Design of siRNA Therapeutics from the Molecular Scale

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Abstract: While protein-based therapeutics is well-established in the market, development of nucleic acid therapeutics has lagged. Short interfering RNAs (siRNAs) represent an exciting new direction for the pharmaceutical industry. These small, chemically synthesized RNAs can knock down the expression of target genes through the use of a native eukaryotic pathway called RNA interference (RNAi). Though siRNAs are routinely used in research studies of eukaryotic biological processes, transitioning the technology to the clinic has proven challenging. Early efforts to design an siRNA therapeutic have demonstrated the difficulties in generating a highly-active siRNA with good specificity and a delivery vehicle that can protect the siRNA as it is transported to a specific tissue. In this review article, we discuss design considerations for siRNA therapeutics, identifying criteria for choosing therapeutic targets, producing highly-active siRNA sequences, and designing an optimized delivery vehicle. Taken together, these design considerations provide logical guidelines for generating novel siRNA therapeutics.

Keywords: siRNA therapeutic; RNAi; liver cancer; siRNA design; delivery vehicle design

1. Introduction

The “big data” that is available and being generated on biological systems has greatly expanded the number of potential targets for treating disease. This is especially true for sequencing data yielding targets for proposed genetic therapies. As one such proposed therapy, siRNAs offer unique advantages
that make them an ideal platform for therapeutic development. Perhaps the strongest argument in support of RNAi-based therapeutics is the ability to design the active agent, the siRNA, and its delivery vehicle simultaneously and mostly independent of each other for the knockdown of specific targets in specific tissues (Figure 1). Additionally, unlike protein-based biologics, little process development would be required for scale-up of siRNA-based therapeutics, as the synthesis and purification protocols for nucleic acids are well established.

Effective silencing and the desired therapeutic effect depend upon the targeted delivery of an siRNA that is both specific and active against the intended target. Furthermore, the choice of an appropriate target largely determines the efficacy of an siRNA-based therapeutic. We therefore discuss the design of siRNA therapeutics in three broad categories: i.) selection of a disease and target mRNA, ii.) design of a highly-active siRNA, and iii.) design of the siRNA delivery vehicle. This review will discuss these aspects of siRNA therapeutic design and where relevant, use liver cancer to demonstrate key/fundamental design characteristics.

Figure 1. siRNA design overview. siRNA therapeutic design should, in the short term, begin by addressing diseases with unmet therapeutic need that are treatable by siRNA-based therapeutics. While initial siRNA and delivery vehicle design begins with some design heuristics, as discussed in the following sections, a completely optimized therapeutic will require concomitant, parallel development of each of the constituents followed by experimental validation of the optimized therapeutic.
2. Choosing a Target for siRNA Mediated Silencing

Selecting a disease to target with any new therapeutic modality begins by choosing a disease for which current therapeutics do not work satisfactorily, thereby avoiding direct competition of the new therapeutic with existing, successful drugs. As the majority of drugs accumulate in the liver, diseases of the liver provide relatively easier targets for early-stage development of any therapeutic. With that in mind, we have chosen advanced liver cancer, specifically hepatocellular carcinoma (HCC), as an example for development of our hypothetical siRNA-based therapeutic. Liver cancer is the 6th most common cancer and the third leading cause of cancer related deaths, with ~750,000 new cases diagnosed annually worldwide [1]. Moreover, it has one of the highest rates of mortality, with only a 14% 5-year survival rate in the United States [2]. The high mortality rate highlights two needs, for earlier diagnosis of the disease and for improved treatments of advanced-stage liver cancer, the most common of which is hepatocellular carcinoma (HCC) [2]. The only approved treatment for advanced stage HCC is Sorafenib, a tyrosine kinase inhibitor having anti-angiogenic activity. Unfortunately, use of Sorafenib only increases median survival rate from 7.9 to 10.7 months over the placebo group [3]. While a number of clinical trials are currently underway using tyrosine kinase inhibitors, monoclonal antibodies, and one siRNA based treatment [4–7], continued development of therapeutics of all kinds for HCC is warranted.

For any disease, multiple proteins are potentially feasible drug targets. In order to select a particular protein target to knock down, it is useful to evaluate, if available, kinetic information about the transcriptional and translational regulation of the target under normal and diseased conditions. Typical siRNA-mediated knockdown only reduces protein levels to ~20% of baseline, and this effect is transient, lasting from a few hours to a couple of weeks before protein levels recover [8,9]. Thus, an siRNA therapeutic should only target mRNAs where this incomplete, transient knockdown is sufficient to achieve a therapeutic effect.

To achieve a maximal therapeutic effect, it is also important to select a target where: (i) the protein half-life is shorter than that of the siRNA [8,10], (ii) the mRNA transcription rate is slower than the rate of siRNA-complex turnover [11], (iii) the concentration of mRNA is sufficiently low to permit silencing at minimal siRNA concentrations [12–14], and (iv) no feedback mechanism will upregulate transcription in response to the knockdown. Satisfying these conditions is possible through both, selecting appropriate target genes and engineering of siRNAs for maximal activity and stability. Engineering of the siRNA will be discussed in the next section.

In the development of an actual therapeutic, we would determine all of the necessary kinetic parameters experimentally. We would then select the target with the shortest protein half-life and confirm that its mRNA half-life is not extraordinarily short. Additional experiments that might be required would be to determine the basal levels of the mRNA targets (molecules/cell), as low expression level targets are more amenable to siRNA-mediated silencing. Using HCC as our example system, we examined five targets that have been previously identified and verified as HCC drug targets (Table 1). We were able to locate published values for protein and some mRNA half-lives [10,15–20]. VEGFR was found to have the shortest protein half-life, 70 min, and the known transcript half-lives were all between ~2–9 h [20], a small variability relative to the protein half-lives. Based on protein half-life alone, we concluded that VEGFR would be the most amenable HCC target for the development of an siRNA therapeutic.
### Table 1. Overview of some HCC targets.

| Target       | Gene Function          | Current Clinical Drugs Targeting                                                                 | Protein Half-life | References |
|--------------|------------------------|---------------------------------------------------------------------------------------------------|-------------------|------------|
| VEGFR/VEGFR2 | Angiogenesis           | Sorafenib, Brivanib, Sunitinib, Cediranib, BIBF1120, Pazopanib, Regorafenib, E7080, TSU-68, Vandetanib, Ramucirumab, IMC-1121B | 70 min            | [5,15,21]  |
| EGFR         | Signal Transduction    | Erlotinib, Cetuximab, Gefitinib, Lapatinib, Vandetanib                                            | 10 h              | [5,16,21]  |
| Bcr-ABL      | Cell Proliferation     | Destanib                                                                                            | 40 h              | [5,10,17,21] |
| MEK          | Signal Transduction    | AZD6244                                                                                             | 6 h               | [5,18,21]  |
| PDGFR        | Angiogenesis/Signal    | Sorafenib, Brivanib, Linifanib, BIBF1120, Pazopanib, TSU-68                                         | 3 h               | [5,19,21]  |

### 3. siRNA Design Considerations

siRNA mediated silencing possesses three characteristics that are desirable for a therapeutic modality: (i) it operates post-transcriptionally, (ii) the specificity of knockdown is high, and (iii) the same target mRNA can be inhibited by many different siRNA sequences. As siRNA sequences can have widely varying activities, significant effort has been spent identifying factors that enhance siRNA activity and reduce off-targeting and non-specific effects [22,23]. These features can be split coarsely into two categories, those related to siRNA activity and those related to siRNA specificity. siRNA activity is influenced by, among other factors, strand selection, the structure of the mRNA target region, base preferences, and overall siRNA G/C content. In comparison, siRNA specificity depends on strand selection, immunogenicity, and uniqueness of the target sequence. Designing an effective siRNA depends upon proper weighting of each of these factors; siRNA selection algorithms typically weight these factors based upon analyses of siRNA activity data [24]. As it stands, the rules for selecting active siRNAs continue to evolve, and it behooves the researcher to design siRNAs with features that are most strongly predictive of high activity. This section will discuss siRNA design considerations that are substantiated by biochemical evidence.

#### 3.1. Details of the RNAi Mechanism

A canonical siRNA is a 21 nt duplex with 19 internal base pairs, 5’ phosphates, and dinucleotide 3’ overhangs [25–30]. When exogenous siRNAs enter the cytoplasm, they are first recognized by a protein complex called the RISC Loading complex (RLC), which is responsible for properly orienting the siRNA duplex as it hands off the guide strand to the active RNA Induced Silencing Complex (RISC) [30–35]. The RLC is a ribonucleoprotein complex minimally made up of Argonaute 2 (Ago2), Dicer, and TAR RNA Binding Protein (TRBP) [30,33,36–38]. The RLC orients the siRNA in the complex, and, in doing so, selects, sometimes incorrectly, one strand to serve as the guide strand that will target RISC to the mRNA [33,39–42]. The passenger strand (i.e., the strand not selected by the RLC) is concomitantly cleaved and removed [33,43–46]. The active RISC then binds to its target mRNA by Watson-Crick base pairing. In a similar fashion to how the passenger strand is removed, RISC, specifically the Ago2 RNaseIII, cleaves the target at the center of the region complementary to
the siRNA, resulting in degradation of the mRNA [28,42,47]. RISC is a multiple turnover enzyme that can then target other complementary mRNAs [42,46–49]. Additional details of the RNAi mechanism and siRNA-mediated silencing can be found in other sources [50,51].

3.2. Differential Terminal Hybridization Stability

For siRNAs to silence the intended target, they must be oriented by the RLC to ensure incorporation of the intended guide strand into the active RISC [30,33,37,38,40,46]. Incorporation of the unintended strand (i.e., the intended passenger strand) leads to the formation of a RISC that cannot cleave the intended target (reducing the activity of the siRNA therapeutic) and that can cause off-target effects (reducing the specificity of the siRNA) [39,40,52]. The difference in the activity of one strand relative to the other is termed functional asymmetry [40]. Functional asymmetry is a function of multiple factors, including RISC stability, RISC turnover rate, and, most directly, biased incorporation of one siRNA strand into RISC [33,48,53].

Biased strand incorporation occurs due to the recognition of differences in the termini of the siRNAs by the RLC proteins [33,37–40,54,55]. Based on early studies of functional asymmetry and biased strand loading [40], it was concluded that differential terminal hybridization stability, the difference in hybridization free energy between the two ends of the siRNA, was predictive of functional asymmetry due to biased strand loading [33,40]. These studies determined that the strand whose 5’ end is less stably hybridized (higher hybridization free energy) to the complementary strand is preferentially loaded into RISC [33,37,38]. Initially, differential hybridization stabilities were calculated using the four terminal base pairs at each end of the siRNA [40,56]. More recent work suggests that using only one nearest neighbor provides a more predictive calculation [54,57]. Nonetheless, when selecting candidate siRNAs, it is recommended to reject siRNAs with an unfavorable differential stability. The remaining candidates can then be pared down further using the sequence characteristics and other design criteria described below.

3.3. 5’ Nucleotide Preference

A number of positional base preferences, within the siRNA, are shown to be correlated to siRNA activity [22,58–68]. Most common among these are preferences affecting nucleotides at or near the 5’-termini of the siRNA strands [22,54,58,60,62,69]. To date, there is incomplete consensus among datasets as to what the most predictive base preferences are, nor is there strong biochemical evidence explaining the reason for specific base preferences. The exception is the two 5’ terminal nucleotides [22,37,54,58,59,61,62,65,66], wherein Ago2 was identified as having a nucleotide specificity loop that shows significantly higher affinity interactions with U and A bases and lower affinity interactions with C and G bases [53]. By classifying the pool of siRNA strands according to their 5’-terminal nucleotides, the pool of candidates can be further limited to those sequences with the nucleotides that give the greatest likelihood of high activity [37,54].
3.4. mRNA Target Region

mRNA secondary structure precludes siRNA binding through steric hindrance of RISC binding and cleavage [70,71]. Conveniently, mRNA secondary structure can be easily, and relatively accurately, predicted in silico, using the nearest neighbor approach to predict the most thermodynamically stable structures [57,61,72–75]. If more certainty about the structure is desired/required, experimental structural analyses can be used to refine the predicted structures [71,76–81]. That said, the actual mRNA structure in a living cell is dynamic and any approach will provide only incomplete information that can guide choices of potential target regions. Using the available structural information, it is recommended, given a choice, to target regions of greater accessibility, especially at the 5’ and 3’ ends of the target region [75].

3.5. Immunogenicity

siRNAs can cause a number of immunogenic and cytotoxic responses, some of which arise due to their dsRNA structure and others that are sequence specific [82–85]. Immune receptors for siRNAs reside on the surface of the cell, within endocytotic vesicles, and within the cytosol [83]. While it is possible to prevent interaction of the siRNA with cell surface and endosomal receptors by protecting/inhibiting accessibility to the siRNA with a delivery vehicle (see section 4.1 below for additional details), it is still worthwhile to design siRNAs that avoid the use of immunostimulatory sequence motifs recognized by these receptors.

In general, cytosolic receptors for RNA are uniform in their expression across all cell types and recognize mainly structural features as opposed to sequence motifs [83]. Among these receptors are OAS1, PKR, and RIG-I [83,86–91]. The canonical siRNA structure does not activate PKR or RIG-I; these receptors are more sensitive to dsRNA longer than 30 bp or having 5'-triphosphates [87,89,92]. Unlike PKR and RIG-I, OAS1 is also activated by a sequence motif, NNWW(N9)WGN, in dsRNA as short as 19bp [86].

Cell surface and endosomal receptors for RNA, mainly the toll-like receptors (TLRs) [83,93], recognize specific sequence motifs, and their expression varies by cell type [83,94]. TLR3 (cell surface and endosomal) is a receptor for dsRNA [95,96]. TLRs 7 and 8 (endosomal) are receptors for ssRNA and are primarily responsible for recognition of specific RNA sequences, termed pathogen-associated molecular patterns (PAMPs) [94,96–105]. The recognition of siRNAs by TLR3 remains an area of investigation, as its activation was shown in clinical trials using naked siRNAs [103]. However, TLR3 in vitro was not activated by the canonical naked siRNA [94]. The recognition of siRNAs by TLRs 7 and 8 requires endocytosis of the siRNA followed by duplex melting within the acidic vesicle exposing the ssRNA bases to the TLR receptors, initiating an immune response [102,106]. When designing an siRNA, the immunostimulatory motifs to avoid include: GUCCUUCAA [107], UGUGU [98], UGU [98], UCA [108], GU-rich sequences (Heil 2004), AU-rich sequences [105], and U-rich sequences [102]. In addition to the immunostimulatory sequences, the UGGC motif has been shown to be cytotoxic via non-immune mechanisms [109].
3.6. Non-Specific Effects

Specificity of an siRNA begins with the selection of a sequence that is unique in the transcriptome of the target cells. However, sequences should also be chosen with consideration given to the possibility of off-target effects resulting from partial complementarity and miRNA-like targeting [84]. Repetitive sequences should also be avoided, in particular, the GGGG motif because it forms a G-quartet secondary structure [110].

miRNA-like targeting occurs when siRNAs have seed region complementarity with the 3’ UTR of an mRNA, resulting in translational repression of the untargeted transcript [111–117]. Moreover, mRNAs that are regulated by miRNAs are also more susceptible to miRNA-like off-targeting due to extended 3’ UTRs and the preexistence of miRNA target sites [118]. Avoiding miRNA-like targeting effects is complicated by an inability to accurately predict seed sequences [116,118,119]. miRNA-like targeting is influenced by the surrounding sequence of the target, the position of the target in the mRNA, and the repetitiveness of the target sequence [114,116,120,121]. As such the best way to account for miRNA-like targeting is to avoid seed sequences that have already been identified (http://www.mirbase.org) [122], so as not to unnecessarily limit the sequence space by avoiding false positives. A number of other tools exist that can predict off-targeting and may prove useful in determining the overall likelihood an siRNA will have strong off-target effects [123,124].

Methods to manipulate the siRNA to avoid off-target effects are not well established. However, there are a number of algorithms that do take off-targeting into account beyond a simple BLAST search [64,123,125]. Nonetheless, off-target effects are particularly challenging to avoid for a number of reasons. First, mismatches are tolerated within the duplex, however where mismatches are tolerated is only partially known (Figure 2) [126–128]. Second, predicting miRNA-like targeting is inaccurate and prediction of seed region interactions often leads to overestimation of off-targeting [118]. Lastly, siRNAs can cause translational repression, post-transcriptional silencing, or P-body based degradation and/or repression [24,129–135], making the cause of the off-target effects difficult to interpret. Still, as with immunogenicity, the only way to fully ensure that off-target effects do not occur is by post-hoc analysis using techniques such as RNA microarrays or parallel sequencing to quantify the levels of all untargeted transcripts [136–138] and antibody microarrays and mass spectrometry to check for off-target translational repression [139,140].

Genetic variation should also be considered, particularly single nucleotide polymorphisms (SNPs). Target regions with as little as one nucleotide difference can change target knockdown efficiency significantly [128,141]. Thus, in the short term, it makes sense to avoid targeting regions where SNPs occur to ensure the broad utility of any therapeutic. Going forward, the existence of inexpensive sequencing and synthesis technologies may allow the design of patient-specific siRNA therapeutics, accounting for each patient’s unique genotype.

From our perspective, the problem of avoiding non-specific effects reverts, largely, to the problem of designing an siRNA with the greatest specific activity. The most active siRNAs can be used at the lowest possible concentrations, decreasing off-target effects while still achieving a therapeutic effect [14]. Pooling siRNAs against a single target has been suggested as a means to improve specificity of silencing [142]. This effect is principally attributed to the reduced concentration of any given siRNA in the pool. However, we expect that design of the most active and specific RNA using guidelines such as
those presented here will maximize the therapeutic window beyond what is possible by pooling. Thus, we would consider accounting for the factors described here as being of lesser importance relative to those that are predictive/indicative of the highest specific activity of a sequence.

**Figure 2.** Tolerance for mismatches between an siRNA and its target. siRNA seed region (bases 2–8) [112,143] and splice site (bases 10–11) [127,132,144] are the least tolerant of mismatches because of their active role in silencing. The 1st nucleotide [53,145], the nucleotides of the 3’overhang (bases 20–21) [146,147], and bases 17–19 [144,148] are the most tolerant of mismatches, because their ability to base pair is at least partially blocked by Ago2. Position and base specific mismatches are tolerated at positions 8–16 [141].

3.7. Other siRNA Design Criteria

Other factors shown to influence siRNA activity include the G/C content (i.e., the overall duplex stability) of the siRNA [22,39,149], the secondary structure of the guide strand [150,151], internal repeats [22], palindromic sequences [152], and positional base preferences along the siRNA [22,58–68,153]. More recently, additional structural criteria, even to the level of tertiary structure [154], have been identified as valuable in predicting siRNA activity. While each of these factors may be important in siRNA design, either their overall influence is thought to be minimal compared to the other selection criteria or there is a lack of consensus on how to implement the feature as a selection criterion. As such, selection of siRNAs using the mentioned criteria should be done as a second order set of rules to distinguish only the most active and specific siRNAs.
3.8. Non-Canonical siRNA Structural Designs

Using non-canonical siRNA structures in large part changes the point of entry into the RNAi pathway or changes the way in which the RNAi proteins interact with the non-canonical siRNA [155]. From the bottom up, ssRNAs are capable of being loaded by Ago2, albeit inefficiently, to form an active RISC in vitro [47,144,156,157] and in vivo when chemically modified [158,159]. By only providing one siRNA strand to enter RISC, proper strand selection is no longer a design characteristic. Other structures are designed to bias strand incorporation by altering the length of the passenger strand (asymmetric interfering RNAs (aiRNAs) and asymmetric short-duplex siRNAs (asiRNAs) [160–162]), the length of the 3’ overhang (fork-siRNAs (fsiRNAs) [163]), or by assembling a duplex with a segmented passenger strand (small internally segmented interfering RNAs (sisiRNAs) [164]). A more recently tested structure utilizes a “bulge” at the second position of the guide strand, creating a perturbation at the first base involved in the seed region [165]; the use of this modification to the canonical siRNA structure was shown to decrease off-targeting by miRNA-like activity. Other non-canonical structures exploit the ability for longer RNAs to enter RISC more efficiently; these structures are called Dicer substrate RNAs (DsiRNAs) [166–169].

3.9. Incorporation of Chemical Modifications

Chemical modifications in siRNAs can increase their stability (specifically with regards to nuclease degradation), minimize immunogenicity, and, to the extent possible, improve the activity of the siRNA [85,102,107,170–175]. Chemical modifications of the siRNA focus on changing the phosphodiester backbone, ribose sugar, nucleotide base, and 2’-OH ribose group. Effective use of chemical modifications requires the substitution of the new chemical moiety at a position within the siRNA where the additional group or structural alteration does not inhibit normal siRNA function. In general, the rationale behind chemical modifications is to incorporate small perturbations in the siRNA structure to prevent recognition and/or binding of the siRNA by nucleases and the immune receptors for RNA. The most common modifications include altering the 2’-OH group to a 2’-O-methyl or 2’-F to prevent recognition of the RNA by nucleases and TLR7 and TLR8 [85,173,176–181]. Unfortunately, no rules exist defining which chemical modifications are most useful and how they are best applied.

The 3’ overhangs are also a common location for chemical modifications for two reasons: (i) they may provide a site of attack for endoribonucleases and (ii) chemical modifications, even bulky ones, are typically well tolerated at these positions [182]. Phosphorothioate and phosphorodithioate modifications to the backbone of the siRNA generate siRNAs with nuclease resistance, but the number and positions of modifications are important in retaining siRNA activity [176,183,184]. A more comprehensive understanding of the RNAi mechanism as well as the effects of various chemical modifications will aid in rational design of siRNAs with an even greater therapeutic index. A more extensive review discussing chemical modifications of siRNAs can be found elsewhere [185].

4. Delivery

siRNAs are hydrophilic, due to their anionic backbone, and do not readily diffuse across cellular membranes. Moreover, naked siRNAs are rapidly filtered from circulation, degraded, and can initiate
immune/inflammatory responses (as stated above) [7,83]. Thus, delivery vehicles must be used to protect/conceal the siRNA while facilitating its transport to the cytoplasm of the targeted cells. By varying characteristics such as size, charge, shape, chemistry, and the means of directing the vehicle-siRNA complexes to the target cells, well-designed delivery vehicles can markedly increase the therapeutic efficacy of a given siRNA [186]. There are many different types of delivery vehicles that have been developed for siRNA-based therapeutics, with lipoplexes and polyplexes currently being the most commonly applied, each possessing different physical and chemical characteristics, as well as offering distinct advantages and disadvantages (Figure 3). The following sections describe a design approach in which the desired properties of the delivery vehicle are selected based on the unique characteristics of the targeted disease. As an illustrative example, these guidelines are applied to the development of a hypothetical HCC therapeutic. The following sections describe these various vehicle types and outline approaches for the design of an siRNA delivery vehicle.

**Figure 3.** Types of non-viral delivery vehicles being utilized in developing siRNA therapeutics. For a more comprehensive review of each particle the following are suggested: Polyplexes [187–189], Lipoplexes [190–192], Carbon Nanotubes [184,193–195], Ceramic Nanoparticles [196–198], Magnetic Nanoparticles [199,200] and Metallic Nanoparticles [201–203]. Additional sources discussing the breadth of delivery vehicles that have been tested to date: [6,7,204–206].

### 4.1. Accessing the Cell Cytoplasm

The purpose of the delivery vehicle is to protect the siRNA and deliver it to the cytoplasm of the target cells where it can be recognized by the proteins of the RNAi pathway. To reach the cytoplasm, vehicle-siRNA complexes can, as is the case with many lipoplexes, cross the membrane directly [207] or
be internalized by endocytosis followed by escape from the vesicle, often by vesicle rupture [208–210]. Vehicle-siRNA complexes are formed in two ways: self-assembly by electrostatics (typical) and covalent attachment (rare). For self-assembled complexes, siRNAs attach on the surface or are fully encapsulated depending on the structure and flexibility of the vehicles. The fully formed complex reduces siRNA degradation by serum nucleases, recognition by toll-like receptors (TLRs), and other molecules of the innate immune system [83]. siRNAs that are not fully encapsulated are more likely to be recognized by serum nucleases and TLRs. While the affinity of the vehicle for the siRNA is critical for complex formation, the complex must dissociate upon cell entry to allow the siRNA to be bound by the pathway proteins [211].

The various endocytotic mechanisms take up species of particular sizes as follows: lipid rafts (40–50 nm), caveolae-mediated endocytosis (50–60 nm), clathrin-mediated endocytosis (<200 nm), macropinocytosis (500–10,000 nm), and phagocytosis (<10,000 nm) [212,213]. Current data suggests that delivery leading to siRNA silencing can occur through multiple uptake mechanisms depending on the vehicle type [186,214,215]. Additionally, all endocytotic pathways efficiently incorporate complexes with a net positive charge [216].

When taking these factors into account, the endocytotic pathways used most frequently by the diseased cells will dictate both the characteristics of the chosen delivery vehicle. For our hypothetical case of liver cancer, HCCs primarily use pinocytosis for the uptake of extracellular components. As such, our vehicle-siRNA complexes should have a maximum diameter of 10,000 nm and a net positive charge [217]. These design requirements do not eliminate any candidate vehicle types (Figure 3) from potentially being used in a therapeutic for the treatment of HCCs.

4.2. Routes of Systemic Delivery

Each tissue is connected to multiple transport systems (e.g., circulatory, lymphatic, etc.). Delivery to the intended target can be enhanced by pairing the characteristics of a therapeutic complex to the characteristics of the transport system that will deliver the complex to the target cells. [218,219]. The goal then becomes to maximize the residence time of the therapeutic complex in a given transport system before it is degraded or sequestered. For diseases in tissues that are difficult to access by transport systems, e.g., prostate, eye, neck, and brain, localized delivery is often more appropriate [205].

The circulatory system is the most common route for therapeutic delivery. In the circulatory system, particles are actively filtered based on size. Particles less than 10 nm in size are filtered by the kidneys within hours of injection, and particles larger than 200 nm are retained within the spleen [220,221]. Thus, to improve the bioavailability of the complexes, it is optimal to design complexes to between 50–200 nm [222].

Bioavailability can be further improved by avoiding recognition and clearance of the complexes by phagocytes. While particle size can be manipulated to avoid phagocytosis, the size range and how much size changes affect clearance differ among particle types [223]. Shape has been shown to have a more consistent effect, with shapes containing large tangential planes (e.g., rods) being phagocytosed more efficiently than particles of uniform diameter (e.g., spheres) [224–226]. Complexes of high zeta potential (>25 mV) are also phagocytosed more efficiently than complexes with potential below
15 mV [227]. PEGylation and the use of hydrophilic polymer coatings have also been shown to inhibit phagocytosis [228–230].

Based on the intent to reach HCCs in the liver as well as metastatic cells, the cardiovascular system provides the best system for delivery. Bioavailability within the system is thus optimized using spherically shaped complexes, 50–200 nm in diameter, with a 0 to 15 mV zeta potential, with PEGylation as needed to achieve the desired circulating half-life. These complex characteristics would reduce clearance from circulation, increasing their access to HCCs.

4.3. Delivery Specifically to Target Cells

Having designed the complex for maximal bioavailability, consideration must now be given to trafficking out of the transport system to the target cells. For instance, tumors, including HCC, are susceptible to enhanced permeability and retention (EPR) which results in an increased uptake and sequestration of particles 100–500 nm in size from the circulation (“passive” targeting) [231–233]. Designing the size of complexes to be 100–200 nm will result in enhanced accumulation in the vicinity of an HCC tumor, without compromising other design characteristics.

Delivery vehicles can also be modified with surface ligands (e.g., peptides, antibodies, or aptamers) that guide and aid uptake by the target cells (“active targeting”) [234]. While easily applied for complexes with encapsulated siRNA, complexes with surface attached siRNA will have reduced surface area for siRNA attachment with the addition of each ligand. Active targeting has proven effective in HCC models [235–237], but the strategies for functionalizing delivery vehicles with ligands are not well defined. In situations where targeting cannot be improved through molecular approaches, magnetic nanoparticles can be directed to a specific target region via external magnetic fields, though this can be logistically difficult [200].

4.4. Methods of Administration

Multiple approaches can be applied for local or systemic administration to the body. Current therapeutics favor localized delivery (intranasal, intraocular, intratumoral, etc.) for their specificity in reaching a target tissue [238], though this is difficult for relatively remote tissues where the disease may be systemic, as for metastatic HCC. In such cases, the circulatory system can be used to access cells at nearly any location in the body, with the possible exception of the brain due to the blood-brain barrier. For access to the circulatory system, transdermal and gastrointestinal absorption can be used, but intravenous (IV) injection is optimal for the immediate delivery of known concentrations of a therapeutic [239]. For IV therapeutics, complexes should be designed to be stable for long-term storage in a saline or intravascular compatible solution. While systemic delivery always has the potential to allow non-specific effects outside the target tissue, these effects can be mitigated through the inclusion of cell-specific targeting moieties that limit uptake by non-targeted cells while not preventing access to targeted cells at remote locations.
4.5. Selection of a Specific Delivery Vehicle for Targeting HCCs

Each of the variety of delivery vehicle types has unique characteristics that are advantageous for particular applications. However, some vehicle types may not perform as well as others in our case of targeting HCCs. Carbon nanotubes are readily phagocytosed \[195\]. Ceramic, magnetic, and metallic delivery vehicles have issues with long term accumulation and cytotoxicity \[198,201\]. Thus, the delivery vehicles most likely to be successful for this application are lipoplexes or polyplexes (Figure 4) \[207,235,239,240\].

Figure 4. Design characteristics of vehicle-siRNA complexes. A variety of characteristics can be manipulated to improve the bioavailability and biodistribution of vehicle-siRNA complexes while reducing their cytotoxicity and immunogenicity.

Further comparison of these two platforms can be based on available experimental data, in particular published results from clinical trials. One type of lipoplex, stable nucleic acid lipid particles (SNALPs), is currently being used in 6 of 10 clinical trials for systemic delivery of siRNAs and has achieved therapeutic efficacy at low doses \emph{in vivo} (ED\(_{50}\) = 0.3 mg/kg) \[6,7,190,191,241\]. Cytotoxicity and pulmonary inflammation related to SNALPs raise concerns for their therapeutic application and has been the result of clinical trials being terminated \[242,243\]. Polyplexes are currently being applied in one clinical trial for the systemic delivery of siRNA with therapeutic effects at ED\(_{50}\) = 2 mg/kg \emph{in vivo} and TD\(_{50}\) = 27 mg/kg \emph{in vivo} \[244–246\]. Recent studies have shown that biodegradable bonds
within the polymer have further reduced toxicity and improved polyplex residence time due to decreased recognition by phagocytes [187,247–249], even though, no toxicity was observed in clinical trials [244]. The choice between a lipoplex or polyplex platform reduces to the preference of increased delivery efficiency (lipoplexes) or reduced toxicity (polyplexes).

5. Conclusions

Current data permits mechanism-driven design of both siRNAs and their delivery vehicles, at least to some extent. Perhaps one of most exciting aspects of siRNA design is the ability to target so many different disease states once an effective delivery vehicle exists for a given tissue type, as exemplified by the SNALP and polyplex platforms currently in clinical trials. In the future, the “plug and play” nature of these combinations of siRNA and vehicle could potentially support the development of personalized therapeutics on a patient by patient basis. Still more comprehensive methods for siRNA sequence selection and modification will be driven by a more complete understanding of the RNAi mechanism. Continuing advances in vehicle chemistry and careful analysis of vehicle-siRNA complex structures will improve the specificity and efficiency of siRNA therapeutics.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Ferlay, J.; Shin, H.R.; Bray, F.; Forman, D.; Mathers, C.; Parkin, D.M. Globocan 2008 v2.0, cancer incidence and mortality worldwide: Iarc canerbase no. 10. Available online: http://globocan.iarc.fr/ (accessed on 26 November 2012).
2. American cancer society. Cancer facts & figures 2012. Available online: http://www.cancer.org/research/cancerfactsfigures/cancerfactsfigures/cancer-facts-figures-2012/ (accessed on 26 November 2012).
3. Llovet, J.M.; Ricci, S.; Mazzaferro, V.; Hilgard, P.; Gane, E.; Blanc, J.F.; de Oliveira, A.C.; Santoro, A.; Raoul, J.L.; Forner, A.; et al. Sorafenib in advanced hepatocellular carcinoma. *N. Engl. J. Med.* 2008, 359, 378–390.
4. Zhu, A.X.; Duda, D.G.; Sahani, D.V.; Jain, R.K. Hcc and angiogenesis: Possible targets and future directions. *Nat. Rev. Clin. Oncol.* 2011, 8, 292–301.
5. Villanueva, A.; Llovet, J.M. Targeted therapies for hepatocellular carcinoma. *Gastroenterology* 2011, 140, 1410–1426.
6. Burnett, J.C.; Rossi, J.J.; Tiemann, K. Current progress of sirna/shrna therapeutics in clinical trials. *Biotechnol. J.* **2011**, 6, 1130–1146.

7. Haussecker, D. The business of rnai therapeutics in 2012. *Mol. Ther. Nucleic Acids* **2012**, 1, e8.

8. Wei, J.; Jones, J.; Kang, J.; Card, A.; Krimm, M.; Hancock, P.; Pei, Y.; Ason, B.; Payson, E.; Dubinina, N.; *et al.* Rna-induced silencing complex-bound small interfering rna is a determinant of rna interference-mediated gene silencing in mice. *Mol. Pharmacol.* **2011**, 79, 953–963.

9. Bartlett, D.W. Insights into the kinetics of sirna-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* **2006**, 34, 322–333.

10. Spiller, D.G.; Giles, R.V.; Broughton, C.M.; Grzybowski, J.; Ruddell, C.J.; Tidd, D.M.; Clark, R.E. The influence of target protein half-life on the effectiveness of antisense oligonucleotide analog-mediated biologic responses. *Antisense Nucleic Acid Drug Dev.* **1998**, 8, 281–293.

11. Larsson, E.; Sander, C.; Marks, D. Mrna turnover rate limits sirna and microrna efficacy. *Mol. Syst. Biol.* **2010**, 6, 1–9.

12. Kennedy, S.; Wang, D.; Ruvkun, G. A conserved sirna-degrading rnase negatively regulates rna interference in c. Elegans. *Nature* **2004**, 427, 645–649.

13. Bian, Y.; Zhou, W.; Zhao, Y.; Li, X.; Eng, W.; Hao, R.; Yang, Q.; Huang, W. High-dose sirnas upregulate mouse eri-1 at both transcription and posttranscription levels. *PLoS ONE* **2011**, 6, e26466.

14. Caffrey, D.R.; Zhao, J.; Song, Z.; Schaffer, M.E.; Haney, S.A.; Subramanian, R.R.; Seymour, A.B.; Hughes, J.D. Sirna off-target effects can be reduced at concentrations that match their individual potency. *PLoS ONE* **2011**, 6, e21503.

15. Calera, M.R.; Venkatakrishnan, A.; Kazlauskas, A. Ve-cadherin increases the half-life of vegf receptor 2. *Exp. Cell Res.* **2004**, 300, 248–256.

16. Schlessinger, J. The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry* **1988**, 27, 3119–3123.

17. Dhut, S.; Chaplin, T.; Young, B.D. Bcr-abl and bcr proteins: Biochemical characterization and localization. *Leukemia* **1990**, 4, 745–750.

18. Wang, P.Y.; Rao, J.N.; Zou, T.; Liu, L.; Xiao, L.; Yu, T.X.; Turner, D.J.; Gorospe, M.; Wang, J.Y. Post-transcriptional regulation of mek-1 by polyamines through the rna-binding protein hur modulating intestinal epithelial apoptosis. *Biochem. J.* **2010**, 426, 293–306.

19. Keating, M.T.; Williams, L.T. Processing of the platelet-derived growth factor receptor. Biosynthetic and degradation studies using anti-receptor antibodies. *J. Biol. Chem.* **1987**, 262, 7932–7937.

20. Yang, E.; van Nimwegen, E.; Zavolan, M.; Rajewsky, N.; Schroeder, M.; Magnusco, M.; Darnell, J.E. Decay rates of human mrnas: Correlation with functional characteristics and sequence attributes. *Genome Res.* **2003**, 13, 1863–1872.

21. Llovet, J.M.; Bruix, J. Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* **2008**, 48, 1312–1327.

22. Reynolds, A.; Leake, D.; Boese, Q.; Scaringe, S.; Marshall, W.S.; Khvorova, A. Rational sirna design for rna interference. *Nat. Biotechnol.* **2004**, 22, 326–330.
23. Jackson, A.L.; Bartz, S.R.; Schelter, J.; Kobayashi, S.V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P.S. Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. 2003, 21, 635–637.

24. Naito, Y.; Ui-Tei, K. Sirna design software for a target gene-specific RNA interference. Front. Genet. 2012, 3, 102.

25. Bernstein, E.; Caudy, A.A.; Hammond, S.M.; Hannon, G.J. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001, 409, 363–366.

26. Zamore, P.D.; Tuschl, T.; Sharp, P.A.; Bartel, D.P. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000, 101, 25–33.

27. Nykänen, A.; Haley, B.; Zamore, P.D. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 2001, 107, 309–321.

28. Elbashir, S.M.; Martinez, J.; Patkaniowska, A.; Lendeckel, W.; Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. 2001, 20, 6877–6888.

29. Lima, W.F.; Murray, H.; Nichols, J.G.; Wu, H.; Sun, H.; Prakash, T.P.; Berdeja, A.R.; Gaus, H.J.; Crooke, S.T. Human Dicer binds short single-strand and double-strand RNA with high affinity and interacts with different regions of the nucleic acids. J. Biol. Chem. 2009, 284, 2535–2548.

30. Sakurai, K.; Amarzguioui, M.; Kim, D.; Alluin, J.; Heale, B.; Song, M.; Gatignol, A.; Behlke, M.A.; Rossi, J.J. A role for human Dicer in pre-RISC loading of siRNAs. Nucleic Acids Res. 2011, 39, 1510–1525.

31. Liu, Q.H.; Rand, T.A.; Kalidas, S.; Du, F.H.; Kim, H.E.; Smith, D.P.; Wang, X.D. R2d2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. Science 2003, 301, 1921–1925.

32. Tomari, Y.; Du, T.; Haley, B.; Schwarz, D.; Bennett, R.; Cook, H.; Koppetsch, B.; Theurkauf, W.; Zamore, P. RISC assembly defects in the Drosophila RNAi mutant armitage. Cell 2004, 116, 831–841.

33. Tomari, Y.; Matranga, C.; Haley, B.; Martinez, N.; Zamore, P.D. A protein sensor for siRNA asymmetry. Science 2004, 306, 1377–1380.

34. Chendrimada, T.; Gregory, R.; Kumaraswamy, E.; Norman, J.; Cooch, N.; Nishikura, K.; Shiekhattar, R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 2005, 436, 740–744.

35. Hammond, S.M.; Bernstein, E.; Beach, D.; Hannon, G.J. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 2000, 404, 293–296.

36. MacRae, I.J.; Ma, E.; Zhou, M.; Robinson, C.V.; Doudna, J.A. In vitro reconstitution of the human RISC-loading complex. Proc. Natl. Acad. Sci. USA 2008, 105, 512–517.

37. Gredell, J.A.; Dittmer, M.J.; Wu, M.; Chan, C.; Walton, S.P. Recognition of siRNA asymmetry by TAR RNA binding protein. Biochemistry 2010, 49, 3148–3155.

38. Noland, C.L.; Ma, E.; Doudna, J.A. Sirna repositioning for guide strand selection by human Dicer complexes. Mol. Cell 2011, 43, 110–121.

39. Khvorova, A.; Reynolds, A.; Jayasena, S.D. Functional siRNAs and miRNAs exhibit strand bias. Cell 2003, 115, 209–216.

40. Schwarz, D.; Hutvagner, G.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P. Asymmetry in the assembly of the RNAi enzyme complex. Cell 2003, 115, 199–208.
41. Hamilton, A.; Baulcombe, D. A species of small antisense rna in posttranscriptional gene silencing in plants. Science 1999, 286, 950–952.
42. Elbashir, S.M.; Lendeckel, W.; Tuschl, T. Rna interference is mediated by 21- and 22-nucleotide mas. Genes Dev. 2001, 15, 188–200.
43. Rand, T.A.; Petersen, S.; Du, F.; Wang, X. Argonaute2 cleaves the anti-guide strand of sirna during risc activation. Cell 2005, 123, 621–629.
44. Leuschner, P.J.F.; Ameres, S.L.; Kueng, S.; Martinez, J. Cleavage of the sirna passenger strand during risc assembly in human cells. EMBO Rep. 2006, 7, 314–320.
45. Matranga, C.; Tomari, Y.; Shin, C.; Bartel, D.; Zamore, P. Passenger-strand cleavage facilitates assembly of sirna into ago2-containing rna enzyme complexes. Cell 2005, 123, 607–620.
46. Yoda, M.; Kawamata, T.; Paroo, Z.; Ye, X.; Iwasaki, S.; Liu, Q.; Tomari, Y. Atp-dependent human risc assembly pathways. Nat. Struct. Mol. Biol. 2010, 17, 17–23.
47. Rivas, F.V.; Tolia, N.H.; Song, J.J.; Aragon, J.P.; Liu, J.D.; Hannon, G.J.; Joshua-Tor, L. Purified argonaute2 and an sirna form recombinant human risc. Nat. Struct. Mol. Biol. 2005, 12, 340–349.
48. Haley, B.; Zamore, P.D. Kinetic analysis of the rna enzyme complex. Nat. Struct. Mol. Biol. 2004, 11, 599–606.
49. Liu, J.D.; Carmell, M.A.; Rivas, F.V.; Marsden, C.G.; Thomson, J.M.; Song, J.J.; Hammond, S.M.; Joshua-Tor, L.; Hannon, G.J. Argonaute2 is the catalytic engine of mammalian rna. Sci. Signal. 2004, 305, 1437–1437.
50. Snead, N.M.; Rossi, J.J. Biogenesis and function of endogenous and exogenous sirnas. Wiley Interdiscip. Rev. RNA 2010, 1, 117–131.
51. Carthew, R.W.; Sontheimer, E.J. Origins and mechanisms of mirnas and sirnas. Cell 2009, 136, 642–655.
52. Grimm, D.; Streetz, K.L.; Jopling, C.L.; Storm, T.A.; Pandey, K.; Davis, C.R.; Marion, P.; Salazar, F.; Kay, M.A. Fatality in mice due to oversaturation of cellular microrna/short hairpin rna pathways. Nature 2006, 441, 537–541.
53. Frank, F.; Sonenberg, N.; Nagar, B. Structural basis for 5'-nucleotide base-specific recognition of guide rna by human ago2. Nature 2010, 465, 818–822.
54. Walton, S.P.; Wu, M.; Gredell, J.A.; Chan, C. Designing highly active sirnas for therapeutic applications. FEBS J. 2010, 277, 4806–4813.
55. Betancur, J.G.; Tomari, Y. Dicer is dispensable for asymmetric risc loading in mammals. RNA 2012, 18, 24–30.
56. Hutvagner, G. Small rna asymmetry in rna: Function in risc assembly and gene regulation. FEBS Lett. 2005, 579, 5850–5857.
57. Lu, Z.J.; Mathews, D.H. Efficient sirna selection using hybridization thermodynamics. Nucleic Acids Res. 2008, 36, 640–647.
58. Ui-Tei, K.; Naito, Y.; Takahashi, F.; Haraguchi, T.; Ohki-Hamazaki, H.; Juni, A.; Ueda, R.; Saigo, K. Guidelines for the selection of highly effective sirna sequences for mammalian and chick rna interference. Nucleic Acids Res. 2004, 32, 936–948.
59. Jagla, B.; Aulner, N.; Kelly, P.D.; Song, D.; Volchuk, A.; Zatorski, A.; Shum, D.; Mayer, T.; de Angelis, D.A.; Ouaffelli, O.; et al. Sequence characteristics of functional sirnas. *RNA* 2005, 11, 864–872.

60. Huesken, D.; Lange, J.; Mickanin, C.; Weiler, J.; Asselbergs, F.; Warner, J.; Meloon, B.; Engel, S.; Rosenberg, A.; Cohen, D.; et al. Design of a genome-wide sirna library using an artificial neural network. *Nat. Biotechnol.* 2005, 23, 995–1001.

61. Ladunga, I. More complete gene silencing by fewer sirnas: Transparent optimized design and biophysical signature. *Nucleic Acids Res.* 2006, 35, 433–440.

62. Shabalina, S.A.; Spiridonov, A.N.; Ogurtsov, A.Y. Computational models with thermodynamic and composition features improve sirna design. *BMC Bioinformatics* 2006, 7.

63. Amarzguioui, M.; Prydz, H. An algorithm for selection of functional sirna sequences. *Biochem. Biophys. Res. Commun.* 2004, 316, 1050–1058.

64. Gong, W.; Ren, Y.; Xu, Q.; Wang, Y.; Lin, D.; Zhou, H.; Li, T. Integrated sirna design based on surveying of features associated with high rnai effectiveness. *BMC Bioinformatics* 2006, 7, 516.

65. Takasaki, S.; Kotani, S.; Konagaya, A. An effective method for selecting sirna target sequences in mammalian cells. *Cell Cycle* 2004, 3, 788–793.

66. Holen, T. Efficient prediction of sirnas with sirnarules 1.0: An open-source java approach to sirna algorithms. *RNA* 2006, 12, 1620–1625.

67. Takasaki, S. Selecting effective sirna target sequences by using bayes’ theorem. *Comput. Biol. Chem.* 2009, 33, 368–372.

68. Katoh, T.; Suzuki, T. Specific residues at every third position of sirna shape its efficient rnai activity. *Nucleic Acids Res.* 2007, 35, e27.

69. Seitz, H.; Tushir, J.S.; Zamore, P.D. A 5’-uridine amplifies mirna/mirna* asymmetry in drosophila by promoting rna-induced silencing complex formation. *Silence* 2011, 2, 4.

70. Brown, K.M.; Chu, C.Y.; Rana, T.M. Target accessibility dictates the potency of human risc. *Nat. Struct. Mol. Biol.* 2005, 12, 469–470.

71. Ameres, S.L.; Martinez, J.; Schroeder, R. Molecular basis for target rna recognition and cleavage by human risc. *Cell* 2007, 130, 101–112.

72. Mathews, D.; Sabina, J.; Zuker, M.; Turner, D. Expanded sequence dependence of thermodynamic parameters improves prediction of rna secondary structure. *J. Mol. Biol.* 1999, 288, 911–940.

73. Ding, Y.; Chan, C.Y.; Lawrence, C.E. Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res.* 2004, 32, W135-W141.

74. Tafer, H.; Ameres, S.L.; Obernosterer, G.; Gebeshuber, C.A.; Schroeder, R.; Martinez, J.; Hofacker, I.L. The impact of target site accessibility on the design of effective sirnas. *Nat. Biotechnol.* 2008, 26, 578–583.

75. Gredell, J.; Berger, A.; Walton, S. Impact of target mrna structure on sirna silencing efficiency: A large-scale study. *Biotechnol. Bioeng.* 2008, 100, 744–755.

76. Vickers, T.A.; Koo, S.; Bennett, C.F.; Crooke, S.T.; Dean, N.M.; Baker, B.F. Efficient reduction of target rnas by small interfering rna and rna-se dependent antisense agents. A comparative analysis. *J. Biol. Chem.* 2003, 278, 7108–7118.

77. Bohula, E.A.; Salisbury, A.J.; Sohail, M.; Playford, M.P.; Riedemann, J.; Southern, E.M.; Macaulay, V.M. The efficacy of small interfering rnas targeted to the type 1 insulin-like growth
factor receptor (igf1r) is influenced by secondary structure in the igf1r transcript. J. Biol. Chem. 2003, 278, 15991–15997.

78. Overhoff, M.; Alken, M.; Far, R.K.-K.; Lemaitre, M.; Lebleu, B.; Sczakiel, G.; Robbins, I. Local rna target structure influences sirna efficacy: A systematic global analysis. J. Mol. Biol. 2005, 348, 871–881.

79. Schubert, S.; Grünweller, A.; Erdmann, V.A.; Kurreck, J. Local rna target structure influences sirna efficacy: Systematic analysis of intentionally designed binding regions. J. Mol. Biol. 2005, 348, 883–893.

80. Shao, Y.; Chan, C.Y.; Maliyekkel, A.; Lawrence, C.E.; Roninson, I.B.; Ding, Y. Effect of target secondary structure on rnai efficiency. RNA 2007, 13, 1631–1640.

81. Yoshinari, K.; Miyagishi, M.; Taira, K. Effects on rnai of the tight structure, sequence and position of the targeted region. Nucleic Acids Res. 2004, 32, 691–699.

82. Sledz, C.A.; Holko, M.; de Veer, M.J.; Silverman, R.H.; Williams, B.R.G. Activation of the interferon system by short-interfering rnas. Nat. Cell Biol. 2003, 5, 834–839.

83. Samuel-Abraham, S.; Leonard, J.N. Staying on message: Design principles for controlling nonspecific responses to sirna. FEBS J. 2010, 277, 4828–4836.

84. Jackson, A. Recognizing and avoiding sirna off-target effects for target identification and therapeutic application. Nat. Rev. Drug Discov. 2010, 9, 57–67.

85. Robbins, M.; Judge, A.; Liang, L. 2'-o-methyl-modified rnas act as tlr7 antagonists. Mol. Ther. 2007, 15, 1663–1669.

86. Kodym, R.; Kodym, E.; Story, M.D. 2'–5'–oligoadenylate synthetase is activated by a specific rna sequence motif. Biochem. Biophys. Res. Commun. 2009, 388, 317–322.

87. Manche, L.; Green, S.R.; Schmedt, C.; Mathews, M.B. Interactions between double-stranded rna regulators and the protein kinase dai. Mol. Cell. Biol. 1992, 12, 5238–5248.

88. Bevilacqua, P.C.; Cech, T.R. Minor-groove recognition of double-stranded rna by the double-stranded rna-binding domain from the rna-activated protein kinase pkr. Biochemistry 1996, 35, 9983–9994.

89. Marques, J.T.; Devoosse, T.; Wang, D.; Zamanian-Daryoush, M.; Serbinowski, P.; Hartmann, R.; Fujita, T.; Behlke, M.A.; Williams, B.R. A structural basis for discriminating between self and nonsulf double-stranded rnas in mammalian cells. Nat. Biotechnol. 2006, 24, 559–565.

90. Kato, H.; Takeuchi, O.; Mikamo-Satoh, E.; Hirai, R.; Kawai, T.; Matsushita, K.; Hiiragi, A.; Dermody, T.S.; Fujita, T.; Akira, S. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-i and melanoma differentiation-associated gene 5. J. Exp. Med. 2008, 205, 1601–1610.

91. Gantier, M.P.; Williams, B.R.G. The response of mammalian cells to double-stranded rna. Cytokine Growth Factor Rev. 2007, 18, 363–371.

92. Nallagatla, S.R.; Hwang, J.; Toroney, R.; Zheng, X.; Cameron, C.E.; Bevilacqua, P.C. 5'-triphosphate-dependent activation of pkr by rnas with short stem-loops. Science 2007, 318, 1455–1458.

93. Takeda, K.; Kaisho, T.; Akira, S. Toll-like receptors. Annu. Rev. Immunol. 2003, 21, 335–376.
Weber, C.; Müller, C.; Podzuweit, A.; Montino, C.; Vollmer, J.; Forsbach, A. Toll-like receptor (tlr) 3 immune modulation by unformulated small interfering rna or DNA and the role of cd14 (in tlr-mediated effects). *Immunology* 2012, 136, 64–77.

Alexopoulou, L.; Holt, A.C.; Medzhitov, R.; Flavell, R.A. Recognition of double-stranded rna and activation of nf-kappab by toll-like receptor 3. *Nature* 2001, 413, 732–738.

Kariko, K.; Bhuyan, P.; Capodici, J.; Weissman, D. Small interfering rnas mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J. Immunol.* 2004, 172, 6545–6549.

Heil, F.; Hemmi, H.; Hochrein, H.; Ampenberger, F.; Kirschning, C.; Akira, S.; Lipford, G.; Wagner, H.; Bauer, S. Species-specific recognition of single-stranded rna via toll-like receptor 7 and 8. *Science* 2004, 303, 1526–1529.

Judge, A.D.; Sood, V.; Shaw, J.R.; Fang, D.; McClintock, K.; MacLachlan, I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic sirna. *Nat. Biotechnol.* 2005, 23, 457–462.

Jackson, A.L.; Burchard, J.; Leake, D.; Reynolds, A.; Schelter, J.; Guo, J.; Johnson, J.M.; Lim, L.; Karpilow, J.; Nichols, K.; et al. Position-specific chemical modification of sirnas reduces “off-target” transcript silencing. *RNA* 2006, 12, 1197–1205.

Diebold, S.S.; Kaisho, T.; Hemmi, H.; Akira, S.; Reis e Sousa, C. Innate antiviral responses by means of tlr7-mediated recognition of single-stranded rna. *Science* 2004, 303, 1529–1531.

Diebold, S.S.; Massacrier, C.; Akira, S.; Paturel, C.; Morel, Y.; Reis e Sousa, C. Nucleic acid agonists for toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur. J. Immunol.* 2006, 36, 3256–3267.

Goodchild, A.; Nopper, N.; King, A.; Doan, T.; Tanudji, M.; Arndt, G.M.; Poidinger, M.; Rivory, L.P.; Passioura, T. Sequence determinants of innate immune activation by short interfering rnas. *BMC Immunol.* 2009, 10.

Kleinman, M.E.; Yamada, K.; Takeda, A.; Chandrasekaran, V.; Nozaki, M.; Baffi, J.Z.; Albuquerque, R.J.C.; Itaya, M.; Pan, Y.; Appukuttan, B.; Gibbs, D.; Yang, Z.; Karikó, K.; Ambati, B.K.; Wilgus, T.A.; DiPietro, L.A.; Sakurai, E.; Zhang, K.; Smith, J.R.; Taylor, E.W.; Ambati, J. Sequence- and target-independent angiogenesis suppression by sirna via tlr3. *Nature* 2008, 452, 591–597.

Reynolds, A.; Anderson, E.M.; Vermeulen, A.; Fedorov, Y.; Robinson, K.; Leake, D.; Karpilow, J.; Marshall, W.S.; Khvorova, A. Induction of the interferon response by sirna is cell type- and duplex length-dependent. *RNA* 2006, 12, 988–993.

Forsbach, A.; Nemorin, J.-G.; Montino, C.; Müller, C.; Samulowitz, U.; Vicari, A.P.; Jurk, M.; Mutwiri, G.K.; Krieg, A.M.; Lipford, G.B.; Vollmer, J. Identification of rna sequence motifs stimulating sequence-specific tlr8-dependent immune responses. *J. Immunol.* 2008, 180, 3729–3738.

Sioud, M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded sirnas is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* 2005, 348, 1079–1090.

Hornung, V.; Guenthner-Biller, M.; Bourquin, C.; Ablasser, A.; Schlee, M.; Uematsu, S.; Noronha, A.; Manoharan, M.; Akira, S.; de Fougerolles, A.; Endres, S.; Hartmann, G.
specific potent induction of ifn-α by short interfering rna in plasmacytoid dendritic cells through tlr7. Nat. Med. 2005, 11, 263–270.

108. Jurk, M.; Chikh, G.; Schulte, B.; Kritzler, A.; Richardt-Pargmann, D.; Lampron, C.; Luu, R.; Krieg, A.M.; Vicari, A.P.; Vollmer, J. Immunostimulatory potential of silencing rnas can be mediated by a non-uridine-rich toll-like receptor 7 motif. Nucleic Acid Ther. 2011, 21, 201–214.

109. Fedorov, Y.; Anderson, E.M.; Birmingham, A.; Reynolds, A.; Karpilow, J.; Robinson, K.; Leake, D.; Marshall, W.S.; Khvorova, A. Off-target effects by sirna can induce toxic phenotype. RNA 2006, 12, 1188–1196.

110. Shafer, R.H.; Smirnov, I. Biological aspects of DNA/rna quadruplexes. Biopolymers 2000, 56, 209–227.

111. Doench, J.G.; Petersen, C.P.; Sharp, P.A. Sirnas can function as mirnas. Genes Dev. 2003, 17, 438–442.

112. Lambert, N.J.; Gu, S.G.; Zahler, A.M. The conformation of microrna seed regions in native micrornps is prearranged for presentation to mrna targets. Nucleic Acids Res. 2011, 39, 4827–4835.

113. Gu, S.; Jin, L.; Zhang, F.; Huang, Y.; Grimm, D.; Rossi, J.J.; Kay, M.A. Thermodynamic stability of small hairpin rnas highly influences the loading process of different mammalian argonautes. Proc. Natl. Acad. Sci. U. S. A. 2011, 108, 9208–9213.

114. Bartel, D. Micrnas: Target recognition and regulatory functions. Cell 2009, 136, 215–233.

115. Lewis, B.P.; Shih, I.H.; Jones-Rhoades, M.W.; Bartel, D.P.; Burge, C.B. Prediction of mammalian microrna targets. Cell 2003, 115, 787–798.

116. Lin, X.; Ruan, X.; Anderson, M.G.; Mcdowell, J.A.; Kroeger, P.E.; Fesik, S.W.; Shen, Y. Sirna-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Res. 2005, 33, 4527.

117. Lai, E.C. Micro rnas are complementary to 3’ utr sequence motifs that mediate negative post-transcriptional regulation. Nat. Genet. 2002, 30, 363–364.

118. Schultz, N.; Marenstein, D.R.; De Angelis, D.A.; Wang, W.-Q.; Nelander, S.; Jacobsen, A.; Marks, D.S.; Massagué, J.; Sander, C. Off-target effects dominate a large-scale rna screen for modulators of the tgf-β pathway and reveal microrna regulation of tgfbr2. Silence 2011, 2, 3.

119. Didiano, D.; Hobert, O. Perfect seed pairing is not a generally reliable predictor for mirna-target interactions. Nat. Struct. Mol. Biol. 2006, 13, 849–851.

120. Doench, J.G.; Sharp, P.A. Specificity of microrna target selection in translational repression. Genes Dev. 2004, 18, 504–511.

121. Broderick, J.A.; Salomon, W.E.; Ryder, S.P.; Aronin, N.; Zamore, P.D. Argonaute protein identity and pairing geometry determine cooperativity in mammalian rna silencing. RNA 2011, 17, 1858–1869.

122. Kozomara, A.; Griffiths-Jones, S. Mirbase: Integrating microrna annotation and deep-sequencing data. Nucleic Acids Res. 2011, 39, D152–D157.

123. Sigoillot, F.D.; Lyman, S.; Huckins, J.F.; Adamson, B.; Chung, E.; Quattrochi, B.; King, R.W. A bioinformatics method identifies prominent off-targeted transcripts in rna screens. Nat. Methods 2012, 9, 363–366.
124. Sudbery, I.; Enright, A.J.; Fraser, A.G.; Dunham, I. Systematic analysis of off-target effects in an rna screen reveals micrornas affecting sensitivity to trail-induced apoptosis. **BMC Genomics** 2010, 11, 175.

125. Anderson, E.M.; Birmingham, A.; Baskerville, S.; Reynolds, A.; Maksimova, E.; Leake, D.; Fedorov, Y.; Karpilow, J.; Khvorova, A. Experimental validation of the importance of seed complement frequency to sirna specificity. **RNA** 2008, 14, 853–861.

126. Snove, O.; Holen, T. Many commonly used sirnas risk off-target activity. **Biochem. Biophys. Res. Commun.** 2004, 319, 256–263.

127. Holen, T.; Moe, S.E.; Sorbo, J.G.; Meza, T.J.; Ottersen, O.P.; Klungland, A. Tolerated wobble mutations in sirnas decrease specificity, but can enhance activity in vivo. **Nucleic Acids Res.** 2005, 33, 4704–4710.

128. Schwarz, D.S.; Ding, H.; Kennington, L.; Moore, J.T.; Schelter, J.; Burchard, J.; Linsley, P.S.; Aronin, N.; Xu, Z.; Zamore, P.D. Designing sirna that distinguish between genes that differ by a single nucleotide. **PLoS Genet.** 2006, 2, 1307–1318.

129. Jackson, A.L.; Burchard, J.; Schelter, J.; Chau, B.N.; Cleary, M.; Lim, L.; Linsley, P.S. Widespread sirna “off-target” transcript silencing mediated by seed region sequence complementarity. **RNA** 2006, 12, 1179–1187.

130. Saxena, S.; Jönsson, Z.O.; Dutta, A. Small rnas with imperfect match to endogenous mrna repress translation. Implications for off-target activity of small inhibitory rna in mammalian cells. **J. Biol. Chem.** 2003, 278, 44312–44319.

131. Scacheri, P.C.; Rozenblatt-Rosen, O.; Caplen, N.J.; Wolfsberg, T.G.; Umayam, L.; Lee, J.C.; Hughes, C.M.; Shannugam, K.S.; Bhattacharjee, A.; Meyerson, M. Short interfering rnas can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. **Proc. Natl. Acad. Sci. USA** 2004, 101, 1892–1897.

132. Aleman, L.M.; Doench, J.; Sharp, P.A. Comparison of sirna-induced off-target rna and protein effects. **RNA** 2007, 13, 385–395.

133. Liu, J.; Valencia-Sanchez, M.A.; Hannon, G.J.; Parker, R. Microrna-dependent localization of targeted mrnas to mammalian p-bodies. **Nat. Cell Biol.** 2005, 7, 719–723.

134. Teixeira, D.; Sheth, U.; Valencia-Sanchez, M.A.; Brengues, M.; Parker, R. Processing bodies require rna for assembly and contain nontranslating mrnas. **RNA** 2005, 11, 371–382.

135. Behm-Ansmant, I.; Rehwinkel, J.; Izaurralde, E. Micrornas silence gene expression by repressing protein expression and/or by promoting mrna decay. **Cold Spring Harb. Symp. Quant. Biol.** 2006, 71, 523–530.

136. Semizarov, D.; Frost, L.; Sarthy, A.; Kroeger, P.; Halbert, D.N.; Fesik, S.W. Specificity of short interfering rna determined through gene expression signatures. **Proc. Natl. Acad. Sci. USA** 2003, 100, 6347–6352.

137. Persengiev, S.P.; Zhu, X.; Green, M.R. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering rnas (sirnas). **RNA** 2004, 10, 12–18.

138. Wilson, P.A.; Plucinski, M. A simple bayesian estimate of direct rai gene regulation events from differential gene expression profiles. **BMC Genomics** 2011, 12, 250.

139. Sydor, J.R.; Nock, S. Protein expression profiling arrays: Tools for the multiplexed high-throughput analysis of proteins. **Proteome Sci.** 2003, 1, 3.
140. Pan, S.; Aebersold, R.; Chen, R.; Rush, J.; Goodlett, D.R.; McIntosh, M.W.; Zhang, J.; Brentnall, T.A. Mass spectrometry based targeted protein quantification: Methods and applications. J. Proteome Res. 2009, 8, 787–797.

141. Huang, H.; Qiao, R.; Zhao, D.; Zhang, T.; Li, Y.; Yi, F.; Lai, F.; Hong, J.; Ding, X.; Yang, Z.; et al. Profiling of mismatch discrimination in rnai enabled rational design of allele-specific sirnas. Nucleic Acids Res. 2009, 37, 7560–7569.

142. Kittler, R.; Surendranath, V.; Heninger, A.-K.; Slabicki, M.; Theis, M.; Putz, G.; Franke, K.; Caldarelli, A.; Grabner, H.; Kozak, K.; et al. Genome-wide resources of endoribonuclease-prepared short interfering rnas for specific loss-of-function studies. Nat. Methods 2007, 4, 337–344.

143. Parker, J.S.; Parizotto, E.A.; Wang, M.; Roe, S.M.; Barford, D. Enhancement of the seed-target recognition step in rna silencing by a piwi/mid domain protein. Mol. Cell 2009, 33, 204–214.

144. Birmingham, A.; Anderson, E.M.; Reynolds, A.; Ilsley-Tyree, D.; Leake, D.; Fedorov, Y.; Baskerville, S.; Maksimova, E.; Robinson, K.; Karpilow, J.; Marshall, W.S.; Khvorova, A. 3’ UTR seed matches, but not overall identity, are associated with rnai off-targets. Nat. Methods 2006, 3, 199–204.

145. Boland, A.; Trittischler, F.; Heimst Am Dt, S.; Izaurralde, E.; Weichenrieder, O. Crystal structure and ligand binding of the mid domain of a eukaryotic argonaute protein. EMBO Rep. 2010, 11, 522–527.

146. Ma, J.-B.; Ye, K.; Patel, D.J. Structural basis for overhang-specific small interfering rna recognition by the paz domain. Nature 2004, 429, 318–322.

147. Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. Nucleic acid 3’-end recognition by the argonaute2 paz domain. Nat. Struct. Mol. Biol. 2004, 11, 576–577.

148. Sashtial, D.G.; Doudna, J.A. Structural insights into rna interference. Curr. Opin. Struct. Biol. 2010, 20, 90–97.

149. Vert, J.-P.; Foveau, N.; Lajaunie, C.; Vandenbrouck, Y. An accurate and interpretable model for sirna efficacy prediction. BMC Bioinformatics 2006, 7, 520.

150. Patzel, V.; Rutz, S.; Dietrich, I.; Köberle, C.; Scheffold, A.; Kaufmann, S.H.E. Design of sirnas producing unstructured guide-rnas results in improved rna interference efficiency. Nat. Biotechnol. 2005, 23, 1440–1444.

151. Köberle, C.; Kaufmann, S.H.E.; Patzel, V. Selecting effective sirnas based on guide rna structure. Nat. Protoc. 2006, 1, 1832–1839.

152. Hossbach, M.; Gruber, J.; Osborn, M.; Weber, K.; Tuschl, T. Gene silencing with sirna duplexes composed of target-mrna-complementary and partially palindromic or partially complementary single-stranded sirnas. RNA Biol. 2006, 3, 82–89.

153. Vermeulen, A.; Behlen, L.; Reynolds, A.; Wolfson, A.; Marshall, W.S.; Karpilow, J.; Khvorova, A. The contributions of dsrna structure to dicer specificity and efficiency. RNA 2005, 11, 674–682.

154. Sciaiola, S.; Cao, Q.; Orozco, M.; Faustino, I.; Stanton, R.V. Improved nucleic acid descriptors for sirna efficacy prediction. Nucleic Acids Res. 2012.

155. Snead, N.M.; Rossi, J.J. Rna interference trigger variants: Getting the most out of rna for rna interference-based therapeutics. Nucleic Acid Ther. 2012, 22, 139–146.

156. Martinez, J.; Patkaniowska, A.; Urlaub, H.; Lührmann, R.; Tuschl, T. Single-stranded antisense sirnas guide target rna cleavage in rnai. Cell 2002, 110, 563–574.
157. Holen, T.; Amarzguioui, M.; Babaie, E.; Prydz, H. Similar behaviour of single-strand and double-strand sirnas suggests they act through a common rnai pathway. *Nucleic Acids Res.* **2003**, *31*, 2401–2407.

158. Lima, W.F.; Prakash, T.P.; Murray, H.M.; Kinberger, G.A.; Li, W.; Chappell, A.E.; Li, C.S.; Murray, S.F.; Gaus, H.; Seth, P.P.; *et al.* Single-stranded sirnas activate rna in animals. *Cell* **2012**, *150*, 883–894.

159. Haringsma, H.J.; Li, J.J.; Soriano, F.; Kenski, D.M.; Flanagan, W.M.; Willingham, A.T. Mrna knockdown by single strand rna is improved by chemical modifications. *Nucleic Acids Res.* **2012**, *40*, 4125–4136.

160. Chu, C.-Y.; Rana, T.M. Potent rnai by short rna triggers. *RNA* **2008**, *14*, 1714–1719.

161. Sun, X.; Rogoff, H.A.; Li, C.J. Asymmetric rna duplexes mediate rna interference in mammalian cells. *Nat. Biotechnol.* **2008**, *26*, 1379–1382.

162. Chang, C.I.; Yoo, J.W.; Hong, S.W.; Lee, S.E.; Kang, H.S.; Sun, X.; Rogoff, H.A.; Ban, C.; Kim, S.; Li, C.J.; *et al.* Asymmetric shorter-duplex sirna structures trigger efficient gene silencing with reduced nonspecific effects. *Mol. Ther.* **2009**, *17*, 725–732.

163. Hohjoh, H. Enhancement of rnai activity by improved sirna duplexes. *FEBS Lett.* **2004**, *557*, 193–198.

164. Bramsen, J.B.; Laursen, M.B.; Damgaard, C.K.; Lena, S.W.; Babu, B.R.; Wengel, J.; Kjems, J. Improved silencing properties using small internally segmented interfering rnas. *Nucleic Acids Res.* **2007**, *35*, 5886–5897.

165. Dua, P.; Yoo, J.W.; Kim, S.; Lee, D.-K. Modified sirna structure with a single nucleotide bulge overcomes conventional sirna-mediated off-target silencing. *Mol. Ther.* **2011**, *19*, 1676–1687.

166. Amarzguioui, M.; Lundberg, P.; Cantin, E.; Hagstrom, J.; Behlke, M.A.; Rossi, J.J. Rational design and in vitro and in vivo delivery of dicer substrate sirna. *Nat. Protoc.* **2006**, *1*, 508–517.

167. Collingwood, M.A.; Rose, S.D.; Huang, L.; Hillier, C.; Amarzguioui, M.; Wiiger, M.T.; Soifer, H.S.; Rossi, J.J.; Behlke, M.A. Chemical modification patterns compatible with high potency dicer-substrate small interfering rnas. *Oligonucleotides* **2008**, *18*, 187–200.

168. Tanudji, M.; Machalek, D.; Arndt, G.M.; Rivory, L. Competition between sirna duplexes: Impact of rna-induced silencing complex loading efficiency and comparison between conventional-21 bp and dicer-substrate sirnas. *Oligonucleotides* **2010**, *20*, 27–32.

169. Foster, D.J.; Barros, S.; Duncan, R.; Shaikh, S.; Cantley, W.; Dell, A.; Bulgakova, E.; O’Shea, J.; Taneja, N.; Kuchimanchi, S.; *et al.* Comprehensive evaluation of canonical versus dicer-substrate sirna in vitro and in vivo. *RNA* **2012**, *18*, 557–568.

170. Turner, J.J.; Jones, S.W.; Moschos, S.A.; Lindsay, M.A.; Gait, M.J. Maldi-tof mass spectral analysis of sirna degradation in serum confirms an rnase a-like activity. *Mol. Biosyst.* **2006**, *3*, 43–50.

171. Volkov, A.A.; Kruglova, N.y.S.; Meschaninova, M.I.; Venyaminova, A.G.; Zenkova, M.A.; Vlassov, V.V.; Chernolovskaya, E.L. Selective protection of nuclease-sensitive sites in sirna prolongs silencing effect. *Oligonucleotides* **2009**, *19*, 191–202.

172. Hong, J.; Huang, Y.; Li, J.; Yi, F.; Zheng, J.; Huang, H.; Wei, N.; Shan, Y.; An, M.; Zhang, H.; *et al.* Comprehensive analysis of sequence-specific stability of sirna. *FASEB J.* **2010**, *24*, 4844–4855.
173. Allerson, C.R.; Sioufi, N.; Jarres, R.; Prakash, T.P.; Naik, N.; Berdeja, A.; Wanders, L.; Griffey, R.H.; Swayze, E.E.; Bhat, B. Fully 2’-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering rna. J. Med. Chem. 2005, 48, 901–904.

174. Bramsen, J.B.; Laursen, M.B.; Nielsen, A.F.; Hansen, T.B.; Bus, C.; Langkjaer, N.; Babu, B.R.; Hojland, T.; Abramov, M.; Van Aerschot, A.; Odadzic, D.; et al. A large-scale chemical modification screen identifies design rules to generate sirnas with high activity, high stability and low toxicity. Nucleic Acids Res. 2009, 37, 2867–2881.

175. Kenski, D.M.; Butora, G.; Willingham, A.T.; Cooper, A.J.; Fu, W.; Qi, N.; Soriano, F.; Davies, I.W.; Flanagan, W.M. Sirna-optimized modifications for enhanced in vivo activity. Mol. Ther. Nucleic Acids 2012, 1, e5.

176. Braasch, D.A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M.A.; Corey, D.R. Rna interference in mammalian cells by chemically-modified rna. Biochemistry 2003, 42, 7967–7975.

177. Chiu, Y.-L.; Rana, T.M. Sirna function in rnai: A chemical modification analysis. RNA 2003, 9, 1034–1048.

178. Manoharan, M.; Akinc, A.; Pandey, R.K.; Qin, J.; Hadwiger, P.; John, M.; Mills, K.; Charisse, K.; Maier, M.A.; Nechev, L.; et al. Unique gene-silencing and structural properties of 2’-fluoro-modified sirnas. Angew. Chem. Int. Ed. Engl. 2011, 50, 2284–2288.

179. Cekaite, L.; Furset, G.; Hovig, E.; Sioud, M. Gene expression analysis in blood cells in response to unmodified and 2-modified sirnas reveals tlr-dependent and independent effects. J. Mol. Biol. 2007, 365, 90–108.

180. Tluk, S.; Jurk, M.; Forsbach, A.; Weeratna, R.; Samulowitz, U.; Krieg, A.M.; Bauer, S.; Vollmer, J. Sequences derived from self-rna containing certain natural modifications act as suppressors of rna-mediated inflammatory immune responses. Int. Immunol. 2009, 21, 607–619.

181. Fucini, R.V.; Haringsma, H.J.; Deng, P.; Flanagan, W.M.; Willingham, A.T. Adenosine modification may be preferred for reducing sirna immune stimulation. Nucleic Acid Ther. 2012, 22, 205–210.

182. Haupenthal, J.; Baehr, C.; Kiermayer, S.; Zeuzem, S.; Piiper, A. Inhibition of rnase a family enzymes prevents degradation and loss of silencing activity of sirnas in serum. Biochem. Pharmacol. 2006, 71, 702–710.

183. Amarzguioui, M. Tolerance for mutations and chemical modifications in a sirna. Nucleic Acids Res. 2003, 31, 589–595.

184. Yang, X.; Sierant, M.; Janicka, M.; Peczek, L.; Martinez, C.; Hassell, T.; Li, N.; Li, X.; Wang, T.; Nawrot, B. Gene silencing activity of sirna molecules containing phosphorodithioate substitutions. ACS Chem. Biol. 2012, 7, 1214–1220.

185. Bramsen, J.B.; Kjems, J. Development of therapeutic-grade small interfering rnas by chemical engineering. Front. Genet. 2012, 3, 154–154.

186. Wang, J.; Byrne, J.D.; Napier, M.E.; DeSimone, J.M. More effective nanomedicines through particle design. Small 2011, 7, 1919–1931.

187. Elsabahy, M.; Wooley, K.L. Design of polymeric nanoparticles for biomedical delivery applications. Chem. Soc. Rev. 2012, 41, 2545–2561.
188. Siegwart, D.J.; Whitehead, K.A.; Nuhn, L.; Sahay, G.; Cheng, H.; Jiang, S.; Ma, M.; Lytton-Jean, A.; Vegas, A.; Fenton, P.; et al. Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. *Proc. Natl. Acad. Sci. USA* 2011, 108, 12996–13001.

189. Ulery, B.D.; Nair, L.S.; Laurencin, C.T. Biomedical applications of biodegradable polymers. *J. Polym. Sci. B Polym. Phys.* 2011, 49, 832–864.

190. Whitehead, K.A.; Sahay, G.; Li, G.Z.; Love, K.T.; Alabi, C.A.; Ma, M.; Zurenko, C.; Querbes, W.; Langer, R.S.; Anderson, D.G. Synergistic silencing: Combinations of lipid-like materials for efficacious sirna delivery. *Mol. Ther.* 2011, 19, 1688–1694.

191. Semple, S.C.; Akinc, A.; Chen, J.; Sandhu, A.P.; Mui, B.L.; Cho, C.K.; Sah, D.W.Y.; Stebbing, D.; Crosley, E.J.; Yaworski, E.; et al. Rational design of cationic lipids for sirna delivery. *Nat. Biotechnol.* 2010, 28, 172–176.

192. Malam, Y.; Loizidou, M.; Seifalian, A.M. Liposomes and nanoparticles: Nanosized vehicles for drug delivery in cancer. *Trends Pharmacol. Sci.* 2009, 30, 592–599.

193. Mutlu, G.M.; Budinger, G.R.S.; Green, A.A.; Urich, D.; Soberanes, S.; Chiarella, S.E.; Alheid, G.F.; McCreminon, D.R.; Szleifer, I.; Hersam, M.C. Biocompatible nanoscale dispersion of single-walled carbon nanotubes minimizes in vivo pulmonary toxicity. *Nano Lett.* 2010, 10, 1664–1670.

194. Cheung, W.; Pontoriero, F.; Taratula, O.; Chen, A.M.; He, H.X. DNA and carbon nanotubes as medicine. *Adv. Drug Deliv. Rev.* 2010, 62, 633–649.

195. Dobrovolskaia, M.A.; Aggarwal, P.; Hall, J.B.; McNeil, S.E. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. *Mol. Pharm.* 2008, 5, 487–495.

196. Li, Y.; Sun, L.; Jin, M.; Du, Z.; Liu, X.; Guo, C.; Li, Y.; Huang, P.; Sun, Z. Size-dependent cytotoxicity of amorphous silica nanoparticles in human hepatoma hepg2 cells. *Toxicol. In Vitro* 2011, 25, 1343–1352.

197. Slowing, I.I.; Trewyn, B.G.; Giri, S.; Lin, V.S.Y. Mesoporous silica nanoparticles for drug delivery and biosensing applications. *Adv. Funct. Mater.* 2007, 17, 1225–1236.

198. Asafa, T.; Tao, Z. Biocompatibility of mesoporous silica nanoparticles. *Chem. Res. Toxicol.* 2012, 25, 2265–2284.

199. Colombo, M.; Carregal-Romero, S.; Casula, M.F.; Gutierrez, L.; Morales, M.P.; Bohm, I.B.; Heverhagen, J.T.; Prosperi, D.; Parak, W.J. Biological applications of magnetic nanoparticles. *Chem. Soc. Rev.* 2012, 41, 4306–4334.

200. Prijic, S.; Sersa, G. Magnetic nanoparticles as targeted delivery systems in oncology. *Radiol. Oncol.* 2011, 45, 1–16.

201. Khlebtsov, N.; Dykman, L. Biodistribution and toxicity of engineered gold nanoparticles: A review of in vitro and in vivo studies. *Chem. Soc. Rev.* 2011, 40, 1647–1671.

202. Cho, E.C.; Zhang, Q.; Xia, Y. The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles. *Nat. Nanotechnol.* 2011, 6, 385–391.

203. Kong, W.H.; Bae, K.H.; Jo, S.D.; Kim, J.S.; Park, T.G. Cationic lipid-coated gold nanoparticles as efficient and non-cytotoxic intracellular sirna delivery vehicles. *Pharm. Res.* 2012, 29, 362–374.
204. Daka, A.; Peer, D. Rnai-based nanomedicines for targeted personalized therapy. *Adv. Drug Deliv. Rev.* **2012**, *64*, 1508–1521.

205. Parveen, S.; Misra, R.; Sahoo, S.K. Nanoparticles: A boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine* **2012**, *8*, 147–166.

206. Burnett, J.C.; Rossi, J.J. Rna-based therapeutics: Current progress and future prospects. *Chem. Biol.* **2012**, *19*, 60–71.

207. De Ilarduya, C.T.; Sun, Y.; Duezguenes, N. Gene delivery by lipoplexes and polyplexes. *Eur. J. Pharm. Sci.* **2010**, *40*, 159–170.

208. De Planque, M.R.R.; Aghdaei, S.; Roose, T.; Morgan, H. Electrophysiological characterization of membrane disruption by nanoparticles. *ACS Nano* **2011**, *5*, 3599–3606.

209. Khalil, I.A.; Kogure, K.; Akita, H.; Harashima, H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol. Rev.* **2006**, *58*, 32–45.

210. Ding, H.M.; Tian, W.D.; Ma, Y.Q. Designing nanoparticle translocation through membranes by computer simulations. *ACS Nano* **2012**, *6*, 1230–1238.

211. Huotari, J.; Helenius, A. Endosome maturation. *EMBO J.* **2011**, *30*, 3481–3500.

212. Doherty, G.J.; McMahon, H.T. Mechanisms of endocytosis. *Annu. Rev. Biochem.* **2009**, *78*, 857–902.

213. Kerr, M.C.; Teasdale, R.D. Defining macropinocytosis. *Traffic* **2009**, *10*, 364–371.

214. Mayor, S.; Pagano, R.E. Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 603–612.

215. 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*, 468–474.

216. Frohlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomedicine* **2012**, *7*, 5577–5591.

217. Goncalves, C.; Mennesson, E.; Fuchs, R.; Gorvel, J.P.; Midoux, P.; Pichon, C. Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. *Mol. Ther.* **2004**, *10*, 373–385.

218. Brigger, I.; Dubernet, C.; Couvreur, P. Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev.* **2002**, *54*, 631–651.

219. Moghimi, S.M.; Hunter, A.C.; Andresen, T.L. Factors controlling nanoparticle pharmacokinetics: An integrated analysis and perspective. *Annu. Rev. Pharmacol. Toxicol.* **2012**, *52*, 481–503.

220. Venturoli, D.; Rippe, B. Ficoll and dextran vs. Globular proteins as probes for testing glomerular permeability: Effects of molecular size, shape, charge, and deformability. *Am. J. Physiol. Renal Physiol.* **2005**, *288*, F605–F613.

221. Petrov, R.A.; DeSimone, J.M. Strategies in the design of nanoparticles for therapeutic applications. *Nat. Rev. Drug Discov.* **2010**, *9*, 615–627.

222. Allen, T.M.; Cullis, P.R. Drug delivery systems: Entering the mainstream. *Science* **2004**, *303*, 1818–1822.

223. Vonarbourg, A.; Passirani, C.; Saulnier, P.; Benoit, J.P. Parameters influencing the stealthiness of colloidal drug delivery systems. *Biomaterials* **2006**, *27*, 4356–4373.

224. Doshi, N.; Mitragotri, S. Macrophages recognize size and shape of their targets. *PLoS ONE* **2010**, *5*, e10051.
225. Decuzzi, P.; Pasqualini, R.; Arap, W.; Ferrari, M. Intravascular delivery of particulate systems: Does geometry really matter? Pharm. Res. 2009, 26, 235–243.
226. Champion, J.A.; Mitragotri, S. Role of target geometry in phagocytosis. Proc. Natl. Acad. Sci. USA 2006, 103, 4930–4934.
227. He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. Biomaterials 2010, 31, 3657–3666.
228. Dufort, S.; Sancey, L.; Coll, J.L. Physico-chemical parameters that govern nanoparticles fate also dictate rules for their molecular evolution. Adv. Drug Deliv. Rev. 2012, 64, 179–189.
229. Vaishnaw, A.K.; Gollob, J.; Gamba-Vitalo, C.; Hutabarat, R.; Sah, D.; Meyers, R.; de Fougerolles, T.; Maraganore, J. A status report on rna therapeutics. Silence 2010, 1, 14.
230. Strumberg, D.; Schultheis, B.; Traugott, U.; Vank, C.; Santel, A.; Keil, O.; Giese, K.; Kaufmann, J.; Dreis, J. Phase i clinical development of atu027, a sirna formulation targeting pkn3 in patients with advanced solid tumors. Int. J. Clin. Pharmacol. Ther. 2012, 50, 76–78.
231. Lonez, C.; Vandenbranden, M.; Rouysschaert, J.M. Cationic liposomal lipids: From gene carriers to cell signaling. Prog. Lipid Res. 2008, 47, 340–347.
243. Barros, S.A.; Gollob, J.A. Safety profile of rnaI nanomedicines. *Adv. Drug Deliv. Rev.* **2012**, *64*, 1730–1737.

244. Davis, M.E. The first targeted delivery of sirna in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: From concept to clinic. *Mol. Pharm.* **2009**, *6*, 659–668.

245. Heidel, J.D.; Davis, M.E. Clinical developments in nanotechnology for cancer therapy. *Pharm. Res.* **2011**, *28*, 187–199.

246. Rozema, D.B.; Lewis, D.L.; Wakefield, D.H.; Wong, S.C.; Klein, J.J.; Roesch, P.L.; Bertin, S.L.; Reppen, T.W.; Chu, Q.; Blokhin, A.V.; et al. Dynamic polyconjugates for targeted *in vivo* delivery of sirna to hepatocytes. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12982–12987.

247. Panyam, J.; Labhasetwar, V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Deliv. Rev.* **2003**, *55*, 329–347.

248. Reilly, M.J.; Larsen, J.D.; Sullivan, M.O. Histone h3 tail peptides and poly(ethylenimine) have synergistic effects for gene delivery. *Mol. Pharm.* **2012**, *9*, 1031–1040.

249. Zhou, J.; Liu, J.; Cheng, C.J.; Patel, T.R.; Weller, C.E.; Piepmeier, J.M.; Jiang, Z.; Saltzman, W.M. Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery. *Nat. Mater.* **2012**, *11*, 82–90.

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