Expert Opinion

ICS-283: a system for targeted intravenous delivery of siRNA

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ICS-283 was developed within Intradigm Corporation as a system that is designed for the systemic delivery of therapeutic small interfering (siRNA) to sites of pathological angiogenesis. The non-viral siRNA delivery system is based on synthetic nanoparticles, known as TargeTran™ (Intradigm Corporation), which functions as a broad-platform technology to deliver siRNA to specific target cells in diseased tissues. The system is constructed to incorporate different functionalities that address critical needs for successful nucleic acid delivery. The TargeTran synthetic vector is a self-assembling, layered nanoparticle that protects and targets siRNA to specific cell types in pathological tissues. At present, ICS-283 is the only antiangiogenic siRNA delivery system that is designed for intravenous administration to treat angiogenesis-driven diseases.

Keywords: angiogenesis, cationic polymers, DNA, small interfering RNA, sterically stabilised nanoparticles, targeted drug delivery

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1. Therapeutic nucleic acids

Nucleic acids are interesting drug candidates as they can alter cellular phenotypes and thereby combat disease. Regarding the nature of the therapeutic nucleic acids, three classes can be distinguished: aptamers, genes and oligonucleotides.

Aptamers are different from other therapeutic nucleic acids due to the fact that they act by a high-affinity binding to the target molecule, leading to a direct effect on its biological function. Aptamers can bind these molecules with this high affinity and high selectivity by forming complex secondary and tertiary structures based on intricate folding via base-pair interactions. They are usually selected and identified by a process of repeated cycles of binding, isolation and mutation known as systematic evolution of ligands by exponential enrichment (SELEX) [1]. In contrast to both other classes of therapeutic nucleic acids, aptamers generally act extracellularly and their function is dependent on their three-dimensional structure [2,3].

The introduction of genes can be used to correct faulty genes or to gain a new functionality, whereas the delivery of oligonucleotides (such as antisense oligonucleotides, triplex-forming antigene oligonucleotides, small interfering RNA [siRNA] and ribozymes) may be used to create phenotypes where the expression, and thereby function, of a specific gene is lost [4,5].

siRNA seems to have some specific advantages over other oligonucleotide-based approaches due to the fact that gene silencing by double-stranded RNA molecules is a naturally occurring pathway to regulate gene expression, which is likely to be responsible for the high potency, specificity and improved cellular handling. In addition, siRNA is remarkably stable in biological milieus, making it less dependent on chemical modifications in order to be active.

There have also been *in vitro* reports where oligonucleotides can function in gene repair; however, these experiments are at a very early stage of development and the therapeutic prospects are unclear [6,7]. Both genes and oligonucleotides have an intracellular site of action. Genes should be transcribed in the nucleus, whereas the oligonucleotide, depending on transcriptional or post-transcriptional inhibition, should

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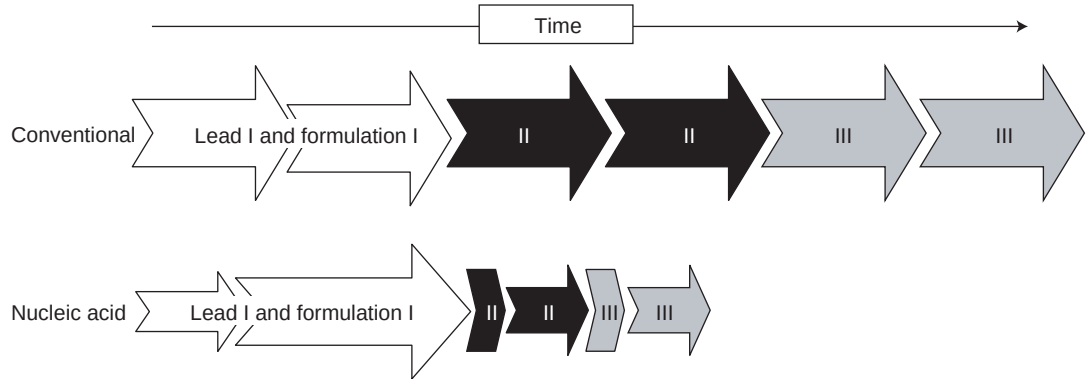

Figure 1. Advantages of therapeutic nucleic acids in drug development. Conventional lead-compound identification and formulation is a time-consuming process that classically requires massive screening for compounds with the desired activity, followed by tailoring of the formulation to the specific physicochemical characteristics of the lead compound. For nucleic acids, lead-compound selection is based on gene sequence and can usually be performed *in silico*, whereas the most essential physicochemical properties of the nucleic acids are already known or can be predicted. As a result, after the first successful lead selection and formulation development process (I), subsequent nucleic acid therapeutics (II/III) require less time.

act in the nucleus or cytoplasm, respectively. Irrespective of the objective being gain-of-function or loss-of-function, the correct localisation of the nucleic acids at their intracellular site of activity is required.

This is a problematic issue as nucleic acids are relatively large molecules, with molecular weights varying between ~ 5,000 Da (5 kDa) for a 15-nucleotides long (15-mer) antisense and almost 2 MDa for 5000 base pairs (5 kb) long plasmids, which is substantially larger than small molecular weight drugs. In addition, they bear a negative charge for each phosphate group in their backbone, strongly reducing their ability to pass over cellular membranes and increasing their hydrodynamic radius. As a result, passive transport of therapeutic nucleic acids over the target cell membrane(s) is poor. As such, the difficulties in intracellular delivery of nucleic acids are the most important obstacle to their pharmaceutical application.

Despite these difficult delivery issues, nucleic acids have characteristics that certainly warrant their development towards application. One of the most attractive properties in this respect is that the application in different diseases would, in principle, only require a change of sequence, whilst at the same time the basic physicochemical properties of the molecules remain the same. Thus, although the formulation of nucleic acids for intracellular delivery may be difficult to accomplish, the same formulation principles can be applied again for subsequent drug development programmes. In this manner, nucleic acid therapeutics allow straightforward lead-compound identification based on sequence selection and, after successful formulation of the first nucleic acid drug, it can be expected that subsequent formulations will easily follow as the molecular properties are alike, substantially reducing the time needed for drug development. This is in sharp contrast to classical drug development where lead compound identification requires massive high-throughput screening efforts and the formulation of a new compound essentially requires a new process to be started (Figure 1) [8-10].

An additional advantage of therapeutic nucleic acid treatment is that, whereas many therapeutic agents can only modulate the function of a subset of proteins, known as 'druggable' targets [11] (a list of these can be found at [101]), this limitation does not apply to nucleic acid therapy.

Taken together, to be able to take advantage of the interesting pharmaceutical properties of nucleic acid drugs, the development of a nucleic acid formulation that is able to realise intracellular entrance is crucial. Over the past two decades, many efforts have been directed towards solving this issue. Three approaches can be distinguished: the use of viral nucleic acid delivery systems [12,13], the use of non-viral nucleic acid delivery systems [14,15] and, for oligonucleotides only, the use of chemically modified nucleic acids [16,17].

Apart from intracellular entry, the development of a successful therapeutic nucleic acid requires several other aspects to be addressed to improve the overall efficiency of the delivery process. An optimal delivery system would, in addition to intracellular entry, protect against the degradative action of nucleases; prevent rapid clearance and elimination by non-target tissues; enable access to the target tissue; induce specific uptake by the target cell population; and promote correct intracellular trafficking to the site of action. Of course, the system needs to be safe and should preferably be cheap to manufacture in large quantities.

2. Nucleic acid delivery

For viral nucleic acid delivery systems, the efficiency of the process after arriving in the target cell is usually not the limiting factor, nor is nuclease protection an important issue as these properties have evolved during millions of years of evolution to

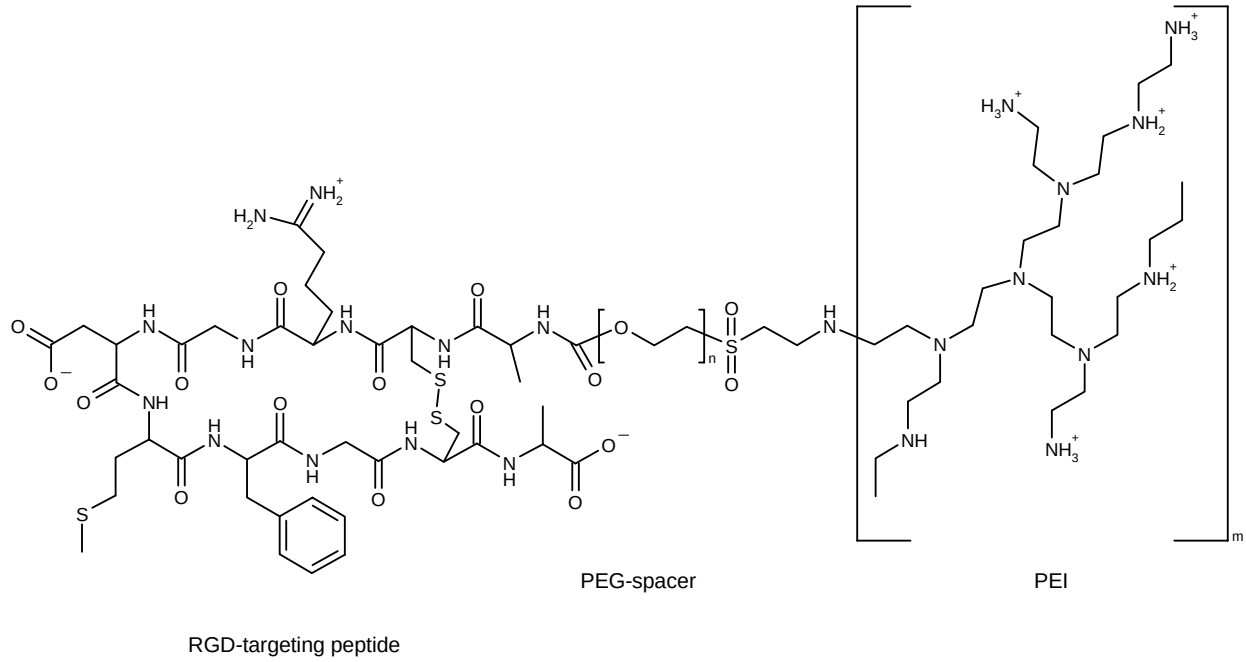

Figure 2. Chemical structure of the TargeTran™ prototype. The construct is designed with three functional regions that are needed to obtain a targeted self-assembling complex with the therapeutic nucleic acid payload. Branched PEI with a molecular weight of 25 kDa (corresponding to 581 monomer units) is used as the cationic complexing polymer and contains on average 25% primary, 50% secondary and 25% tertiary amines. Of the amines, ~ 7% are modified with PEG with an average molecular weight of 3.4 kDa (corresponding to 80 monomer units). The PEG is conjugated to a cyclic peptide containing an RGD-motif.
PEG: Poly(ethylene glycol); PEI: Polyethylenimine; RGD: Arginine, glycine and aspartic acid.

a high degree of sophistication [18-21]. However, viruses usually lack selectivity for the desired target cell type and are rapidly eliminated by the cells of the mononuclear phagocyte system. Furthermore, inflammatory reactions, immunogenicity and oncogenic transformations are important safety considerations for viral vectors that need to be addressed. In addition, their production in quantities that are large enough for clinical use is not a trivial hurdle.

For the chemical modification approach, the attractiveness lies in the avoidance of additional molecules or structures that are needed to enable intracellular delivery [22-24]. Thereby, the entire system would consist of a single compound, which simplifies the drug product. Nevertheless, the chemical modification approach is impossible for plasmids and, for oligonucleotides, it seems to be difficult to achieve the many functionalities that are needed for functional delivery within a single molecule by multiple modifications. The most common backbone modifications, as in the phosphorothioates, increases nuclease resistance but does not improve target cell uptake. For this, other modifications are needed. Usually, extensive modifications lead to the loss of activity, and rapid elimination and distribution to non-target tissues seem difficult to overcome. Finally, it is unclear whether the modified nucleotides after degradation can be incorporated in normal metabolism and what effect this has.

In theory, the non-viral nucleic acid delivery approach offers several advantages, yet one of the biggest hurdles seems to be the efficiency of intracellular delivery of the nucleic acids [25-28]. Nevertheless, rational choices on target cell type and class of nucleic acids has resulted in strong therapeutic effects of non-viral nucleic acid delivery systems in preclinical disease models, as has been reviewed recently [27,29,30]. The focus of this technological overview will be the most developed non-viral delivery systems for therapeutic siRNA delivery: ICS-283. Its carrier system, known as TargeTran™ (Intradigm Corporation), can be used to deliver a variety of nucleic acids and has been developed to overcome most of the limitations that have been identified for the viral and chemical modification approach.

3. TargeTran™

One of the approaches within the non-viral nucleic acid delivery system has been the development of TargeTran, the carrier system in the ICS-283 siRNA delivery technology. By using a modular conjugation strategy, different compounds with different functionalities are connected to create a construct with discrete regions with specific roles. Taken together, the TargeTran chemical structure aims to address all of the delivery needs that are encountered in therapeutic nucleic acid delivery. The chemical structure of the first prototype that has resulted

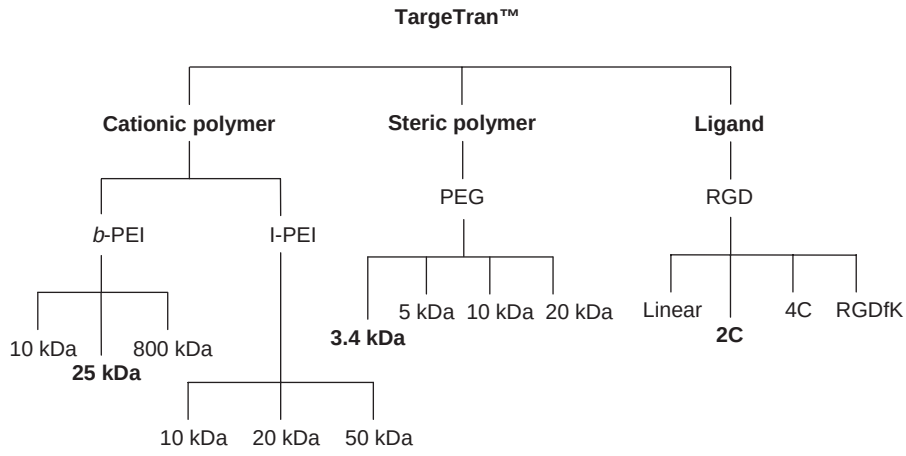

Figure 3. Flexibility in the design of a targeted non-viral nucleic acid carrier system based on the building blocks PEI, PEG and RGD. The choices for the building blocks are primarily based on molecular weight. In addition, for PEI, a branched and a linear version is available with markedly different properties, whereas for the targeting ligand, a linear version has been described as well as a variety of cyclic peptides based on one (2C) or two (4C) disulfide bridges, or a head-to-tail closed version (RGDfK). The blocks that are used in TargeTran™ are indicated in bold.

PEG: Poly(ethylene glycol); PEI: Polyethylenimine; RGD: Arginine, glycine and aspartic acid.

in effective nucleic acid delivery to pathological tissues is shown in Figure 2.

The three major building blocks are the cationic polymer polyethylenimine (PEI), the sterically stabilising polymer PEG and the targeting peptide RGD. Based on these building blocks, an array of different constructs can be synthesised based on the choices that are available for each block (Figure 3). Interestingly, when desired, there are many options to tailor the polymer for novel functionalities by exchanging one or more of the blocks by other blocks with new characteristics. The system as discussed here is designed to carry siRNA to sites of angiogenesis, but in principal the cationic PEI can complex any nucleic acid and the targeting ligand can be adapted to address other target cell types.

Apart from the choices that are available regarding the individual blocks that build the construct, the coupling chemistry and ratio of individual building blocks are important aspects to achieve effective siRNA delivery.

In the prototype, a choice was made for 25 kDa *b*-PEI, 3.5 kDa bifunctional PEG bearing a *N*-hydroxysuccinimide ester for coupling the targeting peptide and a vinylsulfone group for coupling to the PEI, and RGD-2C in a molar ratio of 1:40:40.

3.1 Polyethylenimine

The 25-kD *b*-PEI polymer was chosen for its well-established performance record as a transfection agent, low price and ample availability [31,32]. PEI is known to alter the intravenous fate of nucleic acids that in their free form are rapidly cleared by scavenger receptor-mediated uptake by liver cells and/or by renal excretion. The cationic charges interact electrostatically with the negative charges of the nucleic acids, leading to complex formation. The charge ratio of positive charges (PEI) to negative charges (nucleic acid) that are present during complex formation determines the particle characteristics, such as surface charge and size. When plotting charge ratio against particle size, the mixture has a characteristic peak in particle size around the point where charge neutralisation occurs (charge ratio 1:1). The aggregates form as a result of the attractive forces between particles, which are not sufficiently repelled by the electrostatic forces. At higher or lower charge ratios, particles in the submicron range are formed that do not have the tendency to aggregate by virtue of their net positive or negative surface charge, respectively (Figure 4).

In addition, once taken up by the target cell, PEI is reported to mediate endosomal escape through a 'proton sponge' mechanism, although a debate on the relevance of this mechanism is ongoing [33,34].

3.2 Poly(ethylene glycol)

Certain hydrophilic polymers can bind to or be conjugated to particles and sterically stabilise the surface against interactions with components in the surroundings. PEG is one of the most popular polymers used for this purpose [34-36]. The outstanding steric protection provided by PEG relates to its molecular properties, such as low interfacial energy, conformation, hydrophilicity and high flexibility. In the TargeTran system, 3.4-kD PEG chains are conjugated to PEI. Two main factors govern the steric stabilisation capacity of PEG: molecular weight and density. Longer PEG-chain lengths are more effective in protecting the particle (surface) from aggregation, opsonisation and in reducing the surface charge. But it seems that beyond a molecular weight of 2 kDa, a further increase of the PEG-chain length does not confer additional stabilisation

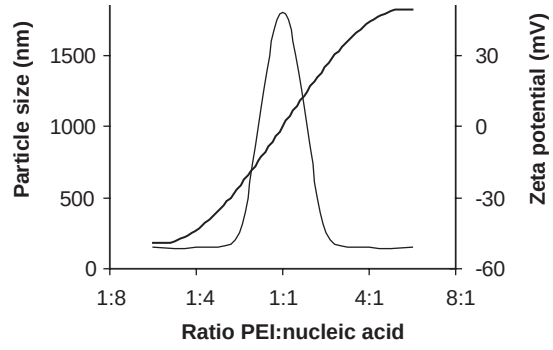

Figure 4. Relationship between charge ratio and resulting particle size (bell-shaped curve) and zeta potential (sigmoidal curve) of the self-assembling polyplexes. The particle size curve shows a characteristic peak around the point where charge neutralisation occurs. The aggregates form as a result of the attractive forces between particles. At higher or lower charge ratios, with corresponding higher or lower surface charge (the zeta potential), electrostatic repulsion ensures colloidal stability.
PEI: Polyethylenimine.

above a density of 3 – 4 mol% (Figure 5). As a result of the steric stabilisation effect, aggregate formation is repressed and colloidal stability can be achieved over a wide range of PEI:nucleic acid ratios. As the PEG coating moves the hydrodynamic plane of shear from the charged particle surface to the edge of the PEG coating, the zeta potential in physiological ionic strength becomes neutral at 2 – 3 nm from the particle surface, as the Debye length in this milieu is only ~ 0.8 nm. Estimates for the thickness of a PEG coating, for PEG with a molecular weight of 2 kDa, range from 3 – 5 nm. As a result of this effective shielding of the surface charge, opsonisation by biomolecules *in vivo* is reduced, as well as charge-mediated cell interactions. In fact, the nanoparticles are effectively rendered more inert, by exposing a neutral surface of flexible hydrophilic polymers that oppose aspecific interactions. To induce uptake by target cells, targeting ligands are coupled to the PEG terminal ends.

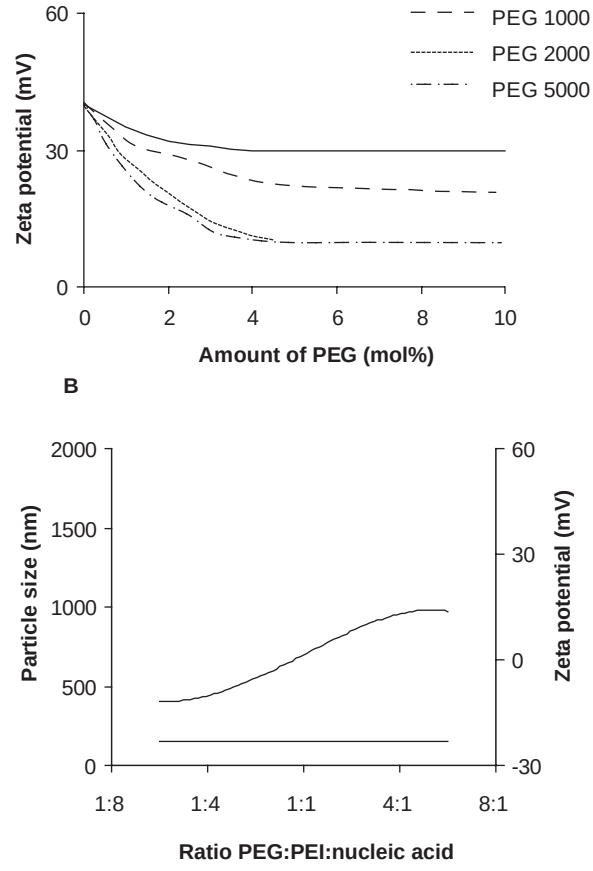

Figure 5. A. Reduction of the zeta potential by PEG-mediated shielding. Higher mol% and higher molecular weight PEG are more effective in shielding the surface charge. Beyond a molecular weight of 2 kDa, however, no additional shielding effect is observed. **B.** Relationship between ratio of PEG-PEI to nucleic acid and resulting particle size (straight line) and zeta potential (curve) of the self-assembling polyplexes. PEG-PEI results in submicron particle size over a broad range of polymer:nucleic acid ratios and zeta potential increases only slowly with increasing amount of cationic polymer.
PEG: Poly(ethylene glycol); PEI: Polyethylenimine.

3.3 RGD peptide

The RGD sequence is a strong recognition signal that is present in natural proteins and peptides that can be employed to obtain preferential binding to either $\alpha_v\beta_3$ integrins (as well as other α_v-integrins) or to other integrin types [37-39]. One of the best investigated non-α_v-integrin is, in this respect, the $\alpha_{IIb}\beta_3$ integrin, which plays an important role in platelet aggregation. The interest in targeting the $\alpha_v\beta_3$ integrins comes from their overexpression on the surface of activated endothelial cells during angiogenesis. RGD peptide ligands offer a way to target activated endothelial cells, one of the crucial cell types in angiogenesis.

The amino acid residues flanking the RGD motif determine the differential affinity and specificity of RGD-containing ligands for each of the various integrin receptors [37,38]. These amino acids may be directly involved in binding to the integrins, but they can also be involved in the conformational changes that affect the spatial orientation of the RGD region. One of the most common modifications that is achieved by the flanking residues to improve the binding properties of RGD peptides is cyclisation. Most importantly, the cyclic structure is more rigid, allowing fixation of the configuration in a preferred conformation for interaction with the target site. Secondly, linear RGD peptides are susceptible to degradation, as a result of the reaction of the aspartic acid residue (D) with the peptide backbone [40]. This reaction is prevented by cyclisation to rigidify the peptide structure. Next to cyclisation and choosing appropriate flanking amino acids to optimise affinity and specificity of the RGD peptide, non-natural D-amino acids and peptidomimetic structures can be incorporated that enhance stability and further improve conformation even more.

Taken together the RGD-PEG-PEI structure aims at achieving:

- PEI: complexation for siRNA binding, nuclease protection, reduction of renal excretion and promoting endosomal escape;
- PEG: promoting colloidal stability, reduction of surface charge and reduction of aspecific cell uptake;
- RGD: targeting activated endothelial cells during angiogenesis.

TargeTran's payload is siRNA, which is chosen because siRNA seems to be more effective in inhibiting gene translation than other oligonucleotide-based approaches. Furthermore, siRNA acts in the cytoplasm, which avoids the need for delivery over the nuclear membrane to achieve activity [41,42].

4. Angiogenesis

The ability to target activated endothelial cells using RGD ligands offers interesting prospects for modifying pathological angiogenesis that occurs during tumour growth and inflammation [43-45]. As the endothelial cells play a pivotal role in the angiogenic process, this cell type is an interesting target cell type for antiangiogenesis-based therapeutic strategies. It is also a cell type that is perfectly accessible to intravenously administered drug delivery systems. Tumour angiogenesis is the result of the ability of tumour cells to stimulate capillary formation to support tumour growth. The primary driving force for tumour angiogenesis is the demand for oxygen and nutrients by the proliferating cancer cells. Folkman and colleagues suggested that once the size of the tumour extends beyond the effective diffusion limit for oxygen, hypoxic conditions occur in the centre of the cell mass [46]. An angiogenic switch then takes place in favour of new blood vessel growth [47]. Normal quiescent blood vessels are primarily composed of two different cell types: endothelial and mural cells. The coverage of the endothelial tubule by mural cells is thought to be important in the maintenance of the quiescent state. For new blood vessel formation to occur, the pericyte coating of the pre-existing tubule must first dissociate. Then the surrounding extracellular matrix is degraded, followed by extravascular fibrin deposition. The endothelial cells are then able to respond to pro-angiogenic signals with proliferation, migration and new tubule formation. After that, remodelling occurs to prune the vessels to fit the needs of the tumour tissue.

Coordinated regulation of the formation of pro- and anti-angiogenic factors is necessary at each stage to ensure the development of a normal, functional vessel. Presumed advantages of a targeted antiangiogenesis-based treatment strategy (as compared with targeted chemotherapy directed towards tumour cells) include broad applicability (all tumour types require neovasculature), amplification of the therapeutic effects (disrupting one blood vessel affects the entire tumour region that is or would be supplied), and limited development of resistance (endothelial cells are genetically stable, unlike tumour cells).

Angiogenesis is also a prominent feature of inflamed tissues, such as in the case of rheumatoid synovitis and several ocular diseases. In these diseases, the new blood vessels are unable to deliver enough oxygen to the inflammatory cell mass, creating a hypoxic environment at the site of inflammation, which in turn activates gene programmes that are associated with angiogenesis, glycolysis and adaptation to pH. These repetitive cycles of hypoxia and reoxygenation together with oxidants that are produced by phagocytic cells promote chronic oxidative stress within the microenvironment at the site of inflammation, leading to the generation of reactive oxygen species that contribute to tissue damage and stimulate the angiogenic process [48].

To devise a successful siRNA-mediated therapeutic strategy, knowledge of the expression profile of angiogenic factors/receptors by the endothelial cells is vitally important. This profile constantly changes as endothelial cells sequentially become activated, dissolve the basal membrane, proliferate, migrate and finally mature into functional blood vessels by remodelling, tube formation and vascular stabilisation. Each phase is associated with expression of specific proteins. Thus, angiogenic endothelial cells are a highly heterogeneous cell population, whose gene expression profile is determined by the phase of angiogenesis that they are in. Consequently, the siRNA should be adapted to the specific mRNA expressed in that particular phase, and targeted to the cells that are going through this specific phase of angiogenesis. The overexpression of the α_v-integrins occurs relatively early during the angiogenic process, the time point when vascular endothelial growth factor is one of the main factors driving the angiogenic process [49-51].

5. Preclinical results

The TargeTran carrier with unmodified chemically synthesised siRNA payload has been tested in *in vitro* and *in vivo* systems to evaluate its cell interaction profile and ability to silence genes [52,53].

On mixing purified TargeTran with an aqueous siRNA solution (in a 2:1, positive to negative charge ratio), the cationic PEI binds to negatively charged siRNA driving self-assembly to form a layered complex with a core consisting of siRNA-PEI, and an outer layer enriched in steric polymer with exposed ligand moieties. The characteristics of the nucleic acids that are used for complexation (oligonucleotides versus plasmids; oligonucleotide sequence; double strandedness versus single strandedness) did not affect the self-assembly process. Figure 6 shows the self-assembly of nanoparticles between nucleic acids and PEI, PEG-conjugated-PEI and TargeTran.

Self-assembly of all polymer (constructs) (PEI, PEG-conjugated PEI or TargeTran) with siRNA resulted in the formation of nanoparticles of ~ 100 nm. PEI nanoparticles, however, bear a strong positive surface charge (35 ± 4 mV, mean ± standard deviation, n = 3). This resulted in promiscuous non-selective cell binding *in vitro*. Functional siRNA transfection *in vitro*

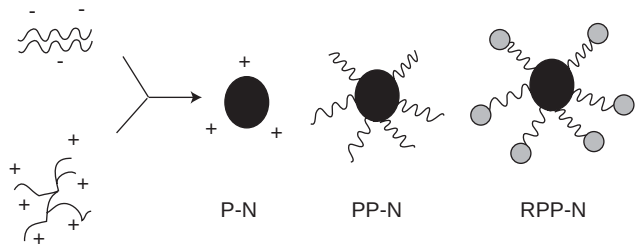

Figure 6. A schematic representation of self-assembly of siRNA and cationic PEI into P-N; PEG-PEI into PP-N; and RGD-PEG-PEI into RPP-N, respectively. The electrostatic interactions between siRNA and PEI drive the formation of the core of the particle. The core is surrounded by a corona enriched in the shielding PEG polymer to the distal end of which targeting ligands can be coupled.

PEG: Poly(ethylene glycol); PEI: Polyethylenimine; P-N: Polyethylenimine nanoparticle; PP-N: Poly(ethylene glycol)-polyethylenimine nanoparticle; RGD: Arginine, glycine and aspartic acid; RPP-N: TargeTran™ nanoparticle; siRNA: Small interefering RNA.

was highly efficient as the silencing of β-galactosidase in SVR-bag4 endothelial cells (constitutively expressing the reporter enzyme) was nearly complete.

Next to the nonspecificity of PEI nanoparticles, another important drawback was the observation that the strong surface charge resulted in aggregate formation following intravenous injection in mice leading to side effects, featured by periods of inactivity and piloerection.

Introducing a protective PEG layer resulted in a low cell interaction of PEG-conjugated-PEI nanoparticles as compared with PEI nanoparticles, which is related to the reduced surface charge (5 ± 4 mV). The near absence of cell interaction obviously decreased silencing efficiencies *in vitro*. However, intravenous injection did not result in aggregate formation nor any other observable side effects in mice.

Coupling of RGD peptide to PEG-PEI was shown to increase cell interaction and transfection efficiency *in vitro* to comparable levels as observed for PEI nanoparticles, whilst maintaining the near neutral surface charge (6 ± 1 mV). Importantly, cell interaction was mediated by the RGD peptide, as endothelial cell binding in the presence of a 100-fold excess of free peptide reduced cell interaction > 90%, which was not something observed for PEI nanoparticles. Importantly, adverse effects following intravenous injection seemed to be absent.

Silencing studies were performed in N2A-tumour bearing mice. In these studies, siRNA-targeting murine VEGFR (mVEGFR)-2 was used, as VEGFR-2 is a pivotal factor in tumour angiogenesis but requires delivery to tumour endothelium to be able to inhibit tumour growth. Efficacy studies with mVEGFR-2 siRNA that was intravenously administered every 3 days (carried by TargeTran), resulted in a strong inhibition of the tumour growth rate, which was siRNA-sequence specific, as control siRNAs failed to inhibit tumour growth. This result indicates that TargeTran-delivered siRNA acts through an angiogenic endothelial cell-specific uptake mechanism.

Reduced tumour growth rate was paralleled by a reduction in blood-vessel density in the tumour region. In addition, the few blood vessels that are visible in the tumour that was treated with mVEGFR-2 siRNA exhibited evidence of erratic branching, supporting further antiangiogenic activity of the delivered siRNA-targeting mVEGFR-2. Taken together, these results suggest that the tumour inhibition by siRNA-targeting mVEGFR-2-TargeTran occurs as a result of effective delivery of the siRNA into tumour vasculature, producing a sequence-specific inhibition of VEGFR-2-expression, leading to the inhibition of tumour angiogenesis and consequently the inhibition of tumour growth. These findings in the N2A-tumour model have been confirmed in other tumour models of human cancer, DLD-1 colon carcinoma and 786-O renal carcinoma (PV Scaria and MC Woodle, unpublished observations).

Inhibition of angiogenesis was also demonstrated in animal models of pathological angiogenesis in the eye. In one model mimicking herpetic keratitis, angiogenesis was induced by inserting biologically active C poly G nucleotides (CpG)-DNA into corneal micropockets, a system that models the mechanism by which herpes simplex virus induces neovascularisation and, ultimately, blindness. Results demonstrate that systemically administered siRNA transported with TargeTran inhibited neovascularisation in the eye. Although the use of siRNA-targeting mVEGFR-2 induced strong inhibitory effects on its own, the antiangiogenic effects were substantially improved when two other siRNA (targeting mVEGF and mVEGFR1) were added to the TargeTran complex in a 1:1:1 molar ratio, respectively. This is an important advantage as TargeTran can enforce identical tissue distribution profiles for different siRNAs by combining them in a single particle; enabling targeting multiple genes to treat complex multifactorial diseases with a cocktail of siRNAs. Inhibition of the neovascularisation in the eye was paralleled by a reduction of VEGF protein levels in the infected cornea. Similar results were obtained in a model of murine ocular neovascularisation induced by hypoxia.

Importantly, siRNA delivered by TargeTran failed to induce an increase of IFN-α levels in the circulation following intravenous administration, indicative of nonspecific immune stimulation by double-stranded RNA.

For siRNA-mediated inhibition of angiogenesis, a number of competing technologies are being developed (Table 1).

6. Expert opinion

siRNA has several advantages compared with other nucleic acid approaches: it is based on a powerful endogenous gene-silencing process, the functional mediators (siRNA molecules) are relatively small and act in the cytosol and can, in principle, be used to silence expression of any gene, independent of the function or location of the gene product. Inhibition of angiogenesis has been the most popular target for therapeutic development of siRNA. Many of the most important pro-angiogenic growth

Table 1. Competing antiangiogenic siRNA delivery technologies. Company development programmes focusing on inhibition of angiogenesis using siRNA*.

Company	Disease	Approach	Stage
Acuity Pharmaceuticals	Age-related macular degeneration	Local treatment: unmodified siRNA against VEGF (Cand5)	Phase II
Alnylam (in collaboration with Merck)	Age-related macular degeneration	Local treatment: chemically modified siRNA	Pre-clinical
Intradigm Corporation	Cancer	Systemic treatment: targeted nanoparticles (ICS-283)	Pre-clinical
Sirna Therapeutics (in collaboration with Allergan)	Age-related macular degeneration	Local treatment: chemically modified siRNA against VEGFR1 (siRNA-027)	Phase I

*Information gathered from publicly available company internet sites ([102-105] per January 1st, 2006).
siRNA: Small interfering RNA; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

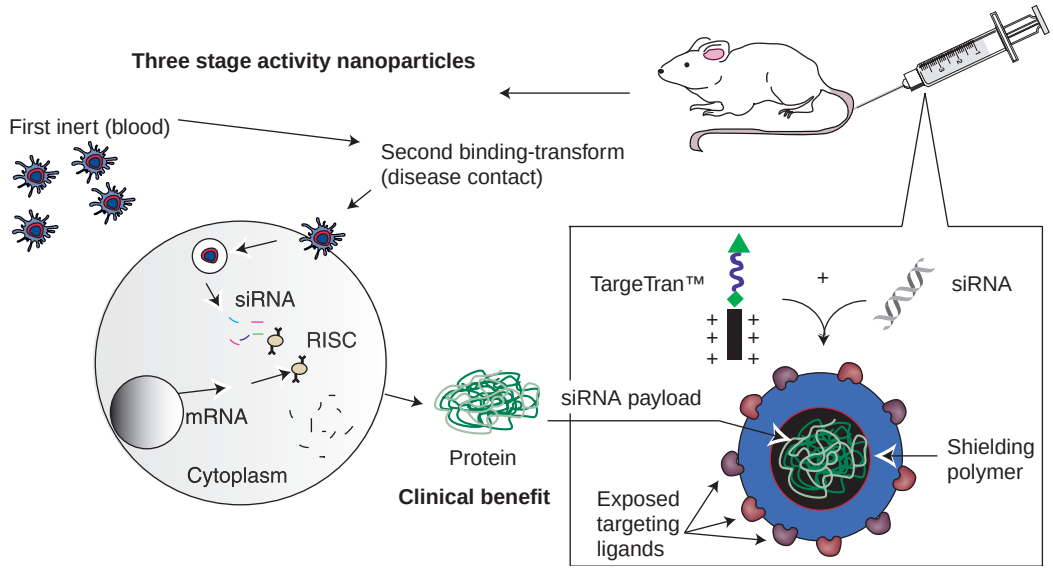

Figure 7. TargeTran™ nanoparticle-mediated siRNA delivery to target cells in pathological tissue adding an extra level of specificity on top of the siRNA-sequence specificity.
RISC: RNA-induced silencing complex; siRNA: Small interefering RNA.

factors (most notably VEGF) and their receptors driving the angiogenic process have been identified over the past few years, allowing rapid lead compound selection. In addition, as angiogenesis proceeds when the balance between pro- and antiangiogenic mediators is distorted, siRNA-induced silencing of pro-angiogenic molecules that are produced in the tumour may shift the balance in favour of angiogenesis inhibition. A beneficial aspect of this approach is that it is sufficient to transfect only a part of the target cell population to shift the balance in favour of angiogenesis inhibition. One of the most important aspects that needs strong research attention to exploit the full potential of siRNA is the *in vivo* delivery to the target cells.

Practically all of the antiangiogenic siRNA delivery technologies are based on local injection of siRNA in the eye. In many therapeutic situations, local treatment is not feasible. The development of ICS-283 based on systemic delivery of siRNA to activated endothelial cells in the form of targeted nanoparticles offers a broad platform technology to cover angiogenesis-driven diseases with an additional level of specificity induced by the targeting ligand (Figure 7). Preclinical models demonstrate that the absolute amounts of siRNA delivered to the angiogenic endothelium are sufficient to induce therapeutic effects, without apparent toxicity. How these observations translate to the clinical situation where doses will be lower and adverse effects are more readily identified are important questions that need to be addressed. Although the system becomes more complex by adding a carrier system to the siRNA, the new system offers siRNA protection, increased target cell uptake and cytosolic localisation, coupled to reduced non-target tissue uptake. Thereby, these added benefits outweigh the increased complexity of the system for applications where local injection is not feasible.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. GOLD L: The SELEX process: a surprising source of therapeutic and diagnostic compounds. *Harvey Lect.* (1995) 91:47-57.
2. NIMJEE SM, RUSCONI CP, SULLENGER BA: Aptamers: an emerging class of therapeutics. *Ann. Rev. Med.* (2005) 56:555-583.
3. YAN AC, BELL KM, BREEDEN MM, ELLINGTON AD: Aptamers: prospects in therapeutics and biomedicine. *Front. Biosci.* (2005) 10:1802-1827.
4. NAKATA Y, KIM TK, SHETZLINE S, GEWIRTZ AM: Nucleic acid modulation of gene expression: approaches for nucleic acid therapeutics against cancer. *Crit. Rev. Eukaryot. Gene Expr.* (2005) 15(2):163-182.
5. LU PY, XIE F, WOODLE MC: *In vivo* application of RNA interference: from functional genomics to therapeutics. *Adv. Genet.* (2005) 54:117-142.
6. YIN WX, WU XS, LIU G *et al.*: Targeted correction of a chromosomal point mutation by modified single-stranded oligonucleotides in a GFP recovery system. *Biochem. Biophys. Res. Commun.* (2005) 334(4):1032-1041.
7. HU Y, PAREKH-OLMEDO H, DRURY M, SKOGEN M, KMIEC EB: Reaction parameters of targeted gene repair in mammalian cells. *Mol. Biotechnol.* (2005) 29(3):197-210.
8. POSNER BA: High-throughput screening-driven lead discovery: meeting the challenges of finding new therapeutics. *Curr. Opin. Drug Discov. Devel.* (2005) 8(4):487-494.
9. OPREA TI, BOLOGA CG, EDWARDS BS, PROSSNITZ ER, SKLAR LA: Post-high-throughput screening analysis: an empirical compound prioritization scheme. *J. Biomol. Screen.* (2005) 10(5):419-426.
10. HANN MM, OPREA TI: Pursuing the leadlikeness concept in pharmaceutical research. *Curr. Opin. Chem. Biol.* (2004) 8(3):255-263.
11. ORTH AP, BATALOV S, PERRONE M, CHANDA SK: The promise of genomics to identify novel therapeutic targets. *Expert Opin. Ther. Targets* (2004) 8(6):587-596.
12. LUNDSTROM K: Latest development in viral vectors for gene therapy. *Trends Biotechnol.* (2003) 21(3):117-122.
13. SINN PL, SAUTER SL, MCCRAY PB Jr: Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors-design, biosafety, and production. *Gene Ther.* (2005) 12(14):1089-1098.
14. DEMENEIX B, HASSANI Z, BEHR JP: Towards multifunctional synthetic vectors. *Curr. Gene Ther.* (2004) 4(4):445-455.
• An excellent overview on the strategies for designing non-viral vectors.
15. RAVI KUMAR M, HELLERMANN G, LOCKEY RF, MOHAPATRA SS: Nanoparticle-mediated gene delivery: state of the art. *Expert Opin. Biol. Ther.* (2004) 4(8):1213-1224.
16. KURRECK J: Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.* (2003) 270(8):1628-1644.
17. MANOHARAN M: RNA interference and chemically modified small interfering RNAs. *Curr. Opin. Chem. Biol.* (2004) 8(6):570-579.
• A good outline of the state-of-the-art of chemical modifications of nucleic acids.
18. ROMANO G: Current development of adeno-associated viral vectors. *Drug News Perspect.* (2005) 18(5):311-316.
19. KO D, HAWKINS L, YU DC: Development of transcriptionally regulated oncolytic adenoviruses. *Oncogene* (2005) 24(52):7763-7774.
20. RALPH GS, BINLEY K, WONG LF, AZZOUZ M, MAZARAKIS ND: Gene therapy for neurodegenerative and ocular diseases using lentiviral vectors. *Clin. Sci. (Lond.)* (2006) 110(1):37-46.
21. NEVE RL, NEVE KA, NESTLER EJ, CARLEZON WA Jr: Use of herpes virus amplicon vectors to study brain disorders. *Biotechniques* (2005) 39(3):381-391.
22. VERMA S, JAGER S, THUM O, FAMULOK M: Functional tuning of nucleic acids by chemical modifications: tailored oligonucleotides as drugs, devices, and diagnostics. *Chem. Rec.* (2003) 3(1):51-60.
23. HENRY SP, GEARY RS, YU R, LEVIN AA: Drug properties of second-generation antisense oligonucleotides: how do they measure up to their predecessors? *Curr. Opin. Investig. Drugs* (2001) 2(10):1444-1449.
24. CRINELLI R, BIANCHI M, GENTILINI L, PALMA L, MAGNANI M: Locked nucleic acids (LNA): versatile tools for designing oligonucleotide decoys with high stability and affinity. *Curr. Drug Targets* (2004) 5(8):745-752.
25. DUFES C, UCHEGBU IF, SCHATZLEIN AG: Dendrimers in gene delivery. *Adv. Drug Deliv. Rev.* (2005) 57(15):2177-2202.
26. SIMONSON OE, SVAHN MG, TORNQUIST E, LUNDIN KE, SMITH CI: Bioplex technology: novel synthetic gene delivery pharmaceutical based on peptides anchored to nucleic acids. *Curr. Pharm. Des.* (2005) 11(28):3671-3680.
27. DE LAPORTE L, CRUZ REA J, SHEA LD: Design of modular non-viral gene therapy vectors. *Biomaterials* (2006) 27(7):947-954.
28. MAO HQ, LEONG KW: Design of polyphosphoester-DNA nanoparticles for non-viral gene delivery. *Adv. Genet.* (2005) 53:275-306.
29. LUNGWITZ U, BREUNIG M, BLUNK T, GOPFERICH A: Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* (2005) 60(2):247-266.
30. NEU M, FISCHER D, KISSEL T: Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J. Gene Med.* (2005) 7(8):992-1009.
31. AKINC A, THOMAS M, KLIBANOV AM, LANGER R: Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* (2005) 7(5):657-663.
32. GROSSE S, ARON Y, THEVENOT G *et al.*: Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations. *J. Gene Med.* (2005) 7(10):1275-1286.
33. REJMAN J, BRAGONZI A, CONESE M: Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol. Ther.* (2005) 12(3):468-474.
34. YOWELL SL, BLACKWELL S: Novel effects with polyethylene glycol modified pharmaceuticals. *Cancer Treat. Rev.* (2002) 28(Suppl. A):3-6.

35. MEHVAR R: Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation. *J. Pharm. Pharm. Sci.* (2000) 3(1):125-136.

36. LEE M, KIM SW: Polyethylene glycol-conjugated copolymers for plasmid DNA delivery. *Pharm. Res.* (2005) 22(1):1-10.

37. TEMMING K, SCHIFFELERS RM, MOLEMA G, KOK RJ: RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. *Drug Resist. Updat.* (2005) 8(6):381-402.

38. TAKAGI J: Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins. *Biochem. Soc. Trans.* (2004) 32(Pt 3):403-406.

39. HORTON MA: Arg-Gly-Asp (RGD) peptides and peptidomimetics as therapeutics: relevance for renal diseases. *Exp. Nephrol.* (1999) 7(2):178-184.

40. BOGDANOWICH-KNIPP SJ, CHAKRABARTI S, WILLIAMS TD, DILLMAN RK, SIAHAAN TJ: Solution stability of linear versus cyclic RGD peptides. *J. Pept. Res.* (1999) 53(5):530-541.

41. MIYAGISHI M, HAYASHI M, TAIRA K: Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* (2003) 13(1):1-7.

42. BERTRAND JR, POTTIER M, VEKRIS A *et al.*: Comparison of antisense oligonucleotides and siRNAs in cell culture and *in vivo*. *Biochem. Biophys. Res. Commun.* (2002) 296(4):1000-1004.

• A concise overview of the importance of angiogenesis in tumour progression.

43. HERBST RS, LORUSSO P, ISOBE T, HURWITZ HI: Angiogenesis pathway inhibitors. *Cancer Chemother. Biol. Response Modif.* (2005) 22:225-245.

44. DVORAK HF: Angiogenesis: update 2005. *J. Thromb. Haemost.* (2005) 3(8):1835-1842.

45. SZEKANECZ Z, KOCH AE: Endothelial cells in inflammation and angiogenesis. *Curr. Drug Targets Inflamm. Allergy* (2005) 4(3):319-323.

46. FOLKMAN J: Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol.* (2002) 29(6 Suppl. 16):15-18.

47. BERGERS G, BENJAMIN LE: Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* (2003) 3(6):401-410.

48. SAUER H, WARTENBERG M: Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. *Antioxid. Redox Signal.* (2005) 7(11-12):1423-1434.

49. ROSEN LS: VEGF-targeted therapy: therapeutic potential and recent advances. *Oncologist* (2005) 10(6):382-391.

50. FERRARA N: VEGF as a therapeutic target in cancer. *Oncology* (2005) 69(Suppl. 3):11-16.

51. CARMELIET P: VEGF as a key mediator of angiogenesis in cancer. *Oncology* (2005) 69(Suppl. 3):4-10.

52. KIM B, TANG Q, BISWAS PS *et al.*: Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. *Am. J. Pathol.* (2004) 165(6):2177-2185.

53. SCHIFFELERS RM, ANSARI A, XU J *et al.*: Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* (2004) 32(19):e149.

Websites

101. http://function.gnf.org/druggable/index.html Genomics Institute of the Novartis Research Foundation website.

102. http://www.acuitypharma.com Acuity Pharmaceuticals website.

103. http://www.alnylam.com Alnylam Pharmaceuticals website.

104. http://www.intradigm.com Intradigm Corporation website.

105. http://www.sirna.com Sirna Therapeutics website.

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