Targeted Delivery of RNA-Cleaving DNA Enzyme (DNAzyme) to Tumor Tissue by Transferrin-Modified, Cyclodextrin-Based Particles

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Received 04/02/04; Accepted 04/09/04
This manuscript has been published online, prior to printing, for Cancer Biology & Therapy Volume 3, Issue 7. Definitive page numbers have not been assigned. The current citation for this manuscript is:

ABSTRACT

Short nucleic acid sequences specific to oncogene targets such as bcl-2, bcr-abl, and c-myc have been shown to exhibit specific anti-cancer activity in vitro through antigen or antisense activity. Efficient in vivo delivery of oligonucleotides remains a major limitation for the therapeutic application of these molecules. We report herein on the preparation of transferrin-modified nanoparticles containing DNAzymes (short catalytic single-stranded DNA molecules) for tumor targeting as well as their biodistribution using various methods of administration in the mouse. Linear, β-cyclodextrin-based polymers are complexed with DNAzyme molecules to form sub-50 nm particles termed “polyplexes”. The surface properties of the cyclodextrin-containing polyplexes are modified by exploiting the ability of the β-cyclodextrin substructure and adamantane to form inclusion complexes. Accordingly, conjugates of adamantane with poly(ethylene glycol) (PEG) are prepared and combined with the polyplexes. The adamantane form inclusion complexes with the surface cyclodextrins of the polyplexes to provide a sterically stabilizing layer of PEG. The stabilized polyplexes are also modified with transferrin for increasing targeting to tumor cells expressing transferrin receptors. The preparation, characterization, and in vitro application of these nanoparticles are discussed. The transferrin-polyplexes containing fluorescently-labeled DNAzyme molecules are administered to tumor-bearing nude mice and their biodistribution and clearance kinetics are monitored using a fluorescence imaging system. Four methods of administration are studied: intraperitoneal bolus and infusion, intravenous bolus, and subcutaneous injection. DNAzymes packaged in polyplex formulations are concentrated and retained in tumor tissue and other organs, whereas unformulated DNAzyme is eliminated from the body within 24 hours post-injection. Intravenous and intraperitoneal bolus injections result in the highest fluorescent signal (DNAzyme) at the tumor site. Tumor cell uptake is observed with intravenous bolus injection only, and intracellular delivery requires transferrin targeting.

INTRODUCTION

One of the greatest barriers to realizing the potential of antisense oligonucleotides, catalytic nucleic acids (ribozymes, DNAzymes), and small, interfering ribonucleic acids (siRNA) for cancer treatment is their delivery to the site of action.1 Numerous in vitro and in vivo studies have utilized unformulated nucleic acids. This approach tends to require large amounts of the nucleic acid and generally lacks cell specificity. The use of delivery agents has been shown to facilitate cellular uptake in vitro, thus reducing the quantity of nucleic acid required for efficacy. Bennet et al. showed enhanced cellular uptake and nuclear localization of oligonucleotides using a cationic lipid delivery system in vitro.2 Several studies have compared the in vitro delivery of oligonucleotides by lipid and polymer formulations. Jaaskelainen et al claim that polymeric systems are ineffective as oligonucleotide delivery agents while lipid-based systems can provide for efficient cellular delivery.3 In contrast, other investigators have shown that specific polylsines, polyethylenimine,4 and cationic dendrimers5,6 can deliver functional oligonucleotides. Although the conclusions reached may be expression-assay dependent, the overall consensus from literature suggests that lipid-based systems tend to be superior to polycations. A possible explanation may be the lack of intracellular release of oligonucleotides by high molecular weight polycations.

When used in vivo, synthetic delivery systems can suffer from interactions with serum components, cause complement activation and show toxicity that are all associated with their polyvalency.7 These shortcomings have obvious implications for therapeutic applications, although exceptions to this behavior have been noted.4,8 Delivery of short nucleic acids by nonviral systems that overcome these problems and show utility for in vivo applications remains a high priority for investigation since they have the potential to be of therapeutic relevance.
Recently, a new multi-component, cyclodextrin-based nucleic acid delivery system has been described. The first component of this system is a short (approximately five repeat units giving on average ten charges per polymer chain), cyclodextrin-containing polycation (CDP, Fig. 1A) that can self-assemble with nucleic acids via electrostatic interactions and form uniformly sized, sub-100 nm particles termed “polyplexes.” The CDPs are capable of providing the in vitro delivery of plasmids in the presence of serum. The CDPs demonstrate low toxicity in vitro (for CDP6, a CDP containing 6 methylene units between charges, IC_{50} = 1.1 mM to BHK-21 cells) and in vivo (for CDP6, LD_{50} = 200 mg/kg in mice) and may be suitable for oligonucleotide delivery due to their low degree of polymerization (short polycations are known to minimize complement activation). Polyplexes formed using CDPs can be modified to impart stability in biological fluids and cell targeting specificity by surface decoration with adamantane-based modifiers (Figs. 1B and C; Fig. 2B and C). The adamantane (AD) forms an inclusion species with exposed cyclodextrins on the polyplexes by complexation, an enthalpically driven physical phenomenon based on van der Waal’s interactions between the cyclodextrin cavity interior and the molecular surface of the adamantane derivatives. Transferrin-modified, poly (ethylene glycol) (PEG)-stabilized polyplexes (TF-PEG-polyplexes) are prepared by polyplex modification with transferrin-PEG-AD and PEG-AD conjugates (Fig. 2C). Transferrin was chosen as a model ligand for oligonucleotide delivery to tumors because the transferrin receptor (TF-R) is often upregulated in rapidly growing cells. These TF-PEG-polyplexes have been shown to facilitate plasmid delivery and in vitro transgene expression via transferrin receptor-mediated endocytosis.

Joyce and coworkers used in vitro selection to obtain single-stranded DNA capable of catalytic cleavage of RNA. These enzymatic DNAs (DNAzymes) have been successful in site-specific RNA cleavage in both in vitro and in vivo settings. The potential advantages of DNAzymes include their catalytic efficiencies, relative stabilities and cost of production.

Expression of the c-myc proto-oncogene can correlate with cell proliferation. Therefore, c-myc has been investigated as a target for down-regulation by oligonucleotides and catalytic nucleic acids for diseases that involve undesired cell growth such as cancer and restenosis. For example, DNAzymes targeting the translation initiation region of c-myc RNA have been reported to site-selectively cleave full length RNA and downregulate c-myc gene expression in smooth muscle cells.

The objective of this work is to assess the ability of the cyclodextrin-containing polycation delivery system to provide for the in vivo delivery of anti-c-myc DNAzymes to subcutaneous tumors in mice. The study focuses on the preparation of transferrin-modified polypeplexes and their biodistribution in tumor-containing nude mice. This is the first report of oligonucleotide delivery using the CDP system. We show that this delivery system lacks significant toxicity, is able to deliver DNAzymes in the presence of serum, and can target tumor tissue via transferrin-mediated targeting when the construct is systemically injected.

**MATERIALS AND METHODS**

**Cells, Animals and Materials.** The following human cell lines were used in this study: the cervical carcinoma HeLa cell line, cultured in minimum essential medium, the ovarian carcinoma A2780 cell line, cultured in RPMI 1640, and the colorectal carcinoma cell line, HT29, cultured in McCoy’s 5A. Culture media were supplemented with 10% fetal calf serum, 50 µg/ml gentamycin, and 2 mM L-glutamine. Cells were grown at 37°C in a humidified incubator with 5% CO_2. All media and supplements were purchased from Invitrogen (Paisley, UK).

Male NMRI mice were purchased from Janvier (Le Genest-St-Ise, France). All animal experiments were carried out with animal ethical committee approval. The ethical guidelines that were followed met the standards required by the UKCCCR guidelines. Mice were inoculated subcutaneously in the inguinal region with 10^7 cells/200 µl serum-free medium using 26G syringes (BD, 26 GA 3/8 1ml). Mice were used 14-16 days after cell suspension injection.

5’-Fluorescein-labeled and 5’-Cy-3-labeled DNAzymes (5’-label-TGAGGGCGAGGTAGCTAACAGCGTGCGGx-3’ with x = 3’dG5’) were purchased from Eurogentec (Seraing, Belgium). In order to improve stability, a 3’dG5’-3’ thymidylate inversion was incorporated at the 3’ end. CDP-Imid, AD-PEG_9000-AD-glugu-PEG-gal, and AD-PEG_3400-Tf were synthesized following analogous protocols described previously. Fluorescein-labeled transferrin (TF-fluor) was prepared by conjugation of

**Figure 1. Components of linear, cyclodextrin polymer-based delivery system for nucleic acids. (A) CDP-Imid. (B) AD-PEG and AD-glugu-PEG-gal. (C) AD-PEG-Tf.**
As has been shown previously, the adamantane modifiers that contain anionic segment are used to tune the surface charge of the formulated particles. Here, AD-glugu-PEG-gal (glugu is the anionic segment) is employed to modify the nature of the surface charge of the formulated particles since we have already reported that this component can work well to vary particle zeta potentials.

The extent of DNAzyme complexation was monitored by gel electrophoresis. Polyplexes were prepared as described above. Aliquots containing 1 µg DNA were digested with 16 µL with distilled water. 4 µL of sample buffer were added to each aliquot, and transferred to a 1% agarose/TAE (40 mM Tris-acetate/1mM EDTA) gel containing 6 µg ethidium bromide/100 µL. Samples were electrophoresed for 2 hrs at 50 V.

Polyplex morphology was visualized by transmission electron microscopy. Unmodified, PEGylated and transferrin-modified polyplexes containing 1 µg of DNAzyme were prepared. Five microliters of sample were then applied to glow-discharged, 400-mesh carbon-coated copper grids for 45 s, after which excess liquid was removed by blotting. The samples were then negatively stained with 2% uranyl acetate for 45 s before observation. Images were recorded using a Philips 201 transmission electron microscope operated at 80 kV.

In Vivo Analysis. A2780, HT29, and HeLa cells were analyzed for relative levels of transferrin receptor expression. Cells were plated at 1,000,000 well in 6-well plates 24 hrs prior to transfection. Cells were then washed with PBS and exposed to 1 mL of antibiotic-free culture media containing 1% BSA and various concentrations of fluorescein-labeled transferrin (ranging from 25 nM to 0.5 µM) for 1 hr at 37°C. The cells were washed twice with PBS, collected by trypsin treatment, washed twice in FACS buffer (25 mL of Hank's Buffered Salt Solution supplemented with 2 mM MgCl2 and containing 10 µL DNase), and resuspended in Hank's Buffered Salt Solution for analysis by flow cytometry using a FACSCalibur from Becton Dickinson (San Jose, CA).

The polyplexes were tested for their ability to bring DNAzymes into A2780, HT29, and HeLa cells. Cells were plated at 300,000/well in 6-well plates 24 hr prior to transfection. For in vitro analysis, unmodified polyplexes were prepared by mixing CDP-Imid and fluorescein-labeled DNAzyme at various polymer-to-DNA charge ratios as described above. Immediately before the uptake experiments, cells were washed with OptiMEM and exposed to unmodified particles containing 5 µg of DNAzyme in 0.5 mL OptiMEM. After 1 hr at 37°C, cells were then washed twice with PBS, collected by trypsinization, washed twice in FACS buffer and Cell Scrub Buffer (Gene Therapy Systems, San Diego, CA) and analyzed for uptake by flow cytometry.

In Vivo Tumor Delivery of Fluorescently Tagged Oligonucleotides Microscopy. A whole body imaging (WBI) system was used to investigate the in vivo tumor delivery of fluorescently tagged DNAzymes. This imaging system consists of a fluorescence stereomicroscope (Olympus) SZX12 equipped with a green fluorescent protein (GFP; excitation: 485–501 nm; emission: 510 nm) and a red fluorescent protein (RFP; excitation 540–552 nm; emission: 568–643 nm) filter set.

Imaging was performed on C57BL/6 male mice bearing xenografted HT29 and A2780 tumors. After intravenous injection of 0.5 mg/kg CDP-Imid, the fluorescence signal was acquired at 1/60th of a second using a (Jai) CV-M90 3-CCD RGB color camera and analyzed using in-house developed software. The system was based on IMAX Vision software components and LaView (National Instruments).

Intracellular DNAzyme delivery was investigated on tumor sections using fluorescence microscopy. Briefly, at the end of each animal experiment, fluorescent tumors were extracted, cryofixed and sectioned. Twelve-μm
sections were observed using axioplan2 (zeiss) fluorescence microscope coupled to a aixiocam hr (zeiss) ccd camera and high resolution pictures (1300 x 1030 pixels) were captured and further analyzed using axiovision software (zeiss). Intracellular distribution of cy3 labeled dnazyme (red) was investigated using a fitc (green) phalloidin cytoskeleton staining. Intracellular distribution of fitc (green) labeled dnazyme was investigated using a sytox (orange) orange nuclear staining.

**dnazyme administration in vivo.** male nmri mice were treated with 1 mg cy3-conjugated c-myc dnazyme (cy3-dnazyme) or polyplex formulations containing 1 mg of cy3-dnazyme. Several administration methods were assessed: intraperitoneal bolus injection, intraperitoneal infusion, subcutaneous injection and intravenous injection. For intraperitoneal injections, 200 µl of tf-peg-polyplex solution or cy3-dnazyme were injected into the peritoneal cavity. Continuous intraperitoneal delivery was obtained by using osmotic pumps (alzet 2001d) with a flow rate of 8 µl/hr implanted in the peritoneal cavity. The pumps were filled with dnazyme (5 mg/ml) alone or dnazyme encapsulated in various polyplex formulations at the same concentration. The stability of the modified polyplexes was verified by following their particles sizes by dynamic light scattering for 24 hours at 37 ºc. Tf-peg-polyplexes were formulated at various polymer-to-dna charge ratios for 1 hour at 37 ºc. Cells (a2780, ht29 and heLa) were then washing with PBS and cell scrub buffer and collected for flow cytometry analysis. The polyplexes efficiently delivered dnazyme to all cell lines tested. Data are presented as mean ± SD of three samples.

**results**

**dnazyme formulations for in vitro studies.** The nucleic acid delivery system under investigation here consists of two classes of components: linear, cyclodextrin-based polycations that condense nucleic acids to form small nanoparticles and adamantane-based conjugates that modify the surface of the polypelexes. For these studies, a cyclodextrin-based polycation that is end-modified with imidazole (CDP-imid) is used as the nucleic acid condensing agent, adamantane-poly(ethylene glycol) (AD-PEG) serves as a particle stabilizer, adamantane-diglutamate-poly(ethylene glycol)-galactose (AD-glugu-peg-gal) tunes the polypelex surface charge by neutralizing excess cationic charges, and adamantane-poly(ethylene glycol)-transferrin (AD-PEG-Tf) imparts tumor-targeting specificity to the particles via the transferrin ligand. The structures of the components are shown in figure 1.

Polyplexes were prepared by mixing the polymer solutions together and then adding the mixture to the dnazyme in solution. The dnazyme (deoxyribozyme oligonucleotide) is a 33-mer oligonucleotide with a 3' inversion modification that has been shown to impact stability in serum and within cells. The CDP-imid binds to dnazyme via electrostatic interactions and the AD-based modifiers interact with surface cyclodextrin by inclusion complex formation. The dnazyme-binding profile of CDP-imid was determined by gel electrophoresis. CDP-imid was mixed with dnazyme at various charge ratios (moles of positive amine charges on CDP-imid to moles of negative phosphate charges on dnazymes) and run on a 1% agarose gel. Unformulated dna migrates down the gel and is visualized by ethidium bromide, while bound DNA remains in the loading wells of the gel. Complete binding of dnazyme with CDP-imid is observed at a charge ratio of 1 (fig. 3).

In vitro delivery of dnazymes. The dnazyme delivery efficiency of CDP-imid-based particles prepared at various charge ratios of CDP-imid to dnazyme was determined by using a fluorescein-labeled dnazyme (fluor-dnazyme) in the formulations. Polypelexes were prepared at charge ratios ranging from 2 to 10. Particle sizing analysis by dynamic light scattering revealed small, condensed particles, confirming that fluorescein does not interfere with dnazyme condensation (data not shown). Polypelexes were exposed to several cell lines for one hour before cells were washed with PBS and cell scrub buffer (for removing surface-associated polypelexes) and assayed for fluor-dnazyme internalization by flow cytometry. CDP-imid is able to efficiently mediate dnazyme uptake to all cell lines at all charge ratios tested (fig. 4). Polypelexes formulated at a charge ratio of 2 are internalized by over 98% of all tested cell lines (a2780, ht-29 and HeLa).

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Figure 3. CDP-imid and DNAzyme binding. DNAzyme and CDP-imid were mixed at various polymer-to-DNA charge ratios and electrophoresed on an ethidium bromide-containing agarose gel. Encapsulated DNAzyme is observed at polymer-to-DNA charge ratios at and above 1.

Figure 4. CDP-imid-mediated delivery of fluorescein-DNAzyme to various human carcinoma cell lines. Cultured cells were exposed to CDP-imid/fluor-DNAzyme polypelexes prepared at various polymer-to-DNA charge ratios for 1 hour at 37 °C. Cells [A2780, HT29 and HeLa] were then washed with PBS and cell scrub buffer and collected for flow cytometry analysis. The polypelexes efficiently delivered DNAzyme to all cell lines tested. Data are presented as mean ± SD of three samples.
Figure 5. Relative expression levels of transferrin uptake by A2780, HT29 and HeLa cell lines. Cultured cells were plated in 6-well plates and exposed to 1 mL of antibiotic-free culture media containing 1% BSA and various concentrations of fluorescein-labeled transferrin (ranging from 25 nM to 500 nM) for 1 hr at 37˚C. Cells were washed with PBS and collected for association with fluorescein-labeled transferrin by flow cytometry analysis. Transferrin uptake was highest in HeLa cells and lowest in A2780 cells. Data are presented as mean ± SD of three samples (in some cases, error bars are too small to be visible).

Unformulated DNAzymes are not readily endocytosed by cells (<0.1% positive cells for all cell lines).

Transferrin Receptor Expression in Various Carcinoma Cell Lines. Several carcinoma cell lines were tested for their relative levels of transferrin receptor expression in order to identify a suitable cell line for a mouse xenograft model. Three human carcinoma cell lines (A2780, HT-29 and HeLa) were exposed to various concentrations of fluorescently-labeled transferrin (Tf-fluor) for 1 hr, washed to minimize nonspecific interactions, and analyzed for levels of transferrin association by flow cytometry. Results are shown as the percentage of total cells with a positive fluorescence signal (Fig. 5). Higher levels of fluorescence indicate more Tf-fluor association that in turn, is likely proportional to the number of cell surface transferrin receptors. Of the cell lines tested, HeLa and HT-29 cells have the highest transferrin uptake: over 85% of cells were associated with Tf-fluor at 50 nM Tf-fluor. The A2780 cells have relatively low levels of transferrin uptake (5% of A2780 cells fluorescence positive at 50 nM Tf-fluor). HT-29 cells also have low background fluorescence and were thus used to develop xenograft tumors in a nude mouse model for in vivo studies.

DNAzyme Formulations for In Vivo Studies. The DNAzyme-containing particles require surface modification to be suitable for in vivo applications. In order to achieve this, the particles were modified by several adamantane (AD)-based conjugates. We have previously shown that polyplexes can be stabilized against salt-induced aggregation by formulation with AD-PEG at 1:1 AD-PEG to cyclodextrin ratios (mole to mole).16 In addition, polyplex surface charge can be tuned by the addition of an adamantane-PEG modifier containing anionic residues such as AD-glugu-PEG-gal. For example, polyplexes formulated at a charge ratio of 3 and modified with 100% AD-PEG have positive zeta potential (+15 mV) while modification with increasing ratios of AD-glugu-PEG-gal to AD-PEG results in particles with negative zeta potentials (Fig. 6). Our preliminary studies as well as reports from others in the field indicate that particles with slightly negative surface charge display prolonged circulation and improved passive tumor targeting.34 Therefore, modification with 40:60 AD-glugu-PEG-gal to AD-PEG gives particles with zeta potentials of ~3 mV and were used for the animal studies. The final component used in the formulation, AD-PEG-Tf, introduces transferrin as a targeting agent.

Figure 6. Tuning of particle zeta potential by modification with AD-glugu-PEG-gal. Polyplexes modified with various amounts of AD-glugu-PEG-gal were prepared as described in the Materials and Methods section. The zeta potential of the particles was determined by particle mobility measurements using a ZetaPals dynamic light scattering detector (Brookhaven Instruments). Zeta potential is reported as the mean ± SD of 4 measurements. Polyplex zeta potential decreases with increasing AD-glugu-PEG-gal modification.

Fully formulated polyplexes were prepared by first mixing the delivery system components (CDP-Imid, AD-PEG, AD-glugu-PEG-gal, AD-PEG-Tf) and then adding the polymer solutions to a solution of DNAzyme. Three types of polyplexes were prepared: unmodified polyplexes (Fig. 2A, CDP-Imid + DNAzyme), PEGylated polyplexes (Fig. 2B, "PEG-polyplex", CDP-Imid + AD-PEG + DNAzyme), and transferrin-modified, PEGylated polyplexes (Fig. 2C, "TF-PEG-polyplex", CDP-Imid + AD-PEG/AD-glugu-PEG-gal + AD-PEG-Tf + DNAzyme). All polyplexes were formulated at a charge ratio of 2.5 (CDP-Imid to DNAzyme charge ratio). PEG-polyplexes include 1:1 AD-PEG/AD-glugu-PEG-Gal (40:60 ratio) to cyclodextrin (by mole) and TF-PEG-polyplexes include 2:100 AD-PEG-Tf to cyclodextrin and 98:100 AD-PEG/AD-glugu-PEG-gal to cyclodextrin (both by mole). The morphologies of the three polyplex formulations were imaged by transmission electron microscopy. Polyplexes, prepared as described, were deposited in solution on glow-discharged, carbon-coated copper grids and negatively stained with uranyl acetate. The three formulations yielded particles with similar morphology and size (Fig. 7). DNAzymes were fully condensed to uniform, spherical particles with diameters ranging from ~30 nm (for unmodified polyplexes) to ~50 nm (for TF-PEG-polyplex).

In Vivo Administration of Nonformulated DNAzymes Compared to DNAzyme-containing Polyplexes Using the CDP-based Delivery System. Male NMRI mice bearing HT-29 tumors were injected in the peritoneal cavity with 1 mg of Cy-3-labelled DNAzyme (Cy3-DNAzyme) either alone or in TF-PEG-polyplex formulations. The fluorescence distributions were monitored by whole body imaging (WBI) using an Olympus fluorescence stereomicroscope attached to a Jai CCD color camera (Fig. 8). Animals were sacrificed 3, 8 and 24 hrs post-injection and tumor, liver, and kidneys harvested for immediate imaging. The organs were then cryofixed to assess efficiency, specificity and retention of DNAzyme delivery to tumor. Intraperitoneal treatment of unformulated DNAzyme leads to a time-dependent decrease in fluorescence intensity in the tumor, liver and kidney. At 3 hrs post-injection, the tumors from mice treated with the unformulated DNAzyme
had higher fluorescence intensity than those treated with the formulated DNAzyme (data not shown). The macroscopic tumor fluorescence intensity was approximately equal in all mice 8 hrs after injection, but by 24 hrs after treatment, clear differences between formulated and unformulated DNAzyme were observed (Fig. 9). WBI of the live mouse showed no fluorescence in the mouse injected with unformulated DNAzyme; however, intense fluorescence was still visible in the peritoneal cavity of mouse injected with TF-PEG-polyplex even 24 hours after injection. The tumors and organs from mice treated with unformulated DNAzyme were not fluorescent while the tumor and organs from mice treated with formulated DNAzymes continued to manifest a bright fluorescence. Therefore, although formulating the DNAzyme does not improve initial delivery specificity, the delivery system enables the DNAzyme to remain in the organs for a longer period of time.

Comparison of Administration Methods. Cy-3-DNAzyme (alone and in TF-PEG-polyplex formulations) was administered to tumor-bearing mice by intraperitoneal infusion, subcutaneous injection, and intravenous injection. Based on the studies described above, all animals were imaged and sacrificed at 24 hrs post-injection and tumors, kidneys, and livers harvested for histology analysis. To determine if a prolonged DNAzyme treatment results in significant improvement in cellular tumor delivery in comparison to a bolus injection, the Alzet minipump system was employed. Osmotic pumps containing formulated or unformulated DNAzyme (at 5 mg DNAzyme/mL) were implanted in the peritoneal cavity. DNAzyme solutions were released at an average flow rate of 8 µL/hr for 24 hours; thus, mice were treated with the same amount of DNAzyme (1 mg) but over a 24 hr period compared with the intraperitoneal bolus studies. WBI of the mice before sacrifice revealed an intense fluorescence in the peritoneal cavity. When comparing the WBI images of the tumors after intraperitoneal bolus treatment and after intraperitoneal infusion, no significant difference was observed. Subcutaneous injection of unformulated DNAzyme and TF-PEG-polyplex near the tumor site did not result in any observable delivery of material to major organs or tumor; all material stayed at the injection site. Administration of material by intravenous injection into the tail vein was completed under low volume (0.20 mL) conditions. As observed with the intraperitoneal bolus administration, WBI imaging of the tumors, kidneys and livers at 24 hours revealed bright fluorescence in mice treated with TF-PEG-polyplex. Mice treated with unformulated DNAzyme had no visible fluorescence remaining in the body at 24 hours post-injection.

Intracellular Delivery Compared Between Intravenous and Intraperitoneal Treatment. As stated above, WBI images of the organs acquired 24 hours after intraperitoneal bolus, intraperitoneal infusion and intravenous bolus injections of TF-PEG-polyplex formulated DNAzyme showed no significant difference between treatments. The intracellular DNAzyme delivery efficiency from the intraperitoneal bolus injection, intraperitoneal infusion, intravenous bolus injection were thus compared by histology analysis. Tumors were sectioned to 12-µm slices. Intracellular distribution of Cy-3-labeled DNAzyme was investigated using a FITC-phalloidin cytoskeleton staining and in a second set of experiments intracellular distribution of FITC-labeled DNAzyme was investigated using a Sytox Orange nuclear staining.

The tumors from the mice that had been treated by either intraperitoneal bolus or intraperitoneal infusion demonstrated accumulation of the DNAzyme in the cap of the tumor (Fig. 10A). No intracellular localization of DNAzyme was observed with intraperitoneal administration. In contrast, the tumors in the mice treated by intravenous injection showed a significant intracellular fluorescence (Fig. 10B) displayed in a colocalization study.
where the fluorescein-phalloidin (green) stained cytoskeleton of the cell overlaps with the Cy3 (red) labeled formulated DNAzyme (Fig. 10B, marked with arrows).

The effect of polyplex formulation on DNAzyme delivery from intravenous injections was therefore studied in more detail. The intratumoral distribution of unformulated DNAzyme and Tf-PEG-polyplex injections were compared first. As shown in Figure 11, the unformulated DNAzyme did not result in an intracellular localization; the only colocalization was found in the fibers of the tissue. In contrast, the tumors treated with the formulated DNAzyme showed an evenly spread cytoplasmic signal of DNAzyme that colocalized with the cell cytoskeleton. The intravenous administration studies were also repeated with fluorescein-labeled DNAzyme. Three DNAzyme formulations (unformulated DNAzyme, PEG-polyplex, and Tf-PEG-polyplex), each containing 1 mg of fluorescent DNAzyme, were administered via low pressure tail vein injections. Mice were sacrificed 24 hrs post-injection and tumors extracted and processed for histology analysis (Fig. 12). Cell nuclei were stained with Sytox orange for contrast with the fluorescein-labeled DNAzyme. Again, the same phenomena were observed as with the Cy3-labeled DNAzyme. Unformulated DNAzyme does not readily enter tumor cells. Only one small area of fluorescence was found in the tumor sample (shown in Fig. 12A). PEG-polyplex is retained in the tumor area but remains extracellular (Fig. 12B). The highest tumor fluorescence was observed with the Tf-PEG-polyplex administration (Fig. 12C). In addition, intracellular fluorescence was observed in several cell patches. Therefore, intravenous administration is the only preferred method of treatment for the cyclodextrin-based delivery system that gives intracellular delivery in tumors. Transferrin targeting is required for successful intracellular delivery of DNAzymes to tumor tissue.

Figure 10. Comparison between intraperitoneal bolus and intravenous bolus routes of administration. (A) Intraperitoneal injections of Tf-PEG-polyplex formulated DNAzyme (Cy3-labeled) resulted in weak fluorescence in the tumor. The majority of the fluorescence was observed in the cap of the tumor. (B) Tf-PEG-polyplex formulated DNAzymes delivered intravenously in mice showed a clear intracellular colocalization between the phalloidin (green)-stained cytoskeleton of the tumor cells and the Cy3 (red)-labeled DNAzyme.
DISCUSSION

Therapeutic oligonucleotides (antisense oligonucleotides, ribozymes, DNAzymes, and siRNAs) are promising anti-cancer agents because of their ability to reduce expression of target proteins. Advantages of such treatment over traditional chemotherapeutics include target specificity and the potential of reduced side effects. For cancer applications, systemic administration of the oligonucleotides is preferred for accessibility to disseminated tumors. However, efficient delivery of these materials to their target site remains a challenge. Injection of unformulated oligonucleotides requires extremely high doses due to nonspecific distribution throughout the body, degradation, and rapid excretion. In this study, we examined the ability of a cyclodextrin-based, polymeric delivery system to provide transport of a c-myc DNAzyme to tumor cells in a nude mouse model using a variety of different administration methods.

The DNAzyme is packaged into nanoparticles by condensation with a cyclodextrin-based polycation (Fig. 2A, CDP-Imid). The imidazolated polymer is used because the imidazole moiety has been shown to enhance endosomal escape, thereby increasing delivery efficiency.\(^9\) CDP-Imid self-assembles with the DNAzyme by electrostatic interactions to form small particles called polyplexes. Complete DNAzyme loading in the polyplexes is confirmed by gel electrophoresis. Solutions of CDP-Imid and DNAzyme are mixed at various molar charge ratios of polymer to nucleic acid. No non-bound DNAzyme is observed at charge ratios of 1 and above, indicating complete binding (Fig. 3). Polyplexes with a positive surface charge are obtained by formulation at charge ratios greater than 1. The positively charged polyplexes can interact with negatively-charged cell surface proteoglycans for uptake by endocytosis.\(^{35}\) The effect of charge ratio on polyplex uptake into three human carcinoma cell lines (A2780, HT-29 and HeLa) was studied by using a fluorescein-labeled DNAzyme with analysis by flow cytometry. Polyplex uptake at all investigated charge ratios (2 to 10) was efficient and rapid (Fig. 4); over 98% of cells have internalized DNAzyme within 15 minutes of exposure. Polyplex uptake studies to HeLa cells were conducted in 10, 20, 30 and 40% serum without any significant impact on delivery efficiencies (data not shown). In contrast, incubation of HeLa cells with unformulated DNAzyme for over 1 hour results in a maximum of 0.1% fluorescein positive cells.

Since free fluorescein readily diffuses through cell membranes, the low intracellular fluorescence detected in this control group indicates that the fluorescein is not readily cleaved from the DNAzyme. Thus, the CDP-Imid is able to package and protect the DNAzyme and provide for intracellular uptake to a variety of cultured cell lines and in serum-containing media.

Several additional considerations arise for particle administrations distal to a desired site. The polyplexes need to be small enough to circulate and diffuse to the target site. In addition, the colloidal particles should be stabilized against salt and serum-induced

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Figure 11. Comparison of tumors from mice treated intravenously with unformulated (A) and Tf-PEG-ppx formulated (B) Cy-3-labeled DNAzyme. The tumors from mice treated with the unformulated DNAzyme only show a weak colocalization in the fibers. In contrast, in the tumors from the mice treated with the formulated DNAzyme, a much brighter and evenly spread colocalization [arrows] can be seen.

Figure 12. Comparison of tumors from mice treated with unformulated Fluorescein-DNAzyme (A), PEG-polyplex (B), and Tf-PEG-polyplex (C). Cell nuclei are stained with Sytox Orange.
aggregation and nonspecific interactions with serum components and undesired cells. Finally, the particles should ideally incorporate a targeting ligand for more specific delivery and retention. Transferrin, an iron-carrying protein, was chosen as the targeting ligand because rapidly growing cells, especially many cancer cells, up-regulate expression of the transferrin receptor in an effort to process more iron. Transferrin has also been used to target plasmids for tumor delivery. In order to identify a suitable cell line for preparing a mouse xenograft tumor model, the relative levels of transferrin uptake in three human carcinoma cell lines (A2780, HT-29 and HeLa) was determined (Fig. 5). The HT-29 and HeLa cell lines have the highest level of transferrin uptake, while the A2780 cell line has very low levels of transferrin uptake. The HT-29 cell line was chosen for the tumor xenograft model for its high transferrin uptake and low intrinsic background fluorescence.

For in vivo studies, polyplexes were prepared at a charge ratio of 2.5 and modified to address the aforementioned concerns for use in vivo by self-assembly with adamantane-based conjugates. The adamantane molecules form inclusion complexes with cyclodextrins on the surface of the polyplexes, thereby decorating the surfaces of the particles as illustrated in Figure 2. PEGylation is a well-established method for stabilizing nanoparticles against salt-induced aggregation and protein binding by providing a protective brush layer. However, PEGylation of cationic nanoparticles does not prevent nonspecific association and uptake by cells; the nanoparticles continue to interact with anionic cell surface proteins. Neutralization of excess surface charge lowers the zeta potential of the particles and reduces nonspecific binding. Studies utilizing polycation/DNA complexes have demonstrated that tumor targeting is facilitated with neutral or negative particles as compared to positively-charged particles that accumulate primarily in the liver.

Positive particle surfaces can be moderated by "charging" with an anionic moiety. The inclusion of two glutamates in the AD-PEG conjugate (AD-glugu-PEG-gal) results in a modifier that can tune particle zeta potential while also providing steric stabilization via PEG. With 40:60 AD-glugu-PEG-gal:AD-PEG, particles have average zeta potential of ~6 mV (Fig. 6); therefore, this ratio was used in particle formulations for in vivo injections. Transferrin-modified polyplexes were prepared by including 2% (by mole to cyclodextrin) of TF-PEG in the PEG-polyplex formulation. Thus, the CDP-Iimid component addresses the first issue of particle size by condensing the nucleic acid to small particles, the AD-PEG component provides steric stabilization in physiologic milieu, AD-glugu-PEG-gal mediates particle surface charge, and the AD-PEG-Tf introduces targeting capability. The three formulations, polyplex, PEG-polyplex, and TF-PEG-polyplex were imaged by electron microscopy (Fig. 7). All formulations result in small and uniform spherical particles. No free DNAzyme is observed in the three formulations, indicating complete encapsulation (also confirmed by DNA binding studies).

The average particle diameter ranges from ~30 nm (polyplex) to ~50 nm (TF-PEG-polyplex). The slight difference in size between the formulations likely represents the effect of modification by PEG and TF-PEG. Polyplexes containing DNAzymes are smaller than plasmid-bearing particles (~80–120 nm) formulated under similar conditions.

Unformulated Cy-3 DNAzyme and TF-PEG-polyplexes containing Cy-3-labeled DNAzyme were administered to mice by intraperitoneal injection. The biodistribution of the DNAzyme was followed by WBI and by whole organ analysis at 3, 8 and 24 hours post-injection. The majority of DNAzyme was eliminated from the body by excretion in the urine, although some fluorescence was also observed in the stool. In addition to strong fluorescence in the tumor and liver, a large amount of DNAzyme was also visualized in the peritoneal cavity of TF-PEG-polyplex-treated mice 24 hours post-injection. The three tissues with visible fluorescence (tumor, liver, and kidney) were extracted and imaged. These tissues all have high transferrin receptor expression (liver has 26 clones/gene, kidney has 16 clones/gene and lung has 14 clones/gene). At 3 and 8 hours post-injection, fluorescence was observed in liver, kidney and tumor in mice injected with both unformulated Cy-3 DNAzyme and TF-PEG-polyplex. However, by 24 hours post-injection, no fluorescence was observed in unformulated Cy-3 DNAzyme-treated mice, whereas significant levels of fluorescence was still observed in tumors of TF-PEG-polyplex-treated mice (Fig. 9). Therefore, while unformulated DNAzyme diffuses relatively rapidly throughout the body, DNAzyme in the cyclodextrin polymer-based nanoparticle delivery system concentrates in and is retained in several target organs (tumor, liver, kidney).

The effect of administration method on biodistribution was studied by comparing tumor, kidney, and liver fluorescence 24 hours following intraperitoneal bolus, intraperitoneal infusion, subcutaneous bolus and intravenous bolus injections of DNAzyme. The high fluorescence level sustained in the peritoneal cavity 24 hours post-intraperitoneal injection of TF-PEG-polyplex formulations suggested a slow release of material from the cavity. Unlike unformulated DNAzyme, the TF-modified particles likely have limited diffusivities due to their size. Indeed, a comparison of intraperitoneal bolus injection and intraperitoneal infusion (via the Alzet pump) of TF-PEG-polypelex revealed similar biodistribution. Subcutaneous injection of formulations did not result in fluorescence outside of the injection site even 24 hours after administration. It is not surprising that the nanoparticles are unable to traverse several tissue layers due to their size and hydrophilicity. In contrast, intravenous administration results in very efficient delivery of material throughout the animal; very little material is retained at the injection site. At 24 hours, the mice treated with TF-PEG-polypelex revealed high tumor, liver, and kidney fluorescence, whereas mice that were administered unformulated DNAzyme had no remaining fluorescence.

A clear effect of polyplex formulation on biodistribution was also observed from the intravenous administration studies. In addition to tumor, liver and kidney fluorescence, animals treated with unmodified polyplexes also showed punctate fluorescence in the lung at all time points (3, 8 and 24 hrs post-injection). In contrast, no lung fluorescence was observed in mice treated with PEGylated polyplexes. The polyplex accumulation in the lung is likely due to trapping of aggregated particles in the lung capillaries. The PEG-stabilized polyplexes are not susceptible to lung accumulation. This phenomenon (lack of lung accumulation with stabilized particles) was also noted by Ogris et al. who compared the organ distribution of unPEGylated plasmid-containing polyplexes and PEG-conjugate polyplexes.

The similar effect observed here indicates that the polyplex modification by inclusion complex formation is also very stable in the circulatory system. Transgene expression achieved from similar particles suggest that the nucleic acids are released intracellularly, perhaps by competitive displacement by high concentrations of ionic proteins (such as histones) in the cell nuclei.

The fluorescent tumors from intraperitoneal bolus, intraperitoneal infusion and intravenous bolus injections of TF-PEG-polypelexes were compared by histology analysis. Polyplexes delivered by intra-peritoneal bolus and intraperitoneal infusion did not penetrate the tumor cap and remained extracellular (Fig. 10A). In contrast, polyplexes administered by intravenous infusion were internalized by tumor cells (as evidenced by colocalization with phallolidin) even...
several cell layers past the vessel locations (Fig. 10B). The various formulations were then injected intravenously and analyzed by histology. Unformulated and PEGylated polyplexes were not efficiently internalized by tumor cells (Fig. 12). Transferrin-modified formulations were successfully internalized in several cell clusters throughout the tissue. Thus, transferrin targeting does not affect biodistribution of material to the tumor; however, targeting is required for uptake and retention by tumor cells. A similar effect was observed in application of transferrin targeting for plasmid delivery; the ratio of plasmid delivered to liver versus tumor is unaffected by targeting but transgene expression is observed only in animals treated with transferrin-targeted polyplexes.\(^{43}\) This study therefore demonstrates the ability of transferrin-modified, cyclodextrin polymer-based polycations to mediate tumor-targeted uptake of DNAzymes from systemic administrations. Advantages of the delivery system include longer tumor retention of DNAzyme and more efficient tumor cell targeting.

**Acknowledgements**

Mark E. Davis is a consultant to and has financial interest in Insert Therapeutics, Inc.

**References**


