

RNA interference in the clinic: challenges and future directions

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Abstract | Inherent difficulties with blocking many desirable targets using conventional approaches have prompted many to consider using RNA interference (RNAi) as a therapeutic approach. Although exploitation of RNAi has immense potential as a cancer therapeutic, many physiological obstacles stand in the way of successful and efficient delivery. This Review explores current challenges to the development of synthetic RNAi-based therapies and considers new approaches to circumvent biological barriers, to avoid intolerable side effects and to achieve controlled and sustained release.

RNA interference (RNAi). Refers to the mechanism of potent and specific gene-silencing caused by double-stranded RNA (dsRNA) produced by endogenous (miRNA) or exogenous (siRNA) sources.

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Since the discovery of RNA interference (RNAi), there has been an explosion of interest and knowledge in using this technology for clinical applications¹. Although small-molecule inhibitors and monoclonal antibodies have led to many successful therapies for cancer^{2,3}, many important cancer therapy targets are difficult to inhibit using these strategies. For example, a small-molecule inhibitor of a specific kinase will not affect its kinase-independent oncogenic functions and so will not restrict the entire function of the protein⁴⁻⁶. Most small-molecule inhibitors are also not specific with regard to target modulation, which can introduce undesirable toxicity. In the case of monoclonal antibodies, the protein might simply be inaccessible if it is not present on the cell surface or in circulation. The use of RNAi in the clinic is attractive as it can circumvent many of these problems and its potential for use as a therapeutic has recently been bolstered by a report of systemic small interfering RNA (siRNA) delivery into human tumours⁷. This study highlights how a molecule, the M2 subunit of ribonucleotide reductase (RRM2), which is difficult to inhibit using conventional approaches, can be targeted using siRNA. Although highly attractive as a therapeutic approach, several hurdles must be overcome to successfully introduce RNAi-based therapies into the clinic. Some of these include efficient and safe systemic delivery, avoidance of undesirable off-target effects, and the development of methods for assessing systemic biodistribution and subcellular localization. In addition, methods for crossing compartmental boundaries and avoiding intracellular trapping are needed. In this Review we discuss the existing challenges and future directions for developing RNAi as a clinical modality for cancer therapy.

Mechanisms of RNAi

The mechanism by which RNAi inhibits the conversion of mRNA into protein has been reviewed elsewhere⁸⁻¹⁰. Briefly, double-stranded RNA (dsRNA) is recognized by an RNase type III enzyme, Dicer, and cleaved into small fragments 21–23 base pairs in length^{11,12}. The dsRNA has sequences that form a sense (passenger) strand and an antisense (guide) strand with respect to the target mRNA (FIG. 1). The dsRNA fragment binds to a protein complex called RNA-induced silencing complex (RISC), and the passenger strand of dsRNA is cleaved and discarded while the guide strand is directed to the 3' untranslated region (UTR) of the complementary target mRNA¹³. When dsRNA is exogenously introduced, referred to as siRNA, a cleavage enzyme within RISC (argonaute 2) degrades the target mRNA, thereby preventing translation^{14,15}. Endogenous non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), also exist in cells and are pre-processed by a nuclear RNase III (Drosha) before export into the cytoplasm by nuclear transport receptor complex, exportin 5–RanGTP^{16,17}. Owing to imperfect matching with 3' UTRs, miRNAs in some instances do not lead to the cleavage of mRNA with the RISC but instead result in translational suppression¹⁸. As our understanding of the role that ncRNAs have in the pathogenesis of cancer has expanded, it has become clear that our ability to harness their potential as an anticancer therapeutic is a formidable task¹⁹.

Current challenges in RNAi delivery

There are several challenges that currently limit the use of RNAi in the clinic. Methods that overcome these are being developed and are discussed below (FIG. 2; TABLE 1).

At a glance

- Many cancer targets are difficult to block with conventional therapies. Although RNA interference (RNAi) as a therapeutic approach is appealing, many challenges to delivery must be overcome.
- Nanoparticles hold promise for the safe and effective intracellular delivery of RNAi-based molecules.
- Physiological barriers and systemic toxicity of nanoparticle-based carrier systems create multiple challenges to bringing RNAi-based therapeutics to the clinic.
- Nanoparticles can be used to help avoid immune-mediated responses to systemic RNAi-based therapy.
- Solutions to improving tumour specificity and the ability to monitor and control short-term and long-term RNAi-based therapies are crucial next steps before clinical use.
- As the technology for delivery improves, so we will also need to improve our understanding of the heterogeneity of RNAi processing in different cancer types.
- Various resistance mechanisms to RNAi-based therapies must be anticipated.

Opsonins

Plasma proteins that act as binding enhancers for phagocytosis.

Reticuloendothelial system (RES)

System composed of scavenging monocytes and macrophages located in the reticular connective tissue (notably the liver, spleen, lung and marrow).

Enhanced permeability and retention (EPR)

The property by which certain sizes of molecules tend to accumulate and remain in tumour tissue more than in normal tissues.

Endocytosis

The active uptake of molecules into a cell by clathrin-dependent and clathrin-independent receptor-mediated endocytosis, pinocytosis and phagocytosis.

Fusogenic lipids

Lipoplexes (containing cationic lipids and nucleic acids) that adopt an inverted hexagonal phase and fuse with anionic membranes resulting in endosomal release.

Fusogenic peptides

Peptides that have cell penetrating properties (such as being highly hydrophobic), which cause cell membrane destabilization and intra-cytoplasmic release.

pH-sensitive lipoplexes

Liposomes that hydrolyse and trigger release of contents owing to subtle drops in pH, such as with endosomal fusion, allowing for endosomal escape.

Intravascular degradation. Naked siRNA is unstable in circulation owing to serum RNase A-type nucleases and rapid renal clearance, leading to degradation and a short half-life²⁰ (FIG. 2). Some investigators have turned towards the chemical modification of the sugars, the backbone or the bases of oligoribonucleotides for stabilization^{21,22}. However, the hydrophobic cell membranes create a challenge for the intracellular delivery of negatively charged polymers. Additionally, once siRNAs are intracellular they only transiently cause gene silencing, as the concentration of the siRNAs decreases with each cell division²³. Nanoparticle carriers have the potential to overcome the challenges of intravascular degradation, and can provide safe and efficient delivery of synthetic dsRNAs. On entering the bloodstream, nanoparticles encounter a complex environment of plasma proteins and immune cells. Nanoparticle uptake by immune cells in circulation, such as monocytes, leukocytes, platelets and dendritic cells, occurs through various pathways and is facilitated by the adsorption of opsonins to the surface of the particle²⁴ (FIG. 2). Additionally, physical and chemical properties of the nanoparticle surface, such as size and surface charge, can lead to haemolysis, thrombogenicity and complement activation, resulting in altered biodistribution and potential toxicity²⁴.

Tissue penetrance and intracellular delivery. For safe and effective delivery of RNAi to the mRNA target of interest, many variables must be negotiated. Although nanoparticles by definition range in size from 1 nm to 1,000 nm in size, it has become clear that intravenously injected particles > 100 nm in diameter are likely to be trapped by the reticuloendothelial system (RES) in the liver, spleen, lung and bone marrow, leading to degradation by activated monocytes and macrophages (FIG. 2). Compared with normal capillaries, tumour vessels are irregularly shaped, defective, leaky and have varying widths²⁵. Higher interstitial fluid pressure in solid tumours can also pose challenges for the diffusion of nanoparticles²⁶ (FIG. 2). Potential strategies to overcome these barriers include using nanoparticles with diameters larger than the fenestrations of normal vessels (from a few nanometers to 150 nm); injecting vectors with

varying radii; and normalizing tumour vasculature, such as with anti-angiogenic agents^{26,27}. Overall, nanoparticles of less than 100 nm in diameter are thought to be optimal for intra-tumoural delivery and for avoiding RES clearance. Another important factor for increased capillary leak is the enhanced permeability and retention (EPR) effect, which can be exploited and enhanced for improved nanoparticle delivery^{28,29} (FIG. 2).

Intracellular trafficking. Many nanoparticles enter cells through endocytosis³⁰. Once endocytosed, intracellular trafficking begins in early endosomal vesicles (FIG. 2). These subsequently fuse with sorting endosomes, which transfer their contents to late endosomes. These late endosomes are acidified (pH ~ 5–6) by membrane-bound proton-pump ATPases. The late endosomal content is relocated to lysosomes, which are further acidified (pH ~4.5) and contain nucleases that degrade RNA³¹. Thus, if endosomal escape does not occur before these later phases, RNAi will not occur. Some mechanisms to enhance endosomal escape include the use of fusogenic lipids, fusogenic peptides, photosensitive molecules, pH-sensitive lipoplexes and pH-sensitive polyplexes³¹. Fusogenic lipids promote endosomal release by increasing the interactions between the liposomal and endosomal membranes. The pH-sensitive materials function by a proton ‘sponge’ effect; as pH inside the endosome drops, protonation on the delivery material allows for an influx of chloride ions and subsequent osmosis, resulting in endosomal rupture and the release of its contents³¹.

The fate of RNAi in biological fluids. Although intracellular barriers to RNAi delivery are being explored in depth, extracellular factors in the microenvironment are less well studied and could be more important. For successful delivery, nanoparticles must extravasate and move through the complex extracellular matrix (ECM) to reach the cancer cells (FIG. 2). The desmoplasia associated with pancreatic cancer is a good example of the difficulties that a complex tumour microenvironment presents in achieving therapeutic drug concentrations³². The biological, chemical and physical properties of the ECM can result in the unpackaging of nanoparticles and the premature release of their contents³³. *In vitro* studies are typically carried out under static conditions that are not reflective of the dynamic forces that are found *in vivo*. Three-dimensional models can be useful to recapitulate ECM barriers to nanoparticle delivery³⁴. The immune system is also an important extracellular barrier. Phagocytosis by macrophages, such as Kupffer cells in the liver or dendritic cells in the lymph nodes, occurs more frequently with particles bearing cationic or anionic charges than with neutral nanoparticles²⁴ (FIG. 2). In addition to phagocytosis by scavenger macrophages, neutrophils can function as extracellular traps for nanoparticles^{35,36}.

Immune-mediated toxicities. Unknown or unexpected toxicities that are related to systemic RNAi delivery must be anticipated. The innate immune response to siRNA is highly contextualized and divided into two groups: Toll-like receptor (TLR)-mediated or non-TLR-mediated

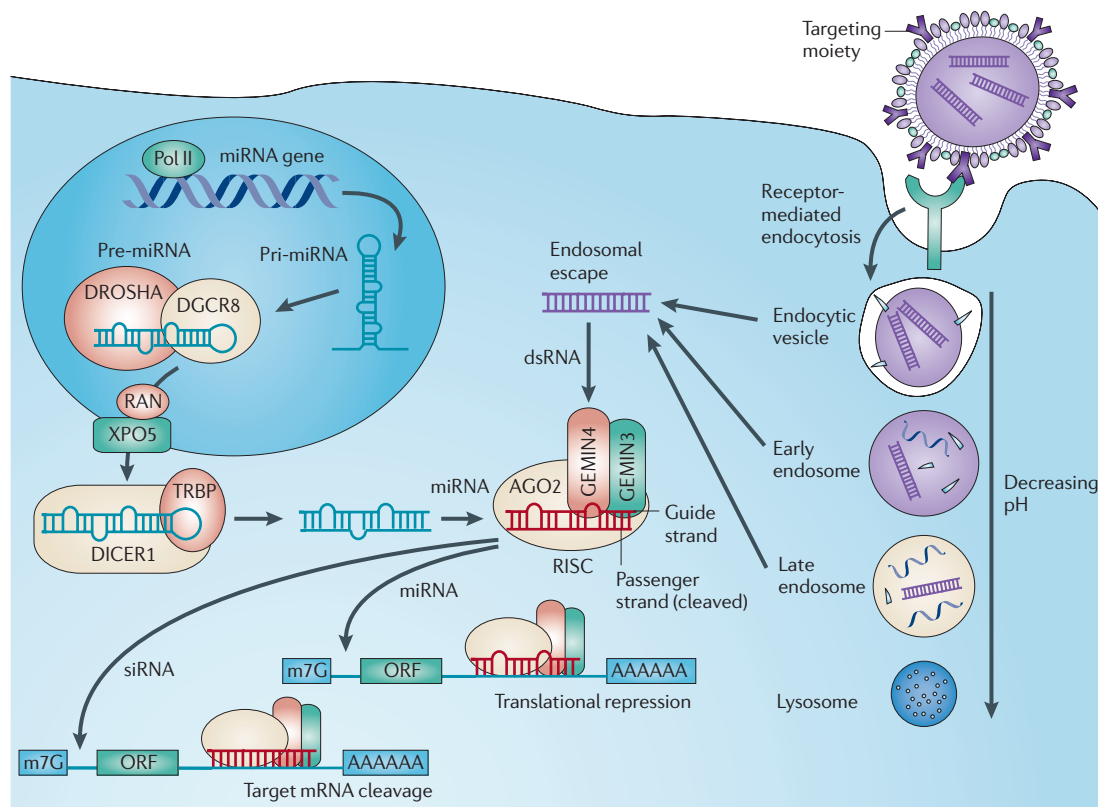


Figure 1 | Mechanism of RNAi following intracellular dsRNA delivery. This demonstrates the intracellular fate of a nanoparticle following receptor-mediated endocytosis. Prior to degradation, the delivered double-stranded RNA (dsRNA) (whether microRNA (miRNA) or synthetic small interfering RNA (siRNA)) must escape the increasingly acidic endosome. After endosomal escape, the dsRNA bypasses Dicer and incorporates into the RNA-induced silencing complex (RISC). This leads to RNA interference (RNAi) by either translational repression or mRNA cleavage. AGO2, argonaute 2; ORF, open reading frame; Pol II, RNA polymerase.

groups. TLR3, TLR7 and TLR8 are activated when engaged with nucleic acids within endosomal and lysosomal compartments. Ligands that activate TLR7 and TLR8 include duplex siRNA, or its corresponding single strand, although this is highly dependent on nucleotide sequence. For example, uridine- and guanosine-rich sequences with either UG dinucleotide or 5'-UGU-3' motifs potentially activate TLR7 and TLR8, and avoidance of such sequences decreases this response³⁷. In addition, AU-rich sequences have been shown to preferentially activate TLR8 (REF. 38). TLR3 is only activated by duplex siRNA, and whether the sequence is important is not yet known³⁹. Following endocytosis from the cell surface, TLR-mediated activation requires endosomal acidification and maturation. TLR7 is predominately expressed in plasmacytoid dendritic cells and B cells, TLR8 in myeloid dendritic cells, monocytes and macrophages, and TLR3 in mature myeloid dendritic cells⁴⁰. TLR3 is also expressed in lung, aorta, dermis, choroidal and umbilical vein endothelial cells. TLR7 and TLR8 signal through the adaptor protein MYD88, which forms a signalling complex with interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK4 and tumour necrosis factor receptor-associated factor 6 (TRAF6). This leads to the nuclear translocation of nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), with downstream activation

of interferon- α (IFN α) and inflammatory cytokines⁴⁰. Additionally, a unique off-target effect of naked siRNA binding to cell-surface TLR3 has been shown to cause anti-angiogenic effects through the activation of IFN γ and interleukin-12 (IL-12)⁴¹. The use of nanoparticles can overcome these effects by shielding the dsRNAs from cell surface interaction.

The non-TLR-mediated innate immune response predominately occurs through the cytoplasmic RNA sensors retinoic acid-inducible gene 1 (RIG1; also known as DDX58) and dsRNA-binding protein kinase (PKR). On dsRNA binding, RIG1 binds to IFN β -promoter stimulator protein 1, which in turn activates interferon regulatory factor 3 (IRF3) and NF- κ B, causing production of IFN β and other inflammatory mediators⁴². PKR is a serine/threonine kinase that is expressed in most mammalian cells and that leads to the phosphorylation of eIF2 α and inhibitor of NF- κ B proteins followed by NF- κ B activation. This non-TLR-mediated pathway can occur in a wide variety of mammalian cells, but the TLR pathway is limited to haematopoietic cells. As nanoparticle targeting improves, innate immune response mechanisms could be better predicted on the basis of the tissue of interest. Some of the chemical modifications to the sugars, backbone or bases of dsRNA (such as modifications to the 2'-OH group in the ribose sugar

pH-sensitive polyplexes
Polyplexes (cationic polymer complexed with nucleic acid) that act as proton 'sponges', preventing acidification after endosomal fusion and allowing influx of counter ions, osmotic swelling and endosome rupture.

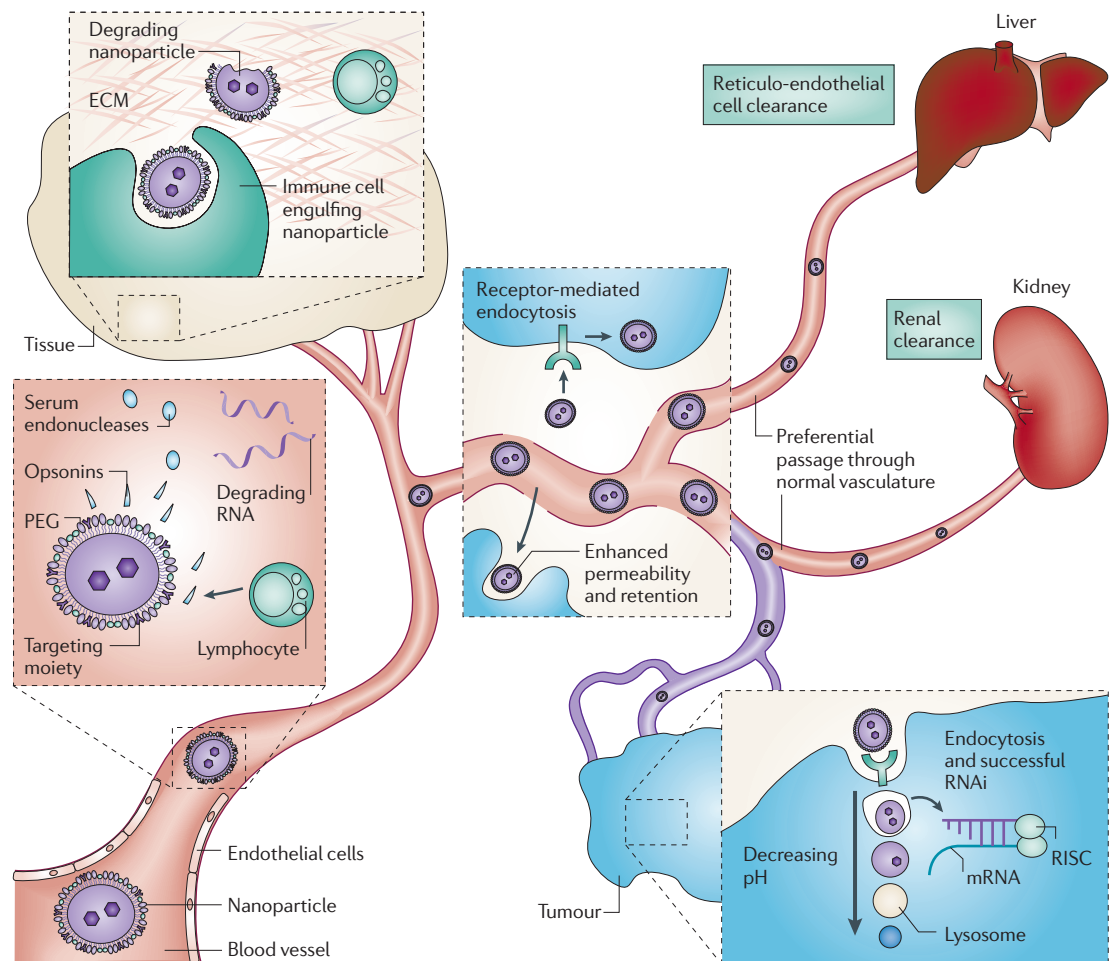


Figure 2 | Overcoming the biological barriers of RNAi delivery. Representation of the fate of a liposomal nanoparticle from the point of intravenous delivery to RNA-induced silencing complex (RISC) incorporation. Intravascular barriers include opsonins that mediate engulfment by macrophages and serum endonucleases that degrade any naked double-stranded RNAs (dsRNAs). Extravascular nanoparticles encounter degradation by immune cells and the extracellular matrix (ECM). Particles preferentially traffic to tumours by the enhanced permeability and retention effect and receptor-mediated endocytosis. Once intracellular, for successful RNA interference (RNAi) naked dsRNAs must undergo endosomal escape for RISC incorporation before acidification by lysosomes. PEG, polyethylene glycol.

backbone, which include 2'-OMe, 2'-F and 2'-deoxy (2'-H) or locked nucleic acid (LNA) modifications) that result in improved intravascular stabilization have also been able to evade innate immune recognition while retaining RNAi activity^{23,40}.

Nanoparticle-mediated toxicities. Although different types of delivery systems have shown efficacy in intratumoural delivery of siRNA, nanoparticle-related toxicity can also pose potential limitations. An ideal system would deliver a dsRNA payload while itself being biocompatible, biodegradable, mostly inert to the immune system and having predictable systemic clearance. Liposomes are phospholipid structures that are assembled when water is added to a dried lipid film, and can range in size from submicrometers to several micrometers in diameter. These can incorporate water-soluble or water-insoluble drugs, therapeutic proteins, DNA and siRNA into their hydrophobic or hydrophilic compartments. Several liposomal-based therapeutics have

been approved for various applications⁴³. Cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methyl sulphate (DOTMA) represent an attractive media for delivery owing to their electrostatic interaction with negatively charged nucleic acids forming lipoplexes. Although useful for *in vitro* experiments, these lipids have been shown to invoke a robust type I and type II interferon response⁴⁴. Cationic lipid-containing liposomes can cause gene expression changes independently of their RNAi payload, raising concerns about safety⁴⁵. Additionally, cationic liposomes have shown dose-dependent toxicity and pulmonary inflammation that arises as a result of reactive oxygen intermediates⁴⁶. As this effect was found to be charge related⁴⁶, neutral nanoliposomes such as 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)⁴⁷ might avoid such toxicities. These nanoliposomes are tenfold more effective in tissue delivery than DOTAP and have no identified toxicities to date^{48,49}. Negatively charged

Table 1 | **Clinical delivery of RNAi: challenges and solutions**

Challenges	Solutions
Biological barriers	
Intravascular degradation	Chemical modification and nanoparticle encapsulation
Tissue penetrance	Exploit abnormal angiogenesis and EPR effect
Intracellular delivery	Exploit receptor-mediated endocytosis
Intracellular trafficking	Incorporate biomaterials that enhance endosomal escape
Extracellular matrix	Optimize physico-chemical properties to escape immune and stromal interactions
Toxicities of RNAi	
Immune response to RNAi	Sequence optimization and oligonucleotide modifications
Carrier toxicity	Use of biodegradable, biocompatible and non-immunogenic materials
Off-target effects	Sequence optimization and oligonucleotide modifications
Oversaturation of RISC	Annotation of 'dose' effects on direct and indirect gene regulation
Tissue specificity	
Healthy versus cancer	Study of long-term RNAi side effects and exploit cancer features for targeting
Predictable delivery	Create carrier libraries annotating delivery efficacy based on healthy tissue and tumour type, and <i>in silico</i> modelling
Monitoring delivery	
Pharmacokinetics	Use of PEGylation or logic-embedded vectors
Pharmacodynamics	Use of bioluminescent imaging and novel biomarkers of delivery and response
Resistance	Study of altered processing machinery and SNP variants affecting RNAi

EPR, enhanced permeability and retention; PEG, polyethylene glycol; RISC, RNA-induced silencing complex; RNAi, RNA interference; SNP, single nucleotide polymorphism.

liposomes can induce an immune response and be subject to electrostatic repulsion by negatively charged cell membranes⁵⁰.

Other lipid and cholesterol formulations, such as poly-electrolyte complex (PEC), along with polyethylenimine (PEI), form micelles that can efficiently deliver siRNA without a detectable IFN α response⁵¹. The conjugation of siRNAs to high-density lipoprotein (HDL) or low-density lipoprotein (LDL) as nanovehicles has also been shown to allow effective delivery to select tissues. HDL-bound siRNAs are predominately taken up by the liver, adrenals, ovaries and kidneys by high-affinity binding to scavenger receptor class B, type I, and LDL-bound siRNAs predominately enter the liver by LDL receptor expression⁵². A fairly new class of lipid-like compounds that is composed of amino-alkylacrylate and amino-acrylamide materials has been termed lipidoids; and these lipidoids have proved safe and well-tolerated in mice and cynomolgus primates^{53,54}. These types of approaches could allow for the identification of not only well-tolerated delivery vehicles, but also the identification of compounds that require less substrate and payload, resulting in an improved therapeutic index.

The use of synthetic and natural polymer carrier systems has been extensively studied. Similar to lipids, their cationic substrate forms electrostatic interactions and allows the incorporation of negatively charged RNA. PEI, dendrimers and natural polymers, including chitosan, cyclodextrins and collagen, are among the more promising synthetic polymers that are currently in development. Although PEI has shown high transfection efficiency and the ability to buffer the acidic endosomal environment by the proton sponge effect,

some intracellular and extracellular cytotoxicity has been observed, and chemical modifications might be necessary before achieving an acceptable toxicity profile for clinical use⁵⁵. Dendrimers consist of a central core molecule from which multiple branching arms stem. Early generation dendrimers, such as polyamidoamine (PAMAM), were cytotoxic owing to cell membrane disruption caused by nanohole formation, membrane thinning and erosion⁵⁶. The biocompatibility of these agents has been improved through surface modifications⁵⁶.

Chitosan is a deacetylated derivative of chitin, an abundant mucopolysaccharide in crustaceans and insects. Its low cost, biodegradability, absence of immunogenicity and high delivery efficacy make it a suitable delivery carrier. Thus far, *in vivo* toxicology studies in mice have shown chitosan to be well tolerated⁵⁷. Tumour-specific delivery of chitosan particles with Arg-Gly-Asp peptide-labelling has recently been demonstrated⁵⁸, as has the ability of these particles to target tumour-associated endothelial cells⁵⁹. Cyclodextrins are cyclic oligomers of glucose with an amphipathic structure. The first targeted siRNA therapy in non-human primates was with a cyclodextrin polycation harbouring a transferrin ligand. This agent only caused increases in IL-6 and IFN levels, and hepatic and renal dysfunction, when administered at high doses⁶⁰. This led to the first Phase I trial of a targeted, nanoparticle-delivered siRNA therapy in humans⁷. Atelocollagen, a highly purified type I collagen that is free of immunogenic telopeptides, has a varied clinical utility, such as the promotion of wound healing and vessel bio-prosthesis. When complexed with siRNA to form nanoparticles it has high delivery efficiency with little immunogenicity⁶¹.

Non-immune off-target effects. One of the bigger hurdles that needs to be cleared to ensure specific transcript silencing is non-immune off-target effects of RNAi. Although siRNA was initially thought to be target gene specific, it soon became clear that cross-hybridization with unintended transcripts that contained partial identity to the siRNA sequence could result in gene silencing (sometimes with as few as 6–8 contiguous nucleotides)⁶². Just as miRNAs usually lead to translational suppression owing to imperfect matching with 3' UTRs¹⁸, siRNA sequences at positions 2–7 of the sense strand best predict off-target translational suppression, suggesting an miRNA-like off-target effect⁶³. Although concomitant silencing of many target genes by a single miRNA is an evolutionarily conserved phenomenon⁶⁴, potential toxicities of such unintended gene silencing can have important phenotypic effects⁶³. Off-target effects of siRNA treatment can account for observed phenotypic changes independently of the target gene of interest⁶⁵. Another unique property of 2'-O-methylation of the lead siRNA strand, other than attenuated intravascular degradation²² and immune stimulation⁴⁰, is a substantial tapering of the observed off-target effects without losing silencing of perfectly complementary targets⁶⁵. As RNAi therapeutics become a clinical reality, the long-term implications of these unintended off-target effects will need to be better understood and controlled.

Oversaturation of an RNAi-silencing complex. On successful intracellular delivery of dsRNA into the cytoplasm, some important barriers can still impede targeted gene silencing. We have shown that cancers can have altered levels of Droscha and Dicer, affecting the processing of precursors of siRNA such as short hairpin RNAs⁶⁶. Low expression levels of Dicer in ovarian, lung and breast cancers correlated with a worse prognosis and poor treatment response⁶⁶. Additionally, other investigators have shown that the high-level expression of short hairpin RNAs in the livers of mice overwhelmed the hepatic RNAi processing machinery, causing hepatic failure and death⁶⁷. Others have shown that the RISC can be oversaturated by competing siRNAs⁶⁸. Some studies suggest that the exogenous introduction of either siRNA or miRNA can lead to competition with endogenous miRNAs for RISC, leading to derepression of miRNA-regulated genes⁶⁹. Thus, as target genes are silenced, derepression of miRNA-regulated genes results in their re-expression, which could have consequences in the tissues of interest. However, whether systemic delivery of dsRNA will be capable of RISC oversaturation is currently unknown.

Limited ability to 'hit the mark'. Although the goal of harnessing RNAi is to attack cancer, some delivery into healthy tissue is unavoidable. Most *in vitro* and *in vivo* experiments rarely exceed a few weeks duration. However, cancer therapeutics must be effective for much longer periods of time: in some cases, years. Although many features of cancer allow for novel and specific targeting, consideration should be given to the unknown long-term consequences of RNAi delivery to healthy tissues, such as hepatic, renal or immune-mediated sequelae.

Once a guide strand is loaded into RISC, the complex is stabilized and able to recycle after multiple rounds of mRNA cleavage⁷⁰. This implies a need for predictable degradation of the guide sequence. However, normal tissues are likely to handle systemically introduced RNAi differently from cancerous tissues⁷¹. For example, intracellular levels of enhanced RNAi-1 nuclease can substantially alter the duration of gene silencing, which can vary from a few days to several months depending on tissue type and expression levels⁷². Cancer cells can have a shorter duration of gene silencing owing to intracellular dilution of the RNAi molecules with each cell division, whereas gene silencing in slowly dividing or senescent cells generally lasts much longer⁷³. Now that targeted nanoparticles delivering RNAi are entering the clinic⁷, it will be important to study the effects of long-term gene silencing in healthy tissues.

Exploiting the features of cancer

Tremendous progress has been made in exploiting the biological and physical features of cancer to enhance nanoparticle delivery. The ability of a nanoparticle to negotiate all known biological barriers, recognize cancer compared with healthy tissue and deliver therapeutic RNAi represent key determinants of therapeutic index. First-generation nanoparticles rely on the EPR effect for passive diffusion into the tumour microenvironment²⁸. As the EPR effect is due to enlarged fenestrations in tumour-associated neovasculature, there is greater nanoparticle accumulation in tumours compared with healthy tissues with normal vasculature^{25,26} (FIG. 2). The main subclasses that reach tumour cells through this mechanism include liposomes, polymers and dendrimers; however, liposomal drug formulations have the longest track record in the clinic. Liposomal doxorubicin entered the cancer clinic in 1995, spawning multiple subsequent liposomal formulations for delivering cytotoxic chemotherapies⁴³. Polymer and dendrimer drug conjugates have followed suit, and many different formulations are currently under clinical investigation⁷⁴. Although several liposomal^{50,75} and polymeric⁵⁷ nanoparticles can be effective delivery vehicles for *in vivo* RNAi, a more tumour-specific approach is desired.

Although second-generation nanoparticles can also arrive in tumours through the EPR effect, surface modifications allow a direct interface with cancer cells. By exploiting a particular epitope, receptor or other unique features of the cancer cell that are absent on healthy tissues, a more sophisticated mode of delivery by receptor-mediated endocytosis is realized³⁰ (FIG. 2). Attaching antibodies to the nanoparticle surface allows for improved surface-specific delivery⁷⁶, but these large moieties can increase cross-sectional area and make navigation across biological barriers more difficult and counterproductive⁷⁷. Aptamer–nanoparticle conjugates have recently been shown to specifically recognize several different cancer types with high efficiency, creating a new method of targeting cell-surface proteins⁷⁸. Through the use of natural ligands, such as folic acid⁷⁹ or transferrin^{76,60}, a 'trojan horse'-type mechanism allows for more directed delivery in cancers overexpressing these receptors.

Aptamer

A DNA or RNA oligonucleotide sequence with a high-affinity binding for specific proteins.

Logic-embedded vector

Vehicles that work in a time-sequential manner to cross biological barriers.

Mesoporous silicon

A biodegradable and biocompatible material made from non-oxidized silicon.

Polyethylene glycol

(PEG). A synthetic polymer that is non-toxic, non-immunogenic and highly water soluble.

The search for greater specificity for cancer using phage display biopanning approaches recently led to the discovery of a radiation-induced cell surface receptor (GRP78) that binds the ligand GIRLRG. Nanoparticles conjugated with GIRLRG were able to specifically target and deliver paclitaxel to radiation-treated cancer cells both *in vitro* and *in vivo*⁸⁰. Further refinement of these targeting techniques, without interfering with nanoparticle arrival or capacity to carry a dsRNA payload, should bring this technology a step closer to the clinic.

Third-generation nanoparticles, such as logic-embedded vectors (LEVs), may have the ability to confront biological barriers in a time- and sequence-dependent manner, resulting in therapeutic release at the desired lesion⁷⁷. These multi-component carriers rely on the material properties of the carriers and their payload⁴³. We recently reported the first *in vivo* multistage siRNA delivery system for sustained gene silencing⁸¹. We used systemically delivered first-stage microparticle carriers that consisted of mesoporous silicon, which enabled the sustained release of second-stage neutral nanoliposomes (DOPC). After a single intravenous administration, the siRNA-targeted gene of interest, *EphA2* (which encodes ephrin type A receptor 2), was inhibited for 3 weeks causing significant antitumour effects in two orthotopic models of ovarian cancer without observable toxicity⁸¹. Although this approach needs further development, it may open the door further for RNAi-based cancer therapeutics.

Systemic versus local delivery. This Review focuses on the systemic delivery of RNAi, but some studies have shown that direct intratumoural injection of nanoparticles containing siRNA can effectively result in gene silencing and tumour response⁵¹. Such an approach may offer higher bioavailability and reduced toxicity from systemic delivery, but many tumours cannot be reached by local delivery. Additionally, local delivery is not a viable option for treating micrometastatic disease. Therefore, although local delivery of RNAi could represent a treatment option for small, easily accessible primary tumours, such as melanoma, breast cancer or cervical cancer, it will probably have a limited role in clinical oncology.

Mathematical and computer modelling

The ability to predict and control the location of RNAi following systemic administration could help to maximize clinical efficacy. Mathematical and computer modelling has begun to shed light on how small modifications in nanoparticles can make dramatic differences to successful RNAi delivery. This has important implications for individualized treatment regimens that are affected by tumour histopathology, location, vasculature, microenvironment and host differences. Design maps provide preliminary multi-dimensional reference tables that show how alterations in size, shape, charge and chemical composition dictate the fate of nanoparticles⁸². These models take into account how the adhesive and endocytic performance of a nanoparticle is altered with changes in geometric (particle radius, for example), biophysical (such as degree of receptor-mediated endocytosis based on ligand-to-receptor surface density ratio) and biological (ligand-receptor

binding affinity, for example) variables under different biological conditions^{30,82}. Given the almost infinite possible nanoparticle combinations⁸³, multiscale *in silico* modelling is being developed to predict how various properties such as tumour growth, normal and tumour-associated vasculature, extracellular matrix, pressure distributions and response to treatment will affect the efficacy of delivery⁸⁴. These reference tables and models will establish a framework for predictable RNAi delivery in the highly plastic and sophisticated environment of malignancy.

Monitoring delivery and therapeutic response

The importance of pharmacokinetics and pharmacodynamics. In simple terms, pharmacokinetics is what the body does to a drug, and pharmacodynamics is what the drug does to the body. Therapeutic index of a drug refers to the ratio of therapeutic effect to toxicity. Thus, the ultimate success of nanoparticle-delivered RNAi will depend on the ability to negotiate biological barriers over a specific duration, selectively 'hit the mark', degrade predictably, be well tolerated and provide a high therapeutic index. Although naked siRNA can be rapidly cleared by the kidneys²⁰, nanoparticles can be cleared quickly through opsonization by highly concentrated phagocytic cells in the RES⁸⁵. This RES clearance seems to be predominately mediated by the phagocytic recognition of specific proteins that are absorbed on the surface of the nanoparticles in circulation. The use of polyethylene glycol (PEG) drug conjugates has proved to be a safe and effective strategy in prolonging the half-life of many US Food and Drug Administration-approved therapeutics. Addition of PEG to nanoparticles substantially mitigates RES uptake because its inherent hydrophilicity and steric repulsion effects reduce phagocyte interactions and complement activation⁸⁶. Although chain length, shape and density of PEG on nanoparticles help to dictate circulation half-life, it is important to emphasize that PEG adds considerable cross-sectional diameter and decreases the ability to add targeting moieties, thus decreasing EPR effect and targeting ability, respectively^{77,86}. As previously described, newer techniques such as the use of LEVs might allow the staged release of secondary particles⁸¹. This technique allows for targeting moieties to be used without the bulkiness of many conjugated PEG particles. An important element of RNAi delivery that must be considered is patient convenience and compliance. As many intravenous cancer therapies are delivered at weekly intervals in an outpatient clinic, successful accrual to clinical trials and patient adherence to treatment schedules will be important considerations in the design concept. Additionally, dose-finding Phase I/II trials will need to rely on models that simultaneously consider both protein expression modulation (efficacy) and dose-specific toxicity. An adaptive Bayesian model termed 'EffTox' is an example of how the probability of treatment efficacy and toxicity can be co-primary end points to determine Phase II dosing⁸⁷.

Biomarkers to monitor nanoparticle delivery and response to RNAi are currently underdeveloped. A recently completed first-in-human Phase I clinical trial used post-treatment biopsies to confirm intratumoural nanoparticle delivery and PCR of cleavage fragments

Quantum dots

Colloidal semiconductor nanocrystals with optical and electronic properties superior to conventional organic fluorophores that can be used for imaging purposes.

to confirm effective RNAi¹⁷. Imaging techniques using quantum dots⁸⁸ incorporated into nanoparticles show promise for high quality *in vivo* imaging and provide a viable means for long-term tracking of nanoparticle delivery⁸⁹. However, quantum dots can be toxic, and techniques to optimize their size and structure will determine their use in the clinic⁹⁰. Various other promising modes of *in vivo* molecular imaging are being developed and are currently in the early stages of clinical assessment⁹¹. Proof of intratumoural delivery still does not confirm endosomal escape, RISC incorporation and successful RNAi. Therefore, there is an urgent need to discover and develop non-invasive biological surrogates of RNAi. Despite the circulating RNases, investigators have been able to capture and amplify circulating mRNAs in cancer patients^{92,93}. Thus, one possible biomarker is circulating mRNA products following RISC cleavage, which could be detected with PCR amplification. Circulating mRNAs are thought to derive from nucleated apoptotic cells, tumour necrosis and nucleic acid shedding by metastases and micrometastases⁹⁴. If validated, these would represent an easy, inexpensive and non-invasive biomarker of RNAi. Another strategy would be to analyse protein expression changes following RNAi-based therapy. Recently, investigators generated isotope tags for relative and absolute quantification (iTRAQ)-labelled peptides from cell lysates following siRNA treatment and verified protein silencing using liquid chromatography–mass spectrometry (LC–MS)/MS⁹⁵.

Mechanisms of resistance. As RNAi enters the clinic, given the heterogeneity of tumours, the development of resistance seems inevitable. Additionally, some cancers could have an inherent resistance to some types of RNAi owing to factors such as ethnicity, somatic mutations,

altered RNAi processing machinery and germline polymorphisms. Significant genetic variation across major ethnic groups exists⁹⁶, which might partly account for variable responses to RNAi. In support of this, a recent report showed that germline single nucleotide polymorphisms (SNPs) within miRNA binding regions at 5' UTRs, coding sequence and 3' UTRs of target mRNAs lead to altered binding affinities⁹⁷. These miRNA–mRNA binding sites occurred at previously annotated SNPs that confer breast cancer susceptibility and lead to altered protein expression⁹⁷. This has important therapeutic implications as it suggests that the delivered miRNAs and siRNAs that target binding regions containing such SNPs could have altered efficacy. Alterations in the RNAi processing machinery such as Dicer, Drosha and RISC complex proteins will need to be further studied and accounted for in RNAi therapeutics^{66,98}. Although exogenous co-expression of the argonaute 2 protein in lung cancer cell lines enhanced RISC cleavage⁹⁹, cancers may adapt to decrease such proteins and effectively inhibit gene silencing. In anticipation of clinically useful RNAi, such resistance mechanisms in cancer will need to be understood.

Beyond the barriers

For more than a decade, there has been tremendous growth in our understanding of RNAi. The potent ability that small dsRNAs have in gene silencing makes them desirable as novel cancer therapeutics, but many biological barriers exist from drug infusion to target mRNA silencing. However, as shown by the first Phase I trial using targeted nanoparticles for RNAi delivery, rapid discoveries are leading us beyond these barriers. We believe, in due time, that the great translational potential of RNAi will continue to navigate such barriers and find its place in the oncology clinic.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

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