molecular pharmaceutics

reviews

The First Targeted Delivery of siRNA in Humans via a Self-Assembling, Cyclodextrin Polymer-Based Nanoparticle: From Concept to Clinic

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Received January 15, 2009; Revised Manuscript Received March 3, 2009; Accepted March 6, 2009

Abstract: Experimental therapeutics developed to exploit RNA interference (RNAi) are now in clinical studies. Here, the translation from concept to clinic for the first experimental therapeutic to provide targeted delivery of synthetic, small interfering RNA (siRNA) in humans is described. This targeted, nanoparticle formulation of siRNA, denoted as CALAA-01, consists of a cyclodextrin-containing polymer (CDP), a polythethylene glycol (PEG) steric stabilization agent, and human transferrin (Tf) as a targeting ligand for binding to transferrin receptors (TfR) that are typically upregulated on cancer cells. The four component formulation is self-assembled into nanoparticles in the pharmacy and administered intravenously (iv) to patients. The designed features of this experimental therapeutic are described, and their functions illustrated.

Keywords: CALAA-01; siRNA; targeted delivery; targeted nanoparticle; clinical trial

Introduction

The development of new therapeutics that attempt to engage a RNAi pathway has opened a new area of drug discovery and development. Traditional drug discovery and development involve large scale screenings of small molecule libraries with initial screening hits subjected to subsequent medicinal chemistry approaches to create enhanced versions. Translation of small molecule experimental therapeutics into the clinic has led to high failure rates for a variety of reasons that include lack of efficacy, poor pharmacokinetics (PK), and so on. Drug discovery and development utilizing RNAi-based mechanisms allow for significantly different strategies than those utilized in traditional drug discovery and development methodologies. They have the potential to provide therapies for many targets that are currently undruggable and to do so with higher success rates in the clinic.

While there are many ways to engage RNAi pathways, the majority of the clinical trials currently being conducted involve small interfering RNA (siRNA). The initial clinical trials employ naked siRNA that is administered locally (to the eye, lung, or skin).[1] The first clinical trial to use systemically administered, naked siRNA is for the treatment of acute renal failure (see www.clinicaltrials.gov for details). Systemically injected, naked siRNA clears quickly through the kidney (within minutes), so this method of administration is much like a "local" administration to the kidney. The naked siRNA may or may not be chemically modified to impart stability and modulate immunostimulatory effects (there are trials using both types of naked siRNA). In 2008, the first targeted delivery of siRNA was accomplished in a human. A phase I trial using the experimental therapeutic CALAA-01 was initiated, and the first patient was treated in May of 2008. This targeted nanoparticle is administered intravenously (iv) for the treatment of solid tumors. Here, I provide the historical development of CALAA-01 (Calando Pharmaceuticals-01) from the design concepts to its clinical application.

Design of a Targeted Delivery System for Nucleic Acids

Delivery systems for nucleic acids that engage the RNAi pathway have benefited from previous work on delivery systems for other nucleic acids such as plasmids (pDNA),

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10.1021/mp900015y CCC: $40.75
Published on Web 03/06/2009

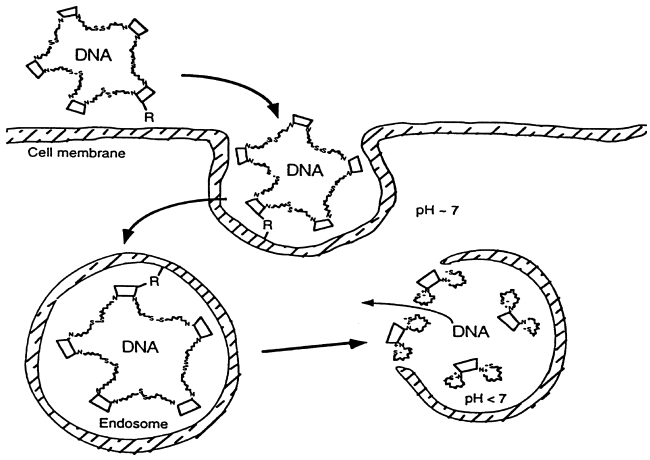

Figure 1. Initial schematic of delivery system in 1996.

oligonucleotides, ribozymes, and DNAyzmes. While there are differences when delivering each of these classes of nucleic acids, there are also common features. We began work in 1996 to develop a targeted delivery system for the systemic administration of nucleic acids. Our objective was to create new experimental therapeutics for the treatment of cancer. The methodology that was taken can be described as a systems approach to the design and engineering of a multifunctional colloidal particle. When the work was initiated, the terminology "nanoparticle" was not generally used. However, this notation has quickly been adopted for colloids in the range of 1−100 nm in diameter, and I will stay with this notation here.

My initial schematic to illustrate the desired concept of a targeted, multifunctional nanoparticle is reproduced in Figure 1 (the first schematic presented in the U.S. provisional patent application). Issues from this initial design that have been carried through to the clinic in 2008 are (i) the use of a cyclodextrin-containing polymer (CDP) formulation that assembles with nucleic acids to give small colloidal particles (nanoparticles), (ii) the formulation of colloidal particles (nanoparticles) having diameters at or below 100 nm, (iii) the use of targeting ligands (R in Figure 1) to bind to cell surface receptors and trigger endocytosis, and (iv) the recognition of acidification as a mechanism to initiate colloidal particle disassembly and escape from the endocytic pathway. While the initial concept was illustrated for pDNA, we realized at that time that the system could work for all forms of nucleic acids, as the CDPs interact with the nucleic acids by electrostatic charge and thus should be sequence- and possibly size-independent.

In the mid-1990s, there were some guidelines for overcoming extracellular and intracellular barriers with non-viral[2] and cationic polymer-based[3] delivery systems. One of the most difficult issues that needed to be addressed was how to create a multifunctional delivery system that could accomplish many tasks at the right place and at the right time. This integration of numerous functions was certainly a limiting factor in the development of non-viral delivery systems. Thus, a primary goal was to develop a fully integrated system that would be capable of transitioning from a research curiosity into an experimental therapeutic that could be used in humans. The synthetic pathways developed to create the components of the delivery system were all defined so they could be scaled up at cGMP conditions. Additionally, formulation methods took into consideration the need for long-term stability. To begin, numerous design issues were addressed. Tables 1 and 2 provide some of the design issues and key features that were considered when creating the targeted delivery system.

Over the course of the development of CALAA-01, there were some modifications to these criteria as new data became available. For example, particle diameters of ∼100 nm were initially considered. After further work from our laboratory as well as other literature reports, the particle diameters were refined to smaller sizes, ∼50−70 nm. This was due to further understanding on requirements for particle movements in tumors.

Finally, the delivery system ended up as a three-component system (CDP, AD-PEG, and AD-PEG-Tf; see Figure 2a) that was developed into a two-vial formulation where the delivery components are contained in one vial and the siRNA in the second vial (see Figure 2b). When the contents of the two vials are combined, they self-assemble into ∼70 nm nanoparticles (see Figure 2b).

These targeted nanoparticles are administered iv to patients where they circulate (too large to escape via the kidney) and localize in tumors (see Figure 3). The transferrin (Tf) on the nanoparticle is able to bind to transferrin receptors (TfR) on cancer cells, and the nanoparticles can be internalized via receptor-mediated endocytosis (Figure 3d). The CDP contains organic groups that are protonated around pH = 6. This "chemical sensing" mechanism then triggers a number of processes that provide escape from endocytic vesicles and nanoparticle release of the nucleic acid into the cytoplasm. Of importance is that each of the delivery components is sufficiently small so that when the nanoparticle disassembles into individual components, those components can be cleared from the body via the kidney. Next, the delivery system, its assembly, and its function will be described in detail.

Components of the Delivery System

CDP. The key to the success of the delivery system is the cyclodextrin-containing polymer. This polymer provides many functions to the overall delivery system. The CDP is

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Table 1. Design Issues Considered (Modified from ref 4)

design criteria	advantages/comments
1. adaptability	1. can modify system in response to new mechanistic information
2. formulation with nucleic acids via self-assembly	2. ease of use and scale-up
3. formulation with any type of nucleic acid and/or combination	3. generality of deliverable, uses electrostatic interactions since these are common to all nucleic acids
4. non-peptide-based	4. no possibility of MHC (major histocompatibility complex) presentations
5. non-antigenic	5. enables repeat dosing
6. complete nucleic acid encapsulation	6. protection of nucleic acid from nucleases and Toll-like receptors on cell surfaces; for example, TLR-3 recognizes double stranded RNA
7. components of system nontoxic and of size to be cleared via the kidney when delivery system disassembles	7. minimize toxicity and provide for excretion *after* disassembly releases the nucleic acid at the target site

Table 2. Key Features of the Delivery System (Modified from ref 4)

features	comments
1. particle size: larger than 10 nm but not larger than 75 $\pm$ 25 nm	1. injectable, minimizes renal clearance (must be larger than 10 nm), access to tumors, movement within tumors
2. particle surface charge: -10 mV to $+10$ mV	2. minimizes phagocytosis, avoids nonspecific interactions, avoids self-aggregation
3. contains targeting ligand on particle surface	3. provides some selectivity for cell type and enhances uptake into cells via receptor-mediated endocytosis
4. endocytic pathway escape mechanism and may also contain a nuclear trafficking component	4. enhances delivery efficiencies, nuclear trafficking not necessary with siRNA

a short polycation (see Figure 4 for basic structure, x is typically around 5). The polycation assembles with nucleic acids (polyanions) primarily via electrostatic interactions. These interactions allow for assembly of the CDP with different types and sizes of nucleic acids. The composite CDP−nucleic acid structures are nanoparticles where the nucleic acids are completely protected from nuclease degradation. The cyclodextrins within the polymer chains that ultimately reside on the surface of the nanoparticles are used for assembling the steric stabilization agent (AD-PEG) and the targeting agent (AD-PEG-Tf) as discussed below. Additionally, the polymer backbone is used as a scaffold for attaching agents (see, e.g., the imidazole groups in the schematic of the CDP in Figure 2a) that assist in the endocytic pathway escape and nucleic acid release (described below).

Cyclodextrins are used in pharmaceutical formulations, and as such, their long-term biocompatibilities are being defined in humans.[5] Because of the low toxicity, the lack of immune stimulation, and the absence of enzyme degradation in humans of cyclodextrins, they were chosen as one of the building blocks for the CDP component. While numerous types of cyclodextrin-containing polymers existed previous to our work, for example, randomly cross-linked cyclodextrin polymers and polymers with grafted cyclodextrins,[5] the CDPs presented here are a new class of polymers in that the cyclodextrin is now part of the polymer backbone of a linear, water-soluble polymer.[5] The CDP polymers are synthesized by a condensation polymerization between a difunctionalized cyclodextrin comonomer and a second, difunctionalized comonomer that contains the charge centers. Since both comonomers are difunctionalized, the polymer formed from their co-condensation is linear. The structure−functional behavior of the CDP type polymers has been investigated in detail,[4,6−12] and only highlights will be presented here.

The basic structure of the linear, cyclodextrin-containing polymers can be represented by the schematic shown in Figure 4a. The CDP that was ultimately found to give the

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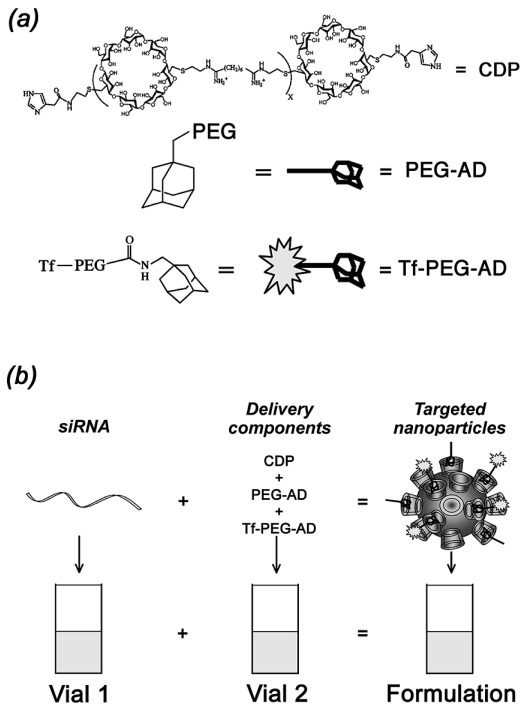

Figure 2. Components and formulation of targeted nanoparticle-containing siRNA. (a) The delivery components are (i) a water-soluble, linear cyclodextrin-containing polymer (CDP), (ii) an adamantane (AD)-PEG conjugate (PEG MW of 5000) (AD-PEG), and (iii) the targeting component that is an adamantane conjugate of PEG (PEG MW of 5000) that has human transferrin (Tf) conjugated at the end opposite to the adamantane (Tf-PEG-AD). (b) The formulation contains two vials, one with siRNA and the other with the delivery components. When the two vials are mixed together, the targeted nanoparticles form via self-assembly of the four components.

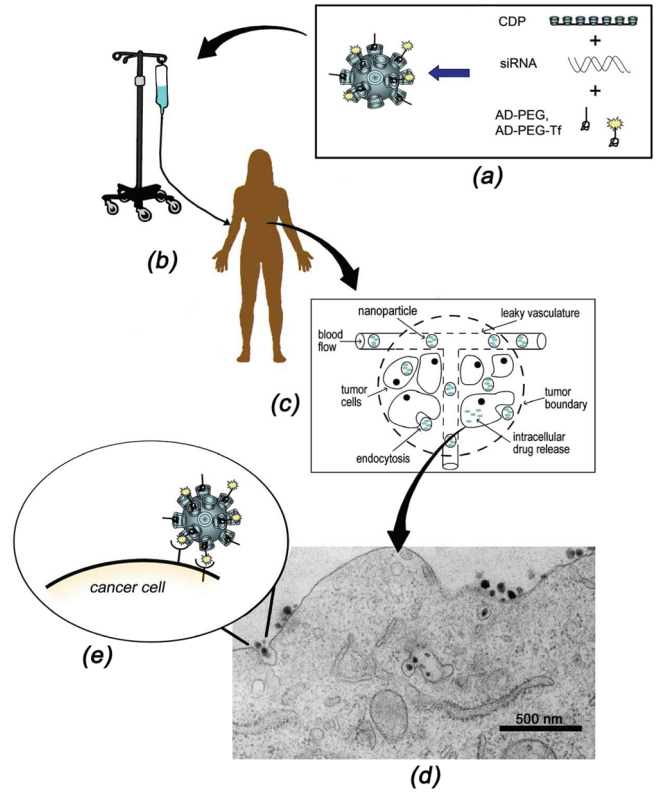

Figure 3. Schematic of how the targeted nanoparticles function. (a) Nanoparticles are assembled from the four components (see Figure 2). (b) Aqueous solutions of nanoparticles are infused into patients. (c) The nanoparticles circulate in the blood of the patient and escape via the "leaking" blood vessels in tumors. (d) Nanoparticles penetrate though the tumor and enter into cells by receptor-mediated endocytosis (transmission electron micrograph of 50 nm nanoparticles entering a cancer cell). Note that the nanoparticles enter and are initially located in vesicles within the cell and must escape and disassemble to deliver their payload. (e) Targeted nanoparticles can have numerous interactions (e.g., Tf with its receptor) on the surface of the cancer cell that then stimulate entrance into the cell.

best overall behavior is illustrated in Figure 4b, and its relationship to the schematic in Figure 4a is provided. Typically, these polycations have ~5−6 repeating units (x = 5, 6) and thus are low molecular weight polymers (can be considered oligomers). This was accomplished by design to provide low toxicity, minimal complement activation and to permit renal clearance in animals and humans. The structural variations investigated were (i) the type of cyclodextrin (A in Figure 4),[4,8,10,12] (ii) the absence of the cyclodextrin (A in Figure 4) (other sugars[4,8,12] and even methylenes[4,8,12] instead of sugars were explored), (iii) the distance between the cyclodextrin and the charge center (S in Figure 4),[4,6,12] (iv) the type of charge center (C in Figure 4), for example, amidines, quarternary amines, and secondary amines,[4,9−12] and (v) the distance between the charge centers (B in Figure 4).[4,7] The structure−functional relationship investigations primarily employed pDNA as the nucleic acid being delivered. The overall conclusions obtained were (i) the presence of the cyclodextrin gave the highest water solubility and the lowest toxicity, (ii) the type of cyclodextrin did not really affect the transfection properties (gene expression and toxicity) and beta-cyclodextrin was chosen because it provided much better properties for large-scale processes of collection and purification of the CDP over both alpha and gamma cyclodextrin, (iii) when the distance between the cyclodextrin and the charge center was small, the polymer would not bind well to the nucleic acid (presumably due to steric effects of the cyclodextrin), (iv) the further the distance between the charge centers, the lower the cellular toxicity; however, the binding to the nucleic acid also declined with increasing distance, and (v) the amidine charge centers provided for lower toxicity and better delivery efficacy. Given these results, the CDP illustrated in Figure 4b provided the best overall properties and was used as the polymer for further formulations.

Nanoparticles formed from polymers and nucleic acids can enter cells via endocytosis. In order to effectively deliver the nucleic acid, the polymer must facilitate endocytic pathway escape and release of the nucleic acid. Certain pH-sensitive polymers such as polyethyleneimine (PEI) are said

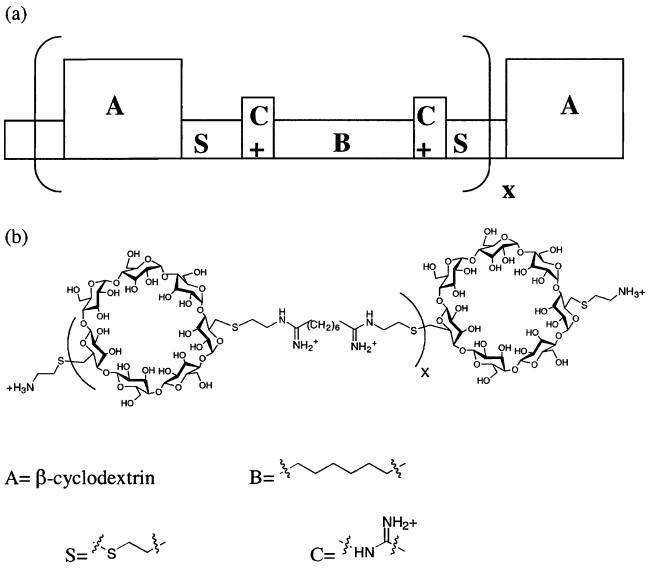

Figure 4. Schematic of linear, water-soluble cyclodextrin-containing polymers. (a) Generalized structure of polymers where A represents a segment of the polymer that comes from one comonomer (typically containing the cyclodextrin), B represents a segment of the polymer that comes from the other comonomer, C is the charge center, and S is the spacer between A and C. (b) Specific example of a polymer with labeled A, B, C, and S. Adapted with permission from ref 12. Copyright 2004 CRC Press.

to exhibit a "proton sponge" effect, since they have titratable amine groups (pK_a values in the range of 4−7) and have the ability to buffer the pH changes as endocytic vesicles acidify.[13] The concept involves the buffering of endocytic vesicles, their swelling due to osmotic pressure increases, and ultimately vesicle rupture to release its contents. In attempts to exploit this proposed pathway of release, several publications have shown that histidylated or imidazolated polycations provided substantial gene delivery over their parent polymers.[14,15] Pun (formerly Hwang) first conjugated histidine to the ends of the CDP and showed enhanced gene delivery over the CDP without end group modification.[16] Subsequently, the end groups were modified to imidazole groups.[4,12] The addition of the imidazole termini did provide buffering of endocytic vesicles in live cells.[17] Investigations into the underlying processes of imidazole-modified CDP behavior to give enhanced delivery efficacies revealed that there are numerous functions occurring simultaneously in addition to the buffering and endocytic vesicle escape.[18] Thus, the precise mechanisms leading to enhanced delivery efficiencies remain elusive. However, there is no question that the addition of imidazole to the CDP does enhance delivery efficacy (whether plasmid or siRNA[18]). The final CDP used for in vivo work does contain imidazole end group modifications as illustrated in Figure 2a.

Steric Stabilization, AD-PEG. Nanoparticles that are polycation−nucleic acid composites are known to be unstable in biological fluids such as blood. One method of preventing self-aggregation and undesired interactions with nonself entities in biological fluids is to employ steric stabilization. Polythethylene glycol (PEG) has been conjugated to different non-viral delivery carriers, and the resulting PEGylated particles have demonstrated increased salt and serum stability. A difficulty with directly PEGylating a polycation is that the PEG can interfere with the binding to the nucleic acid (this is particularly important with siRNA). While there is a significant amount of PEGylation technology that is available, it all relies upon covalent attachment of the PEG to the carrier. We developed a new methodology for steric stabilization with PEG polymers that exploits the presence of the cyclodextrin in the CDP.

The concept of providing steric stabilization to the CDP−nucleic acid nanoparticle involves the fact that cyclodextrins can form inclusion complexes with small molecules (for a review on inclusion complexes and their uses in pharmaceutical applications, see ref 5). Adamantane (AD) has one of the highest association constants with beta-cyclodextrin (on the order of 10^4-10^5 M^{-1} for adamantane carboxylate[19]). A molecular conjugate of AD and PEG can be formed by reacting one end of the PEG with AD. The AD in the AD-PEG conjugate can form an inclusion complex with the beta-cyclodextrin (Figure 5) on the surface of the nanoparticle in order to decorate the surface with AD-PEG to provide steric stabilization (see schematics in Figures 2, 3, and 5).[19] This formulation is the first to create steric stabilization on a nanoparticle with PEG via noncovalent interactions. In order to provide stabilization in 150 mM salt, the molecular weight of the PEG needed to be at least 5000.[19] Lower molecular weight PEGs were not able to provide complete stabilization at a mole ratio of cyclodextrin to AD of 1 to 1, and higher molecular weight PEGs were not investigated.[19,20] Additionally, the AD-PEG conjugate can be prepared such that it includes charge. That is, an anionic region can be placed between the AD and the PEG, for

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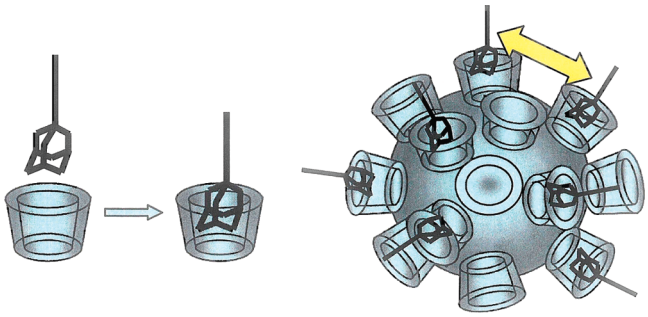

Figure 5. Schematic representations of the adamantane−PEG inclusion complex formation with the cyclodextrin (left) and the PEG−PEG interactions when the AD-PEGs are localized on the surface of the nanoparticles (right).

example, and those conjugates can be used alone or in combination with AD-PEG to tune the surface zeta potential of the nanoparticles. Using a 1:1 ratio of cyclodextrin to AD in the formulation but a combination of AD-PEG and AD−anionic charge−PEG, the zeta potential of the formulated nanoparticles could be tuned from 15 mV at 100% AD-PEG to −25 mV at 0% AD-PEG.[12]

Targeting Agent, AD-PEG-R. The PEGylation of nanoparticles to provide steric stabilization for systemic administration also greatly reduces the ability of the nanoparticles to interact with the surfaces of cells. In order to engage cell surface receptors for receptor-mediated endocytosis, targeting ligands can be added to the nanoparticles. When PEGylating the CDP−nucleic acid nanoparticles with AD-PEG, the cellular uptake and intracellular trafficking were significantly affected over non-PEGylated nanoparticles.[21] Thus, to add a targeting ligand for enhancing specific cellular uptake, another molecular conjugate was developed: AD-PEG-R. Just like with AD-PEG, the AD-PEG-R conjugate can decorate the surface of the CDP−nucleic acid nanoparticle to give a controlled number of targeting ligands (R). Targeting ligands are conjugated to the opposite end of the PEG from the AD (molecular weight of the PEG remained 5000). The length of the PEG allows the targeting agent to be located at the exterior surface of the PEGylated nanoparticle, and the PEG spacer provides sufficient flexibility and distance from the surface of the CDP−nucleic acid core to allow for binding to cell surface receptors with good affinity.[20,22] In fact, several Tf molecules on the surface of the nanoparticles can provide multivalent binding to cell surfaces that have high TfR density.[20] A variety of targeting ligands have been explored. For example, small molecules such as galactose (for targeting hepatocytes)[4,12,19] and proteins such as transferrin (for targeting cancer cells)[20,22] and antibody fragments (see below) have been reported for this system. The targeting ligand that has been investigated in the greatest detail is Tf. The amount of TfR is known to be elevated on the surfaces of malignant cells of many types. Thus, it provides a cell surface receptor that can be targeted for a variety of cancers. Additionally, GMP human Tf is now commercially available (from Mebiopharm in Japan).

Two-Vial Formulation

The formulation of the three delivery system components (CDP, AD-PEG, AD-PEG-R) with a nucleic acid to give a targeted nanoparticle was accomplished as follows. Shortly after the delivery system was developed, we recognized that the nanoparticles could be formulated by adding all the components together at one time (schematically illustrated in Figure 2b). The delivery components were placed in one vial and the nucleic acid in a second vial. When the two solutions (same volumes) are mixed, the nanoparticles self-assemble.[12,20] What is intriguing about this formulation is that the assembly can give nanoparticles at very high concentrations. These self-assembled formulations can occur at nucleic acid concentrations in the mg nucleic acid/mL range.[12,20] A typical formulation involves a 3:1 charge ratio of polymer positive charge to nucleic acid negative charge. This ratio insures that all the nucleic acid is contained within nanoparticles. The nanoparticles have approximately a 1:1 charge ratio,[20] so excess CDP is in solution. Also, since a 1:1 mol ratio between the cyclodextrin and the AD from the total AD-PEG and AD-PEG-R is used, there is excess AD-PEG and AD-PEG-R in solution. Complete compositional analyses of these nanoparticles have been published using siRNA and AD-PEG-Tf.[20] The formulation gave a 70 nm diameter nanoparticle that contained ∼10 000 CDP molecules, ∼2000 siRNA molecules (19 base pair duplexes with 2 base overhangs on either end), ∼4000 AD-PEG, and ∼100 AD-PEG-Tf.[20] The amount of Tf can be tuned from zero to about 5 mol % of the total AD-PEG before Tf-Tf induced aggregation of the particles occurs.[20]

Two features of the self-assembly formulation warrant further discussion. First, the nanoparticle diameters of CDP formulations with siRNA, pDNA, and calf thymus DNA (CT-DNA) all increase with nucleic acid concentration (exceeding 100 nm) when AD-PEG is not included at the time of formulation.[20] However, when AD-PEG is included, the nanoparticle diameters remain constant, independent of nucleic acid concentration at 60−80 nm.[20] Thus, the AD-PEG changes the assembly processes that are occurring and provides a formulation that provides highly concentrated nanoparticles by simple mixing. Second, if the AD-PEG conjugates were providing stability by only the inclusion complex interactions, then the stabilization should disappear upon dilution of the nanoparticles (as would occur upon injection into an animal or human). After years of investiga-

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tion, the reason for the lack of dissociation upon dilution of the AD-PEG from the nanoparticles was determined. By using isothermal titration calorimetry (ITC), the thermodynamics of the interactions between the AD-PEG and either an individual cyclodextrin, a CDP strand, or an assembled CDP−siRNA nanoparticle were measured. The change in enthalpy from binding between the AD-PEG and the three forms of CD mentioned showed that there was a significant increase in the energy of stabilization only when the AD-PEG was binding to the nanoparticle.[20] We hypothesized that this increase in stabilization energy was from favorable interactions between the PEG chains that were closely associated on the surface of the nanoparticle (see Figure 5 for schematic).

As the molecular weight of the PEG was decreased, this stabilization energy declined. Thus, if our hypothesis is correct, then the self-assembly process can be understood further. That is, in the solution of the delivery components, the AD-PEG (and AD-PEG-R) is interacting with the CDP but those interactions are driven entirely by the inclusion complex formation and are weak. When the nucleic acid is added, the multivalent electrostatic interactions between the CDP and the nucleic acid are sufficiently strong to provide for CDP−nucleic acid assembly into nanoparticles (exclude AD-PEG components from the interior). As the nanoparticles are forming, the AD-PEG components form inclusion complexes with surface cyclodextrins, and when they do, then the configuration is stabilized by the extra stabilization energy obtained from the lateral interactions of the high density of PEG organized on the surface of the nanoparticle. Upon dilution, this extra energy of stabilization is the key energy inhibiting disassembly. There is clear evidence that the AD-PEG-Tf remains on the nanoparticles as the effects of the Tf are observable in animals (discussed below).

When dealing with self-assembling systems, there is concern about their stability under "working" conditions. For the present case, the nanoparticles need to be stable in the circulation. Thus, we tested their ability to self-assemble under working conditions as follows. The delivery components were injected iv into the tail vein of mice, and then 1 min later siRNA was injected iv into the same tail vein. A few minutes later, blood was collected and serum run on a gel to determine whether the siRNA had assembled with the CDP in the circulation. Also, the order of injection was reversed. The data obtained are shown in Figure 6. The results clearly show that the siRNA and the CDP self-assemble in the circulation of the mouse. Given that the components self-assemble under "working" conditions, the likelihood that they would disassemble in the circulation is low. In fact, plasma/serum samples collected from numerous animal experiments have not revealed any free siRNA.

In Vivo Delivery of DNAzymes and pDNA in Mice

Initial animal experiments using the CDP, AD-PEG, and AD-PEG-Tf delivery components utilized two different types

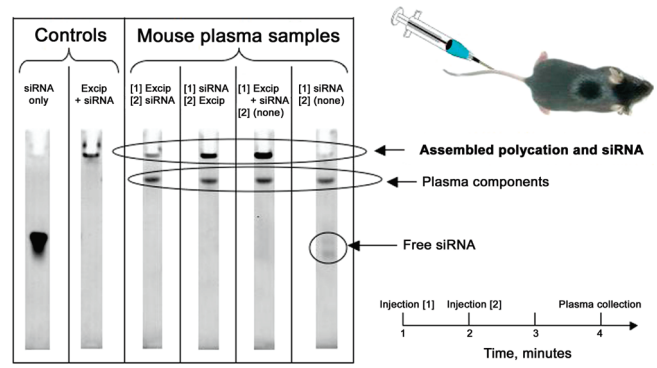

Figure 6. Self-assembly test in the circulation of mice. The delivery components (CDP, AD-PEG, AD-PEG-Tf) are denoted Excip. When the siRNA alone is injected iv into mice, free siRNA is observed on a gel from plasma collected 3 min after injection (last lane). When the Excip are injected and then 1 min later the siRNA injected (or the reverse order), plasma collected at 3 min after the first injection shows no free siRNA (lanes 3 and 4 from the left) and bands indicative of formulated particles (injected formulated particles are in the next to the last lane) are obtained. The mouse experiments were conducted by B. Mack at Caltech, and the gel run by J. Liu at Calando Pharmaceuticals.

of nucleic acids, namely, DNAzymes[23] and pDNA.[24] Results from those two investigations were consistent with one another and provided the basis for siRNA delivery in vivo (next section).

Intravenous injections of nanoparticles containing Cy-3-labeled DNAzymes were monitored by whole animal fluorescence imaging of tumor-bearing mice.[23] Nanoparticles that lacked AD-PEG or AD-PEG-Tf collected in the lungs, presumably due to aggregation. However, when nanoparticles contained AD-PEG, there was no fluorescence observed in the lungs. These results strongly suggest that the PEGylated particles do not aggregate in circulation. Tumor site fluorescence was obtained with nanoparticles containing AD-PEG and those with AD-PEG + AD-PEG-Tf. However, intracellular delivery within the tumor was only observed with the formulation that contained the AD-PEG-Tf.

Intravenous injections of nanoparticles containing pDNA coding for the p53 gene were studied in nude mice bearing PC3 tumors (TfR positive and p53 null).[24] The nanoparticles were formulated with either AD-PEG or AD-PEG + AD-PEG-Tf. By using PCR methods, the biodistribution of the plasmid was determined. It was observed that the amount

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of the plasmid in the tumor was ~10% of the injected dose in the best cases and that there were no differences in the amounts of plasmid localized in the tumors when using nanoparticles that did or did not contain the AD-PEG-Tf. However, using RT-PCR, the amount of mRNA from the delivered p53 gene was measured. No mRNA was observed from the formulation that did not have Tf, while the Tf-containing formulation did yield p53 mRNA.

The results of these two studies showed that amounts of tumor localization of the nanoparticles were independent of the presence of the targeting ligand (Tf). However, in both cases, the data are consistent with the Tf ligand providing greater tumor cell uptake (directly observed in the case of the DNAzyme and implied by the presence of the mRNA for the case with the pDNA). Additionally, it is clear that the nanoparticles need to be PEGylated to provide for circulation without significant lung accumulation.

Movement to siRNA Delivery

The discovery of RNAi and more specifically the demonstration that siRNA could function in mammalian cells without eliciting an immune response opened the pathway for creating human therapeutics with siRNA. Potent siRNA against almost any gene target can be found. However, a major hurdle to the creation of human therapeutics with siRNA is the delivery problem. Thus, it is not surprising that the initial clinic applications of siRNA involve local delivery. Several companies have announced programs to create systemically delivered siRNA, and Calando Pharmaceuticals was the first to enter the clinic with their targeted delivery of siRNA (CALAA-01) for cancer in 2008. While the exact nature of CALAA-01 has not been reported, it is similar to the delivery system outlined here (Figures 2 and 3). Work contributing to the development of CALAA-01 is presented in this section.

Given the success of the CDP-based delivery system for DNAzymes and pDNA, when siRNA was discovered, the CDP-based delivery system was investigated for use with siRNA. Since siRNA does not need to be transported into the nucleus, the intracellular delivery problem is actually less complicated than with pDNA.

Naked siRNA is quickly degraded by serum nucleases. Thus, numerous studies employing siRNA utilize chemical modifications that increase its stability. However, clinical studies with naked siRNA do not always use chemically modified siRNA. Bartlett and Davis showed that the gene inhibition kinetics of unmodified and nuclease-stabilized siRNA were essentially the same when electroporated into cells.[25] Thus, the intracellular half-life of gene inhibition does not appear to be a function of nuclease stability. These authors hypothesized that the stabilization advantages of nuclease-stabilized siRNA originate primarily from the effects prior to and during internalization before the siRNA can interact with the intracellular RNAi machinery.[25] A more important factor that determines the gene inhibition half-life is the cell doubling time.[26] Both in vitro and in vivo data demonstrate that dilution due to cell division and not intracellular siRNA stability governs the duration of gene silencing.[26] Therefore, the use of a delivery system that protects the siRNA from degradation prior to intracellular release should be able to function with siRNA that is not chemically modified although such formulations may produce an innate immune response.

Shortly after the discovery of siRNA function in mammalian cells, there were reports of immune responses in cell cultures. However, Heidel and co-workers showed that naked siRNA could be administered systemically to mice without inducing an interferon response.[27] There are a number of Toll-like receptors that are located on different cell types and even at different locations (on cell surfaces or in the endosome) on various cell types (TLR-3 is located both on the cell surface and intracellularly in human fibroblasts but remains intracellular in other cell types such as monocyte-derived immature dendritic cells). Thus, the biodistribution and cellular uptake of the siRNA could likely affect whether innate immune responses are stimulated in animals and humans. Judge et al. showed that non-viral delivery vehicles (both lipid-based and polymer-based) could induce interferon and inflammatory cytokine responses in mice when formulated with siRNA.[28] This group also reported that selective incorporation of 2′-*O*-methyl modifications of the RNA strands could eliminate these responses in mice.[29]

The initial demonstration of CDP-based delivery system formulated with siRNA (not chemically modified) utilized a disseminated murine model of Ewing's sarcoma.[30] siRNA targeting the breakpoint of the EWS-FLI1 fusion gene was shown to inhibit the gene product in vitro and that this inhibition provided an antiproliferative effect. TC71 cells (TfR positive, EWS-FLI1 positive) constitutively expressing luciferase were injected into mice, and their proliferation was monitored by in vivo bioluminescence imaging. The treatment groups investigated were (i) injection solution (D5W), (ii) naked siRNA, (iii) full formulation (illustrated in Figure

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2) with a control siRNA, (iv) full formulation with the anti-EWS-FLI1 siRNA, and (v) formulation without the AD-PEG-Tf of the anti-EWS-FLI1 siRNA. Only formulation (iv) revealed any antitumor effects (and tumor inhibition of EWS-FLI1 mRNA). Further studies indicated that no innate immune responses were obtained from any of these formulations. Thus, this work provided evidence to show that the correct sequence and the targeting agent were both necessary for biological efficacy. Of importance was the fact that the control sequence contained a known immunostimulatory motif when used with lipid-based delivery systems. Thus, the CDP-based delivery system did not elicit innate immune responses even with known immunostimulatory sequences.

Further investigations with the CDP-based delivery system have been reported. Bartlett et al. showed via in vivo multimodal imaging (PET and bioluminescence) the kinetics of the biodistribution and gene inhibition function of the targeted and nontargeted versions of the nanoparticles.[31] Interestingly, like with the delivery of the pDNA (p53 gene),[24] the amount of siRNA reaching the tumor was the same for the Tf targeted and nontargeted siRNA-containing nanoparticles.[31] The kinetics of the biodistribution were unchanged between targeted and untargeted nanoparticles. However, the luciferase gene inhibition was significantly greater for the Tf targeted nanoparticles. Thus, the primary effect of the Tf targeting ligand is to enhance tumor cell uptake not to change the amount localized in the tumor tissue.[31] This conclusion has been obtained with a lipid-based delivery system as well.[32] That is not to say that a nontargeted nanoparticle does not enter tumor cells, but rather that the targeted version of the nanoparticle should always outperform the nontargeted version. Bartlett and Davis have shown this using a potent siRNA against ribonucleotide reductase subunit 2 (RRM2) in a murine tumor model.[33] The likely reason for the lack of antitumor efficacy with the nontargeted EWS-FLI1 siRNA was that the siRNA used in that study was not highly potent.[30] Given the results from these studies, the CDP-based delivery system was translated into clinical use as described next.

CALAA-01

CALAA-01 is an experimental therapeutic that employs the CDP-based delivery system to formulate siRNA against ribonucleotide reductase subunit 2 (RRM2). This experimental therapeutic was developed under the direction of Dr. Jeremy Heidel at Calando Pharmaceuticals.[34] CALAA-01 employs Tf as the targeting agent, and the siRNA is not chemically modified. The clinical material is a two-vial formulation as illustrated in Figure 2. A potent siRNA against RRM2 was developed that exhibits significant antiproliferative activity in a broad spectrum of cancer types of human, mouse, rat, and monkey.[35] The RRM2 sequence shows complete target site homology in mouse, rat, and monkey, and this greatly assisted in the translation of this siRNA into the clinic. The RRM2 gene product is an established cancer target, and reviews on the history of this antitumor target are available.[36] A pilot safety study in nonhuman primates has been published.[37] This study was the first to show that multidosing of siRNA could be safely accomplished in a nonhuman primate. No complement activation and liver toxicities were observed at any dose level. When administered at 3 and 9 mg siRNA/kg, the nanoparticles were well tolerated. However, at 27 mg siRNA/kg, elevated levels of blood urea nitrogen and creatinine were observed, indicating mild kidney toxicity (was reversible). Additionally, at this dose level, IL-6 levels were increased. Multiple administrations spanning a time frame of 17−18 days (dosing on days 1, 4, and 7 followed by dosing on day 19 for assessing antibody formulation and possible effects on PK) allowed for assessment of anti-nanoparticle antibodies. Low titer, nonclearing (no changes in PK) antibodies to the human Tf on the nanoparticles were obtained. The antibodies were not against the PEGylated nanoparticles. This is important, as clearing antibody generation in humans has been observed with PEGylated liposomes. In total, the multiple, systemic doses of the targeted nanoparticles containing the nonchemically modified siRNA were safely administered to nonhuman primates. Antitumor efficacy has been observed in mouse models with doses of 2.5−5.0 mg siRNA/kg mouse.[30,31,33,38] Using body surface area normalizations, these dose levels

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translate to 0.6−1.2 mg siRNA/kg monkey. Thus, the predicted therapeutic window is quite large based on these data.

CALAA-01 was used to treat the first patient in a phase I clinical trial in May of 2008. The trial is a safety study treating adults with solid tumors who are refractory to standard-of-care therapies. CALAA-01 is administered via a 30 min iv infusion on days 1, 3, 8, and 10 of a 21-day cycle. Further details of the trial can be found at www.clinicaltrials.gov. As of this writing (end of 2008), dose escalations have occurred and the trial is proceeding.

CALAA-01 is the first targeted delivery of siRNA in humans. Comparisons to other nontargeted and targeted experimental and approved therapeutics can be found elsewhere.[39,40] The treatment of patients with CALAA-01 ushers in a new era of targeted experimental therapeutics. Other formulated siRNA therapeutics are sure to follow CALAA-01, and it will be interesting to monitor the progress of these experimental treatments over upcoming years.

Beyond CALAA-01 with CDP-Targeted Delivery of siRNA

In addition to CALAA-01, Calando Pharmaceuticals has announced another siRNA product denoted CALAA-02.[38] CALAA-02 uses the same delivery system as CALAA-01, but the siRNA now targets HIF-2alpha (another cancer target). Hotchkiss and his colleagues working with Heidel at Calando Pharmaceuticals have used the CDP delivery system to coadminister siRNA against Bim and PUMA in a mouse model of sepsis.[41] Effective administration of the antiapoptotic siRNA to lymphocytes reversed the immune cell depletion normally observed in sepsis.

My group at Caltech continues to work with the CDP-based delivery system. Our current efforts involve the development of antibody fragment (Fab and $F(ab')_2$) targeting agents.[42] Fab-containing nanoparticles (Fab-PEG-AD) can be formulated and show multivalency when binding to cell surfaces. In vivo work in mouse models is proceeding at this time.

Conflict of Interest Disclosure

M.E. Davis has been a consultant to and has financial interest in Calando Pharmaceuticals.

Acknowledgment. I thank all my coauthors who are listed on the references provided. I especially thank Dr. Jeremy Heidel who provided the leadership in bringing CALAA-01 from the benchtop to the bedside. Lastly, I thank the patients who willingly participated in the clinical trial.

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