced HCC that extends overall survival by only 2.8 months [5]. This dismal scenario mandates development and evaluation of novel therapeutic strategies for this fatal malady.

Astrocyte elevated gene-1 (AEG-1), also known as Metadherin (MTDH) and LYRIC, plays an important role in regulating hepatocarcinogenesis. We documented that AEG-1 is overexpressed in both mRNA and protein levels in a high percentage (>90%) of HCC patients and a significant percentage of patients harbored genomic amplification of the AEG-1 locus in chromosome 8q22 [6]. AEG-1 is transcriptionally regulated by c-Myc [7], an oncogene frequently upregulated in HCC [8]. The tumor suppressor miRNA miR-375, which is downregulated in HCC patients, targets AEG-1 [9]. Thus AEG-1 overexpression occurs by multiple mechanisms in HCC patients. HCC with more microvascular invasion or pathologic satellites, poorer differentiation, and TNM stages II to III are prone to exhibit higher AEG-1 expression [10]. HCC patients with high AEG-1 expression documented higher recurrence and poor overall survival [11]. Overexpression of AEG-1 in poorly aggressive HCC cell line HepG3, which expresses low level of AEG-1, significantly increases *in vitro* proliferation, invasion and anchorage-independent growth and *in vivo* tumorigenesis, angiogenesis and metastasis in nude mice [6, 12, 13]. As a corollary, transgenic mice with hepatocyte-specific overexpression of AEG-1 (Alb/AEG-1) develop highly aggressive angiogenic HCC with significantly accelerated kinetics upon treatment with the hepatocarcinogen n-Nitrosodiethylamine (DEN) when compared to their Wild-type counterparts [14, 15]. Conversely, knockdown of AEG-1 in highly aggressive human HCC cell line QGY-7703, expressing high levels of AEG-1, significantly abrogates *in vivo*

tumorigenesis [6, 12], and an AEG-1 knockout (AEG-1KO) mouse shows profound resistance to DEN/phenobarbital (PB)-induced initiation and progression of HCC [16]. AEG-1 overexpression profoundly modulates expression of genes associated with proliferation, invasion, chemoresistance, angiogenesis and metastasis in both human HCC cell lines and Alb/AEG-1 hepatocytes [6, 14, 15]. These studies establish an essential role of AEG-1 in hepatocarcinogenesis and identify AEG-1 as a valid target to develop a HCC therapeutic.

We have identified that AEG-1 interacts with retinoid x receptor (RXR) and inhibits retinoic acid (RA)-induced cytotoxicity [17]. AEG-1 contains an 'LXXLL' motif which is employed by transcriptional co-activators, such as SRC-1, to interact with nuclear receptors to activate transcription [18]. In the absence of RA, RXR-RAR heterodimer binds to co-repressors and inhibits transcription [19]. Upon binding of RA to RXR-RAR, there is a conformational change so that co-repressors move out allowing co-activators to bind and activate transcription. In the nucleus AEG-1 interacts with RXR via 'LXXLL' motif and interferes with recruitment of co-activators thereby blocking RA-induced transcription [17]. In cancer cells AEG-1 accumulates in the cytoplasm and entraps RXR thereby preventing its nuclear translocation [17]. Additionally AEG-1 induces phosphorylation-mediated inactivation of RXR by activating ERK [17]. Collectively, these mechanisms result in profound inhibition of RA function when AEG-1 is overexpressed. A combination of a lentivirus expressing AEG-1 shRNA (lenti.shAEG-1) and all-*trans* retinoic acid (ATRA) profoundly and synergistically inhibited growth of subcutaneous human HCC xenografts in nude mice [17].

In this study, we document that hepatocyte-targeted nanoparticle-delivered AEG-1 siRNA in combination with ATRA results in profound synergistic inhibition of orthotopic human HCC xenografts in nude mice. These exciting observations suggest that this combinatorial strategy might be an effective way to counteract HCC.

Materials and methods

Materials

2-[N-Morpholino] ethanesulfonic acid hydrate (MES) buffer, lactobionic acid, poly[ethylene glycol] 2-aminoethyl ether acetic acid, M_n 2100 (PEG), poly[amidoamine], generation 5, provided as methanolic solution (PAMAM), 2-Mercapto ethanol, deuterated water D_2O and deuterated chloroform ($CDCl_3$) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Thermo Scientific (Rockford, IL). Sodium hydroxide (NaOH), acetonitrile (ACN) and formic acid were purchased from Fisher Scientific (Hampton, NH). Ultrapure DNase/RNase-free distilled water was purchased from Invitrogen (Grand Island, NY). 0.22 μm syringe filter was purchased from Millex-GS (Millipore Corporation, Billerica, MA). Formvar® 0.5% solution in ethylene dichloride film was purchased from Electron Microscopy Sciences (Hatfield, PA). Control scrambled siRNA (siCon) was obtained from Dharmacon (Lafayette, CO) while AEG-1 siRNA was obtained from Sigma-Aldrich.

Generation of PAMAM-PEG-Gal

The PAMAM-PEG-Gal delivery system was constructed by conjugating galactose to one end of the polyethylene glycol (PEG) chain and the other end of the PEG chain was conjugated to the poly(amidoamine) (PAMAM) backbone (Fig. 1). The first step in this synthesis was to prepare PEG-Gal. To do this, 5 ml of MES buffer (0.1 M, pH 6) was added to 45.65 mg of EDC (238 μ moles = 10x lactobionic acid), 129.25 mgs of sulfo-NHS (595 μ moles = 2.5x EDC) and 8.53 mgs of lactobionic acid (23.8 μ moles = 10x PEG). This was then stirred for 15 minutes, 167.5 μ L of 2-mercaptoethanol solution was added (2380 μ moles = 10x EDC) and the pH was raised to 7 using 1N NaOH solution. Next, 5 mg of NH₂-PEG-COOH (2.38 μ mole) was added to the solution and stirred at room temperature overnight. Finally, the solution was dialyzed using a 1 kDa dialysis membrane against 1L of distilled water for 48 h (water was changed at 2, 4 and 6 h), lyophilized (Freezone @ 4.5, Labconco Corp., Kansas City, MO) and stored at -20 °C. The next step was to use the PEG-Gal to prepare the PAMAM-PEG-Gal. To achieve this, 5 ml of 0.1 M MES buffer was added to 3.93 mg of EDC (20.50 μ moles = 10x PEG-Gal), 11.13 mgs of sulfo-NHS (51.26 μ moles = 2.5x EDC) and 5 mgs of PEG-Gal (2.050 μ moles = 8% molar equivalent of the terminal amine groups in PAMAM [20], and then stirred for 15 minutes. 14.42 μ L of 2-mercaptoethanol solution was added to quench free EDC [21] (205.0 μ moles = 10x EDC) and the pH was raised to 7 using 1N NaOH solution. 5.764 mg of PAMAM (0.2 μ mole = 25.63 μ moles of amine groups) was then added to the solution and stirred at room temperature overnight. Finally, the solution was dialyzed using 10 kDa dialysis membrane against 1L of distilled water for 48 h (water was changed at 2, 4 and 6 h), lyophilized and stored at -20 °C.

Preparation of the nanoplexes

The nanoplexes were prepared by electrostatic interaction between positively charged PAMAM-PEG-Gal and negatively charged siRNA molecules. Nanoplexes were formulated at different ratios of primary amine groups in PAMAM-PEG-Gal to phosphate groups in siRNA (N/P ratios). PAMAM-PEG-Gal and siRNA solutions were prepared in ultrapure DNase/RNase-free distilled water. Solutions were sterilized by filtering with a 0.22 μ m syringe filter. Finally, an equal volume of the desired concentration of cationic solution was pipetted into the anionic solution and then vortexed immediately for 30 s. The nanoplex solutions were incubated at room temperature for 30 min before use.

Characterization of PAMAM-PEG-Gal

¹H-NMR—Calculation of PEG to Gal molar ratio was performed by dissolving samples in CDCl₃, while PAMAM-PEG ratio was calculated by dissolving samples in D₂O. Characterization was carried out using proton nuclear magnetic resonance (¹H-NMR, Bruker Avance 300).

Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS)—Estimation of PEG presence in the conjugate was confirmed using MALDI-TOF/TOF-MS. Briefly, a matrix of alpha-cyano-4-hydroxycinnamic acid was dissolved in ACN and 0.1% formic acid at ratio of 50/50 (v/v),

then the matrix was mixed with an aqueous solution of the sample at a ratio of 1:1 (v/v). MALDI with positive, reflector mode was used. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Ultraflextreme; Bruker)

Ion trap and time-of-flight mass spectrometry (IT-TOF-MS)—Gal presence in the conjugate was confirmed by injecting aqueous solutions of samples directly into IT-TOF-MS (Shimadzu, Columbia, MD) equipped with an electrospray ionization source in negative ion mode.

Characterization of nanoplexes

Size and zeta potential measurements—Particle size and zeta potential of nanoplexes (with varying N/P ratios) were measured using a Zetasizer Nano ZS particle analyzer via dynamic light scattering (DLS) technique (Malvern Instrument Ltd., Southborough, MA). Size and zeta potential measurements were performed on aqueous solution of the nanoplexes at 25 °C. Size was measured at 173° backscatter detection in disposable polystyrene cuvettes. Zeta potential was measured in a zeta potential folded capillary cell.

Transmission electron microscopy (TEM)—Surface morphology of the prepared nanoplexes was assessed using TEM. A drop (10 µl) of aqueous solution of the nanoplexes (N/P 20) was added for 30 sec on a carbon coated, glow discharged 400-mesh TEM copper grid that was pre-coated with a Formvar®. Whatman® filter paper was then used to remove any excess liquid and the grid was air dried. Negative staining was then performed by adding a drop of freshly prepared 1% w/v phosphotungstic acid solution for 30 sec followed by removing the excess stain as described before. TEM images were captured using JEOL JEM-1230 transmission electron microscope equipped with a Gatan UltraScan 1000 2k × 2k CCD acquisition system. Captured images were processed using ImageJ (Image Processing and Analysis in Java, Version 1.47).

Gel electrophoresis—The ability of the conjugate to bind with siRNA was confirmed using gel electrophoresis assay. PAMAM-PEG-Gal and siRNA at N/P ratios of 0.5, 1, 5, 10, and 20 were mixed with 2× BlueJuice gel loading buffer (Invitrogen). The final solutions were loaded into the wells of a 1% (w/v) agarose gel containing 0.5 µg/mL of ethidium bromide in 1× Tris-acetate-EDTA (TAE) buffer. Gels were exposed to a constant current of 100 mA for 2 h. DNA migration was then visualized with a UV transilluminator (Spectroline, Westbury, NY).

Orthotopic xenograft studies in nude mice

All animal studies have been approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. QGY-luc cells (QGY-7703 cells stably expressing luciferase) were orthotopically implanted by intrahepatic injection in athymic nude mice (6–8 weeks of age) [22]. Tumor growth was monitored by bioluminescence imaging (BLI) with a Xenogen IVIS imager once a week [22]. One week after the establishment of the tumor the mice (n = 8 per group) were treated with PAMAM-siCon, PAMAM-siCon+ATRA, PAMAM-AEG-1si and PAMAM-AEG-1si+ATRA. ATRA was administered i.p. at a dose of 10 mg/kg and 500 µg of siRNA, conjugated with PAMAM-

PEG-Gal at a ratio of 5:1, was administered i.v. per injection. A total of 8 injections were administered twice a week and the mice were sacrificed 2 weeks after the last injection.

Immunohistochemistry

Immunohistochemistry was performed using formalin-fixed paraffin embedded (FFPE) sections as described previously [13]. The sections were blocked in PBST using 10% normal goat serum for rabbit polyclonal antibody, 10% normal horse serum for mouse monoclonal antibody and 10% normal rabbit serum for chicken polyclonal antibody. Primary antibodies were diluted in PBST containing 5% corresponding blocking serum. The primary antibodies used were: AEG-1 (chicken polyclonal; 1:500), PCNA and cleaved caspase-3 (Cell signaling; mouse and rabbit monoclonal, respectively; 1:300). Secondary antibodies were diluted in PBST containing corresponding 2.5% blocking serum. The signals were developed by avidin-biotin-peroxidase complexes with a DAB substrate solution (Vector laboratories).

Total RNA extraction, cDNA preparation and quantitative RT-PCR

Total RNA was extracted from tumor samples using the QIAGEN miRNAeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA preparation was done using ABI cDNA synthesis kit. Real-time polymerase chain reaction (RT-PCR) was performed using an ABI ViiA7 fast real-time PCR system and Taqman gene expression assays according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were represented as the mean $\pm$ Standard Error of Mean (S.E.M) and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test. A p-value of <0.05 was considered as significant.

Results

Synthesis and characterization of PAMAM-PEG-Gal

For the specific purpose of the present studies, we have developed a delivery system for siRNA composed of poly(amidoamine) dendrimers (PAMAM), polyethylene glycol (PEG), and lactobionic acid (Gal) (Fig. 1A). Cationic PAMAM complexes and compacts siRNA, PEG forms a hydrophilic layer outside of the polyplex for steric stabilization, and galactose serves as a cell binding ligand for liver cancer cells that overexpress the asialoglycoprotein receptor for galactose ligands. The utilization of the PEG component and the galactose ligand are expected to significantly improve the pharmacokinetics and tumor binding attributes of the delivery system and improve AEG-1 siRNA therapeutic efficiency. Synthesis of PAMAM-PEG-Gal was carried out utilizing EDC/NHS chemistry in two-step process. First, the carboxylic group of Gal was conjugated to the amine group of PEG via an amide bond to form PEG-Gal. Second, the free carboxylic group of the PEG in PEG-Gal conjugate was further conjugated to the free amine groups in PAMAM via amide bonds to form PAMAM-PEG-Gal (Fig. 1B). Molar ratios of PEG to Gal and PAMAM to PEG-Gal were further confirmed using $^1\text{H-NMR}$ (Fig. 2). PEG, Gal and PEG-Gal $^1\text{H-NMR}$ spectrums in CDCl_3 are shown in (Fig. 2A). Peaks at 3.5 and 4.5 ppm represented 180 H of

PEG (-CH₂CH₂O-) and 1H of Gal, respectively [23, 24]. Integration ratios between both peaks at 3.5 and 4.5 ppm were 180.09:1 which confirmed that the molar ratio of PEG:Gal in the PEG-Gal conjugate was (180/180.09):(1/1) $\approx$ 1:1. It is worth noting here that CDCl₃ was utilized in this study because of the overlapping that occurs between Gal peak at 4.5 ppm and the D₂O peak at 4.4–4.8, when D₂O was used as a solvent. PEG-Gal, PAMAM and PAMAM-PEG-Gal ¹H-NMR spectrums in D₂O are shown in (Fig. 2B). Peaks at 2.3 and 3.5 ppm represented 504 H of PAMAM (-CH₂CONH-) and 180 H of PEG-Gal (-CH₂CH₂O-), respectively [25]. PAMAM:PEG-Gal molar ratios in the PAMAM-PEG-Gal can also be calculated from the integration ratios between peaks at 2.3 and 3.5 ppm, which were equal to 1:2, respectively. These integration ratios showed that PAMAM:PEG-Gal molar ratios were (1/504):(2/180) $\approx$ 1:5.6, indicating that each molecule of PAMAM was conjugated to 5.6 molecule of PEG-Gal. Presence of PEG in the formed conjugates was further assessed through MALDI-TOF/TOF-MS (Fig. 3A). MALDI spectrum showed peaks of mass around 2200 Da, and mass difference between adjacent peaks equivalent to multiples of PEG monomer (-CH₂CH₂O-, 44 Da). These peaks were observed in PEG, PEG-Gal and PAMAM-PEG-Gal, supporting the presence of PEG in the final construct. Finally, the presence of Gal ligand in the final construct was further verified by IT-TOF-MS (Fig. 3B). A distinct Gal peak of m/z 348.9 was found in the final construct of PAMAM-PEG-Gal, verifying the inclusion of Gal in the final construct and supporting the ¹H-NMR data.

Characterization of PAMAM-AEG-1si nanoplex

Complexes of PAMAM-PEG-Gal and AEG-1 siRNA was formed by electrostatic interactions at a series of different N/P ratios. Sizes of the prepared nanoplexes, at different N/P ratios, ranged between 85 and 158 nm in diameter. Increase in the size from 85 to 158 nm was observed when N/P ratio was increased from 0.5 to 1. Higher N/P ratios resulted in constant size $\sim$ 120 nm (Fig. 4A). The zeta potential of the formed nanoplexes changed drastically from -23.3 to +22.5 when N/P ratio changed from 0.5 to 1. N/P ratios >1 resulted in a constant charge of $\sim$ +29.0 (Fig. 4A). TEM analysis showed that complexes were spherical or semi-spherical in shape with porous but otherwise smooth surfaces (Fig. 4B). The ability of the PAMAM-PEG-Gal to efficiently complex with siRNA at low N/P ratios was shown by gel retardation assays. siRNA complexed with PAMAM-PEG-Gal remained in the loading wells and did not migrate into an agarose matrix (1% (w/v) at N/P ratios >1 when an electric field was applied (Fig. 4C).

Combination of PAMAM-AEG-1si and ATRA inhibits orthotopic human HCC xenografts

QGY-7703 human HCC cells express high levels of AEG-1 and generate highly aggressive metastatic HCC in nude mice [6, 12]. We have established stable clones of QGY-7703 cells expressing luciferase (QGY-luc) [22]. QGY-luc cells were orthotopically implanted into the livers of athymic nude mice and tumor development was monitored by bioluminescence imaging (BLI) using Xenogen IVIS imager [22]. One week after the establishment of the tumor the mice (n=8 per group) were treated with PAMAM-siCon, PAMAM-siCon+ATRA, PAMAM-AEG-1si and PAMAM-AEG-1si+ATRA. ATRA was administered i.p. at a dose of 10 mg/kg and 500 μ g of siRNA, conjugated with PAMAM-PEG-Gal at a ratio of 5:1, was administered i.v. per injection. A total of 8 injections were administered twice a week and the mice were sacrificed 2 weeks after the last injection. In the control group the tumor

developed aggressively (Fig. 5A & B). ATRA showed very little effect which might be attributed to high AEG-1 level in QGY-luc cells. PAMAM-AEG-1si showed significant reduction in tumor growth and the combination of PAMAM-AEG-1si+ATRA showed profound and synergistic inhibition so that the tumors were almost undetectable by BLI (Fig. 5A & B). Measurement of liver weight at the end of the experiment corroborated the findings of BLI (Fig. 5C, top panel). Analysis of AEG-1 mRNA in tumor samples by Taqman Q-RT-PCR showed marked decrease in AEG-1 level by PAMAM-AEG-1si when compared to the other groups (Fig. 5C, bottom panel). Histologically, ATRA treatment did not show significant difference from the control group (Fig. 6A, top row). PAMAM-AEG-1si showed some areas of necrosis which might be attributed to inhibition of angiogenesis upon knockdown of AEG-1. The combination of PAMAM-AEG-1si+ATRA showed large areas of necrosis compared to other groups (Fig. 6A, top row). There was marked decrease in AEG-1 expression with PAMAM-AEG-1si (Fig. 6A, 2nd row). Proliferation marker PCNA was markedly decreased in PAMAM-AEG-1si and with PAMAM-AEG-1si+ATRA the decrease was more pronounced (Fig. 6A, 3rd row & Fig. 6B). PAMAM-AEG-1si group showed induction of apoptosis, determined by cleaved caspase-3 staining (Fig. 6A, bottom row & Fig. 6B). However, it was markedly increased in PAMAM-AEG-1si+ATRA group (Fig. 6A, bottom row & Fig. 6B). The expression of ATRA-responsive genes RARB and HOXA1 in the tumor samples were checked at the end of the treatment. While ATRA and PAMAM-AEG-1si alone resulted in small but significant increases in these genes, the combination of PAMAM-AEG-1si+ATRA resulted in robust increase in RARB and HOXA1 expression (Fig. 6C). It should be noted that the growth rate of untreated QGY-luc cells and PAMAM-siCon group was similar (data not shown).

Discussion

Nanoparticles have been proposed as potential means to deliver siRNA to cells. The benefits of nanoparticles include protection of siRNA, good tissue penetration, relatively good cell uptake, and stability [26–28]. Different types of compounds that can be collectively categorized as nanoparticles or nanoplexes have been tested for their ability to deliver nucleic acids to cells [28]. Cationic polymers can bind nucleic acids electrostatically and protect them from degradation and can facilitate uptake to cells [29, 30]. A promising class of cationic polymers that efficiently complex with siRNA is the poly(amidoamine) (PAMAM) dendrimers [31–34]. PAMAM dendrimers have a number of advantages over linear cationic polymers such as PEI [34, 35]. In comparison to linear cationic polymers, PAMAM dendrimers are spherical, monodisperse polymers with a reduced structural density in the intramolecular core [34]. Additionally PAMAM dendrimers also show very low toxicity profiles in mouse studies [36]. The use of PAMAM dendrimers is highly advantageous in gene delivery, but its high positive charge density results in a degree of cytotoxicity and hemolysis [37, 38]. Addition of a PEG layer to PAMAM dendrimers reduce the charge density and thus significantly overcome these drawbacks [39, 40]. In addition, PEG is capable of enhancing the pharmacokinetics of the conjugate through increasing its circulation half-life by reducing interactions with serum proteins [41]. Although pegylation can mitigate the drawbacks of using PAMAM dendrimers, it can also reduce its binding and uptake by targeted tissues (e.g. hepatocytes) [42]. The addition of the Gal ligand to the PEG

layer allows for a specific interaction of the nanoplex with asialoglycoprotein receptors that are over expressed selectively in liver hepatocytes [43], resulting in higher uptake of the nanoplexes in these cells. We successfully synthesized PAMAM-PEG-Gal conjugate using EDC/NHS chemistry. ¹H NMR data showed that PEG:Gal molar ratios were $\approx 1:1$, and PEG:PAMAM ratios were $\approx 5.6:1$, indicating that 4.4% of the surface amine groups of PAMAM (5th generation has 128 surface amine groups) were occupied. It is worth noting here that the targeted ratio of PEG: PAMAM of about 8% of the PAMAM surface amine groups was chosen because Qi et al. showed that higher transfection and acceptable toxicity were achieved compared to 4% and 15% [20]. Presence of PEG and Gal in the final conjugate was further confirmed by MALDI and mass spectrometry. siRNA was complexed with the prepared conjugate through electrostatic interaction [44] between negatively charged phosphate groups of the siRNA and the positively charged amine groups of the PAMAM-PEG-Gal conjugate. TEM images of the prepared nanoplex after drying showed spherical nanoplexes of sizes around 50 nm. DLS measurements in an aqueous environment showed diameters of 131 nm and this increase in hydrodynamic diameter from the DLS measurements further confirms the presence of hydrophilic PEG layers. An increase of the positive charge at higher N/P ratios indicates the condensation of the siRNA completely within the conjugate. This was confirmed using gel retardation assays in which nanoplexes prepared at N/P ratios of 0.5 and 1 showed migration of the siRNA band and this was completely hindered with nanoplexes prepared at higher N:P ratios. An N/P ratio of 5 was chosen for the *in vivo* experiments because: (1) It is the lowest ratio that can condense the siRNA completely and thus higher protection of the siRNA from nucleases will be achieved [44]. (2) The smallest amount of PAMAM will be used and thus the lowest side effects are expected. (3) It was also shown that N/P ratios between 5 and 10 gave the most efficient transfection [40].

We document that hepatocyte-targeted PAMAM dendrimers efficiently delivered AEG-1 siRNA to the target organ confirming the therapeutic utility of this delivery system in targeting AEG-1. Since i.v. administration of any compound first goes to the liver, HCC is the tumor that might derive the highest benefit from gene therapy strategies. A phase I trial for HCC employing i.v. administration of lipid nanoparticle (LNP)-conjugated siRNA for vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) in cancer patients showed several beneficial aspects [45]. Biweekly administration was safe and well-tolerated. The siRNA effectively induced cleavage of targeted mRNA in the liver and demonstrated antitumor activity, including complete regression of metastatic lesion in the liver. This clinical trial, along with positive results from multiple pre-clinical studies targeting different molecules via nanoparticle-delivered siRNA, indicates that this modality of treatment is a viable option for HCC patients [45–47]. Indeed, the FDA has approved a recent clinical trial for nanoparticle-delivered PLK1 siRNA for HCC (<http://investor.tekmirapharm.com/releasedetail.cfm?ReleaseID=850580>). PAMAM-AEG-1si therefore is clinically relevant, especially in the context of HCC. Additionally, our studies, along with studies from various laboratories, have firmly established that AEG-1 positively regulates all aspects of aggressive cancer, and thus it is a bona fide target for therapeutic intervention for a wide variety of cancer [48]. As such PAMAM-AEG-1si, designed to target specific organs, might be of utility for tumors of other organ systems. AEG-1KO mice

are viable and fertile [16] further indicating that AEG-1 inhibition might be an effective anti-cancer strategy without causing harmful effects to normal cells.

Chemoresistance is one of the most profound phenotypes conferred by AEG-1, and indeed AEG-1 inhibition sensitizes killing by diverse therapeutic agents both *in vitro* and in nude mice xenograft models [12, 13]. A variety of mechanisms, including translational upregulation of multidrug resistance gene-1 (MDR1) and upregulation of the transcription factor LSF, contribute to AEG-1-induced chemoresistance in HCC cells [12, 13]. Our proposed combinatorial strategy is based on a molecular interaction between AEG-1 and RXR that we have unraveled and that confers resistance to RA-induced cytotoxicity [17]. We documented that *ex vivo* treatment of human HCC cells with lenti.shAEG-1 along with i.p. injection of ATRA resulted in synergistic and marked inhibition of subcutaneous xenografts in nude mice [17]. We now demonstrate that PAMAM-AEG-1si and ATRA combination profoundly inhibits orthotopic xenografts in nude mice. These exciting findings from two independent experiments mandate that this strategy needs to be evaluated in a more stringent endogenous HCC model so that if found effective this strategy might be translated to the patients.

RA analogs have been evaluated in Phase II/III clinical trials for prevention and treatment of HCC [49–51]. These studies were carried out before AEG-1 was even cloned. One potential reason why RA failed to progress further as a HCC therapeutic might be that overexpressed AEG-1 in a high percentage of HCC patients blocked RA action. ATRA is a routine cancer therapeutic for leukemia [52]. Combination with AEG-1 inhibition might establish ATRA or other RA analogs again as a viable therapeutic option for HCC. Availability of a small molecule inhibitor for AEG-1 would have simplified AEG-1-targeting therapeutic strategies. However, AEG-1 is a scaffold protein which may not be amenable to inhibition by small molecules. Our proposed strategy of targeted nanoparticle-mediated siRNA delivery thus might be the most efficient way to inhibit AEG-1.

Acknowledgments

Present study was supported in part by grants from The James S. McDonnell Foundation and National Cancer Institute Grant R01 CA138540 and R21 CA183954 (DS). DS is the Harrison Endowed Scholar in Cancer Research and Blick Scholar. PBF holds the Thelma Newmeyer Corman Chair in Cancer Research. A.K.S acknowledges support from the National Cancer Institute at the National Institutes of Health (P50 CA97274/UI Mayo Clinic Lymphoma SPORE grant and P30 CA086862 Cancer Center support grant) and the Lyle and Sharon Bighley Professorship. K.E. acknowledges the Egypt Ministry of Higher Education for a graduate fellowship award.

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Figure 1. Generation of PAMAM-PEG-Gal. A. Strategy for targeting siRNA to liver. B. Strategy for preparation of the conjugates.

Figure 2.

Characterization of PAMAM-PEG-Gal by ^1H -NMR (300 MHz). A. ^1H -NMR of PEG, Gal and PEG-Gal using CDCl_3 as a solvent. Integral ratios between PEG ($-\text{CH}_2\text{CH}_2\text{O}-$) and Gal peaks are shown in the insert of the lower panel. B. ^1H -NMR of PEG-Gal, PAMAM and PAMAM-PEG-Gal using D_2O as a solvent. Integral ratios between PEG ($-\text{CH}_2\text{CH}_2\text{O}-$) and PAMAM ($-\text{CH}_2\text{CONH}-$) peaks are shown in the insert of the lower panel. PAMAM structure demonstrated here is for generation zero, while the one utilized in the study is of generation five with 128 surface amine groups.

Figure 3.

Characterization of PAMAM-PEG-Gal by MALDI and mass spectrometry. A. The presence of PEG in the final PAMAM-PEG-Gal construct was verified using MALDI. B. The presence of Galactose ligands in the final PAMAM-PEG-Gal construct was verified using Mass Spectrometry.

Figure 4.

Characterization of the PAMAM-PEG-Gal complexed with AEG-1 siRNA (PAMAM-AEG-1si). A. Size and zeta potential measurements of PAMAM-PEG-Gal and AEG-1 siRNA complexes formed at varying amine to phosphate (N/P) ratios. B. Transmission electron microscopy image of N/P 20 showing spherical morphology and size of PAMAM-AEG-1si complexes. C. Gel electrophoresis assay showing siRNA retention in complexes prepared at varying N/P ratios.

Figure 5.

Combination of PAMAM-AEG-1si and ATRA profoundly inhibits orthotopic human HCC xenograft in nude mice. A. BLI imaging of the mice before and after the indicated treatment. B. Quantification of photon counts from the animals. The data represent mean $\pm$ SEM; *: $p < 0.01$. Eight (8) animals per group were used. C. Liver weight at the end of the treatment (top panel). AEG-1 mRNA expression in the tumors at the end of the treatment by Taqman Q-RT-PCR (bottom panel).

Figure 6. PAMAM-AEG-1si and ATRA combination inhibits proliferation and induces apoptosis. A. H&E (top row) and IHC staining of the tumors using the indicated antibodies. The asterisk indicates area of necrosis. B. Quantification of PCNA- and cleaved caspase-3 positive cells in the tumors. Data represent mean $\pm$ SEM. *: $p < 0.01$. C. Expression of RARB and HOXA1 in the tumors at the end of the treatment by Taqman Q-RT-PCR. Data represent mean $\pm$ SEM. *: $p < 0.05$; #: $p < 0.01$.