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# Short interfering RNA (siRNA): tool or therapeutic?

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Gene silencing by siRNA (short interfering RNA) is a still developing field in biology and has evolved as a novel post-transcriptional gene silencing strategy with therapeutic potential. With siRNAs, virtually every gene in the human genome contributing to a disease becomes amenable to regulation, thus opening unprecedented opportunities for drug discovery. Besides the well-established role for siRNA as a tool for target screening and validation *in vitro*, recent progress of siRNA delivery *in vivo* raised expectations for siRNA drugs as the up-and-coming 'magic bullet'. Whether siRNA compounds will make it as novel chemical entities from 'bench to bedside' will probably depend largely on improving their pharmacokinetics in terms of plasma stability and cellular uptake. Whereas locally administered siRNAs have already entered the first clinical trials, strategies for successful systemic delivery of siRNA are still in a preclinical stage of development. Irrespective of its therapeutic potential, RNAi (RNA interference) has unambiguously become a valuable tool for basic research in biology and thereby it will continue to have a major impact on medical science. In this review, we will give a brief overview about the history and current understanding of RNAi and focus on potential applications, especially as a therapeutic option to treat human disease.

## INTRODUCTION

Over the past 20 years the idea of targeting the molecular level of diseases by modifying gene expression patterns has attracted great interest. Based on the sequencing of the human genome and our burgeoning understanding of the molecular causes of diseases, the possibility of turning off pathogenic genes at will appears to be an appealing approach for treatment of a wide variety of clinical pathologies. Rationale-based drug design for development of compounds suited to specifically switch-off target genes holds promise in finding therapies for complex diseases, such as diabetes or cancer, with fewer side effects.

In 2002, RNAi (RNA interference) as a further mRNA-transcript-targeting strategy came to the attention of the general public by the technique's designation as the 'breakthrough of the year' by *Science* [1]. Since then, the number of publications utilizing this novel technology has increased exponentially, which has led to

a more profound understanding of the underlying mechanism of RNAi and has provided indications for potential therapeutic applications.

## BASICS OF RNAi

The first observations of gene silencing by dsRNA (double-stranded RNA) derived from experiments with plants. In the late 1980s and early 1990s, genetic experiments conducted on petunias yielded bizarre results. Introducing numerous copies of a gene coding for a deep-purple colour led to plants with white or patchy blossoms and not to a darker hue, as would have been expected [2,3]. Somehow, the introduced genes had silenced both themselves and the plant's intrinsic colour-coding genes.

At that time the use of ASOs (antisense oligonucleotides), short single-stranded stretches of DNA or RNA with complementary sequence to their target mRNA, was the state of the art for gene-silencing experiments. However, the observations in petunias were not compatible with the mechanism of gene down-regulation by ASOs.

The puzzling findings of colour alterations in petunias after transfection remained unexplained for nearly a decade, until, in 1998, Fire and co-workers [4] reported that injection of dsRNA into *Caenorhabditis elegans* resulted in potent gene silencing. dsRNA several hundred bases in length not only caused significant gene silencing, but was clearly more active than the corresponding single-stranded antisense molecules, a revolutionary finding in the field of molecular biology. Since the introduced dsRNA molecules interfered with the function of the targeted gene, the process was coined 'RNA interference'.

The unprecedented potency of gene silencing by RNAi prompted intensive research efforts over the next couple of years, leading to our current understanding of the RNAi machinery (for a review, see Mello and Conte [5]). These first mechanistic clues for the RNAi machinery were obtained from experiments using *Drosophila* extracts. In essence, RNAi is initiated by long stretches of dsRNA that undergo processing by an enzyme referred to as Dicer [6] (Figure 1). Dicer cuts the long stretches of dsRNA into duplexes with 19 paired nucleotides and two nucleotide overhangs at both 3′-ends [7]. These duplexes are called siRNA (short interfering RNA). The double-stranded siRNA then associates with RISC (RNA-induced silencing complex), a fairly large (approx. 160 kDa) protein complex comprising Argonaute proteins as the catalytic core of this complex [8]. Within RISC, the siRNA is unwound and the sense strand removed for degradation by cellular nucleases. The antisense strand of the siRNA directs RISC to the target mRNA sequence, where it anneals complementarily by Watson–Crick base pairing. Finally, the target mRNA is degraded by RISC endonuclease activity [9].
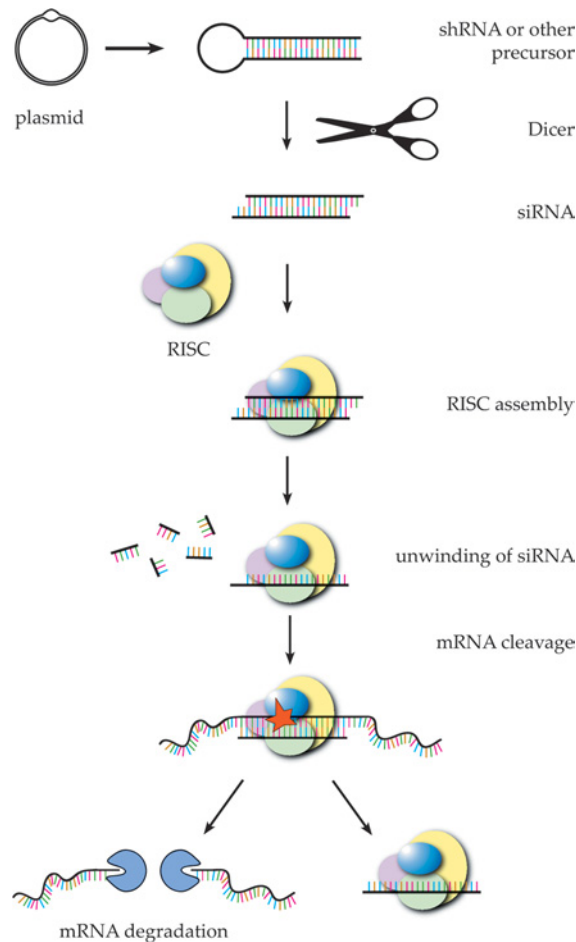


**Figure 1    RNAi machinery**
Long dsRNA precursors derived from endogenous genes or artificially introduced plasmids are cleaved by Dicer yielding siRNA. Alternatively, synthetic siRNA can be transfected into cells. siRNA is incorporated into RISC, followed by unwinding of the double-stranded molecule by the helicase activity of RISC. The sense strand of siRNA is removed and the antisense strand recruits targeted mRNA, which is cleaved by RISC and subsequently degraded by cellular nucleases.

This mechanism is fundamentally different from PTGS (post-transcriptional gene silencing) by ASOs (Table 1). Binding of ASOs to their target mRNA prevents protein translation either by steric hindrance of the ribosomal machinery or induction of mRNA degradation by Rnase H (ribonuclease H) [10].

Although gene silencing by ASOs was known to be a reliable method for targeted gene down-regulation in human cell culture, RNAi was first studied in *C. elegans* and plants and it was unclear whether it would work in mammalian cells too. In 2001, Tuschl and co-workers [11] provided the first evidence that siRNAs can mediate sequence-specific gene silencing in mammalian cells and that the dicing step can be bypassed by the transfection of siRNA molecules into cells. From that time on it was evident that RNAi could be used to study, and maybe even influence, the molecular basis of human disease.

**Table 1    Comparison of antisense oligonucleotides and siRNA**
Rnase H, ribonuclease H.

|  | ASOs | siRNA |
|---|---|---|
| Oligonucleotide chemistry | DNA or RNA | RNA |
| Structure | Single stranded; 16–30 nt | Double stranded; 19–21 nt with 2 nt overhangs |
| Chemical modifications available | Phosporothioate, $2'$-$O$-methoxyethyl, $2'$-$O$-methyl, peptide nucleic acid, locked nucleic acid and morpholino. | Cholesterol conjugate, locked nucleic acids, $2'$-deoxy-$2'$-fluorouridine and $2'$-fluoropyrimidines. |
| Intracellular processing from precursors | No | Yes (by Dicer) |
| mRNA cleavage | Rnase H | RISC |
| Standard doses effective *in vitro* | 50–400 nmol/l | 5–100 nmol/l |
| First *in vivo* use | 1991 [102] | 2002 [48] |
| Clinical trials | Numerous Phase 1–3 clinical trials ongoing; local or systemic administration. | First studies initiated; so far only local administration. |

RNAi represents an outstanding strategy for modulating gene expression. In contrast with other mRNA-targeting strategies, RNAi takes advantage of the physiological gene-silencing machinery, which might explain the excellent potency of RNAi.

The finding that long dsRNA was processed to siRNA prompted a search for interfering RNA encoded by the cellular genome itself. To date, a still increasing number of similar, yet different, short RNA species have been identified called miRNA (microRNA) [12], tiny non-coding RNA [13], heterochromatic RNA [14] or small modulatory RNA [15]. This classification may be somewhat artificial and many processes involving short RNA are still incompletely understood. miRNA, as the best characterized short RNA species, are approx. 70 nt in length and transcribed from their coding genes to form a double-stranded stem–loop hairpin molecule by self-complementary binding [16]. They have been found in all multicellular organisms investigated so far, from *Drosophila* to man. Approx. 250 different miRNA sequences are predicted in humans from genomic screening [17], with some 175 miRNAs already confirmed experimentally [18]. The miRNA hairpin is processed by Dicer and incorporated into RISC [19]. Most mammalian miRNAs inhibit protein synthesis by binding to the 3′ UTR (untranslated region) of target transcripts causing translational arrest [20]. Although even a single base mismatch may completely abrogate siRNA silencing [21], translational inhibition by miRNAs tolerates a mismatch to the mRNA target sequence [22]. As a consequence, a single miRNA is able to regulate many, maybe even hundreds of, different genes [23], which may contribute significantly to siRNA off-target effects discussed below.

## Why do cells need such an elaborate RNAi machinery?

According to our present understanding, RNAi has many different functions to accomplish. RNAi seems to contribute to the silencing of repetitive or transposable genetic elements ('jumping genes') [24]. These 'jumping genes' constitute a great deal of nuclear DNA and RNAi is thought to prevent unwanted and sometimes even dangerous re-insertion of such elements into our genome. Furthermore, RNAi is also considered to be part of a 'nucleic-acid-based immune system' [25], since RNAi has been shown to protect human cells from viral infection by knocking down viral transcripts [26,27]. Remarkably, some viruses have evolved suppressors of RNAi to circumvent the host defence [28,29]. Finally, short RNAs may contribute to genomic imprinting [30] or help in defining tissue-specific gene expression patterns by modulating DNA conformation [31].

## siRNA AS A TOOL FOR *IN VITRO* SCREENING

The leading application for RNAi technology to date is studying the knockdown of genes *in vitro*. For such a purpose, siRNA-mediated gene silencing provides a fast and reliable way to characterize a gene knockdown phenotype *in vitro* with striking potency at relatively low compound costs. Functional genomic approaches delineating a complete signal transduction pathway using multiple siRNAs are feasible as well. As a result, a plethora of studies were performed screening the relevance of candidate genes *in vitro* in diverse medical specialties.

For instance, RNAi was shown to suppress replication of HIV-1 not only in established cell lines, but also in primary peripheral blood lymphocytes [32]. For neurodegenerative diseases, specific siRNA allowed selective suppression of the toxic gain-of-function gene mutation for ALS (amyotrophic lateral sclerosis) [33] and Huntington's disease [34].

Since the RNAi-mediated gene-silencing machinery is also intact in cancer cells [35], multiple *in vitro* siRNA studies have evaluated the knockdown phenotype of

**Table 2**    **Online siRNA design tools**

| URL | Provider |
|-----|----------|
| http://katahdin.cshl.org:9331/RNAi/html/rnai.html | Cold Spring Harbor Laboratories |
| http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.htm | Wistar Institute |
| http://jura.wi.mit.edu/siRNAext/ | Whitehead Institute |
| http://www.ambion.com/techlib/misc/siRNA_finder.html | Ambion |
| http://design.dharmacon.com/ | Dharmacon |
| https://rnaidesigner.invitrogen.com/rnaiexpress/ | Invitrogen |

oncogenes. As a prime example, the effect of targeting the Bcr/Abl kinase oncogene in CML (chronic myeloid leukaemia) cells was tested [36]. The importance of this signalling molecule in the pathogenesis of CML is reflected by the clinical success of inhibiting Bcr/Abl signalling by imatinib (Gleevec™). Silencing the Bcr/Abl fusion protein by siRNA induced a strong apoptotic response in CML cells which was comparable with cell death induced by imatinib [36].

The siRNA approach is also suited for combination therapies targeting known chemoresistance factors (e.g. p53 and bcl-2) with the aim to sensitize tumour cells to cell death [37,38]. For example, P-gp (P-glycoprotein) is a transmembrane phosphoglycoprotein involved in the cellular export of diverse chemotherapeutic drugs such as vinblastine, doxorubicin and paclitaxel. Induction of P-gp gene expression, leading to increased drug efflux, is one of the mechanisms by which cancer cells acquire a multi-drug-resistant phenotype [39]. Silencing of acquired P-gp expression by RNAi re-sensitized multi-drug-resistant breast cancer cells to standard chemotherapeutics [40].

The early *in vitro* experiments rapidly established the role of siRNA-mediated knockdown as a valuable tool for studying the genetic impact on human diseases. However, bearing in mind the complexities of the RNAi machinery, designing siRNAs for experimental gene silencing has to be conducted carefully in order to achieve maximum mRNA knockdown while minimizing undesired effects. Moreover, intramolecular folding of target mRNA or association with other proteins may impede RISC binding. Even if a certain stretch of mRNA proves to be amenable to RISC binding, structural properties of siRNA can hamper RNAi efficacy. In this context, recently published data indicate that thermodynamic stability of siRNA duplexes can severely influence mRNA knockdown [41]. Several guidelines on siRNA design have been published [41], with those recommended by Dr Thomas Tuschl's laboratory being a common reference (http://www.rockefeller.edu/labheads/tuschl/sirna.html). In addition, there are several online siRNA design tools available on the web from academic institutions or commercial siRNA suppliers (Table 2). Of note, some of the most potent siRNA

molecules used are only partially in line with the selection criteria by common siRNA design guidelines and vice versa [42,43]. Therefore the optimal sequence of an siRNA molecule still needs to be determined empirically by comparing the gene-silencing ability of several lead candidate siRNA sequences.

Apart from designing a potent targeted siRNA, the choice of adequate control siRNAs is of outstanding importance in obtaining meaningful data from siRNA experiments. Given the fact that a match of as few as 7 nt [31] can induce a translational block, even the most carefully designed siRNA sequences might show a considerable amount of off-target effects when introduced into cells. For example, exogenously administered siRNAs could be 'mistaken' for miRNAs inducing translational repression. Furthermore, it turned out that RNAi does not only degrade mRNA transcripts or induce translational inhibition, but is also capable of influencing mRNA transcription by induction of heterochromatin formation [14] and DNA methylation [44]. Of note, preliminary data reported recently indicate that siRNA can even induce gene expression in certain settings [45]. A common approach to exclude significant off-target effects of synthetic siRNAs is monitoring the transcriptome of a cell by gene expression profiling [46]. If significant changes in untargeted mRNA sequences occur before knockdown of targeted mRNA could lead to such alterations, one has to assume that the tested siRNA is not exclusively specific. Therefore, for experiments studying the effect of siRNA-induced gene silencing, it is recommended to apply several independent siRNAs targeting different sequences of the target gene as well as non-targeting control siRNAs.

Effective RNAi can be achieved not only by transfection of siRNAs, but also by introduction of a shRNA (short hairpin RNA) expressing plasmid into human cells [47]. The intracellularly transcribed hairpin RNA is processed by Dicer and the generated functional siRNAs are incorporated into RISC. Transfection of cells with shRNA-producing plasmids provides several advantages. The RNAi effect can be more pronounced and long-lived [48] compared with transient transfection by siRNA. Once the plasmid is delivered into the cell, it can produce large numbers of shRNAs and subsequently siRNA molecules, whereas synthetically synthesized siRNAs probably are not amplified in mammalian cells [23]. On the other hand, transfection efficiency of plasmids is often lower than that of synthetically synthesized siRNAs [49].

Besides experiments silencing selected target genes, ambitious large-scale gene knockdown studies have become feasible with the advent of RNAi [50]. High-throughput applications using RNA-mediated interference have emerged in recent years as possibly the most powerful of a 'second wave' of functional genomic technologies [51]. An approach taking advantage of RNAi that might prove particularly suitable for drug
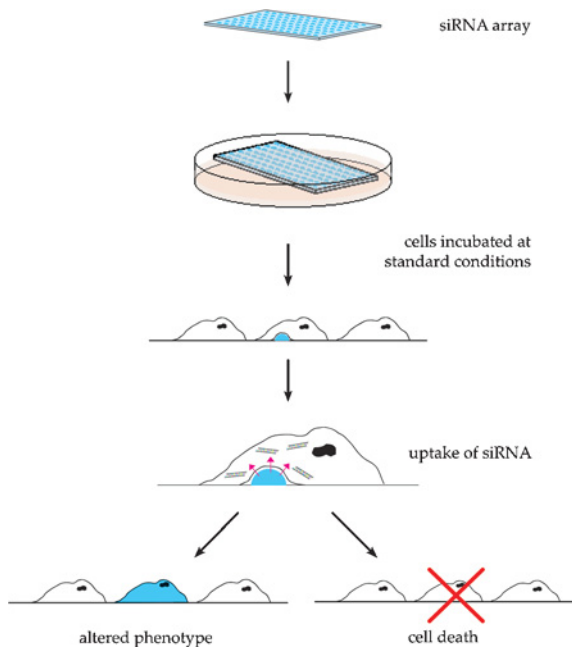
**Figure 2 TCA**

siRNA is arrayed on to a solid support slide and pre-complexed with uptake-enhancing lipids. The array is placed in a cell culture dish, and cells are allowed to overgrow the slide. As cells reach the siRNA spots, they incorporate the liposome–oligonucleotide complex and a potential phenotype becomes detectable.

development is the TCA (transfected cell array; Figure 2). TCAs offer a high-throughput method for target gene identification and phenotyping of studied cells. The principle of TCAs is similar to gene expression microarrays, where hundreds or thousands of short oligonucleotides are spotted on to slides to measure mRNA levels. In a first step, siRNA nucleotides [52] or shRNA [53] targeting different sequences of one or several genes are dissolved in a gelatine solution and arrayed on to a solid support slide using a standard robotic arrayer or piezo-electric dispensing printer. In contrast with gene expression profiling microarrays, the oligonucleotides in the TCAs cannot be immobilized on to the glass surface, as covalent binding would preclude the oligonucleotides from entering the cells. Subsequently, the oligonucleotide array is covered with cells allowing complete overgrowth of the array. As cells reach the siRNA spots, they incorporate the siRNA oligonucleotide, and a potential knockdown phenotype becomes detectable. Because the oligonucleotide is placed on the slide first and transfection of adherent cells starts from the adherent cell surface and is not added from 'above' as in cell culture media, the process is called 'reverse transfection' [49] or 'surfection' [54]. Notably, TCAs enable high-throughput loss-of-function studies of cells within their micro-environment, which might be critical for gene function in some contexts. Another favourable feature is that TCA requires fewer cells per gene tested than standard microwell plate-

based protocols. This is important particularly for human primary cells, where numbers of isolated cells are often limited.

Regardless of whether single gene silencing or TCA is used, spontaneous cellular uptake of unmodified siRNA *in vitro* is poor. Cells are not 'willing' to take up foreign nucleic acids and charged oligonucleotides, such as ASO or siRNA, barely pass the cell lipid layer spontaneously. Thus, for the standard *in vitro* experiment, siRNA needs to be pre-complexed with uptake-enhancing lipids to obtain penetration for intracellular delivery. Transfection efficiency can be monitored by using fluorochrome-coupled siRNAs [55] or detecting reporter gene expression after co-transfection of siRNA and a reporter gene plasmid [53]. Although siRNA uptake issues *in vitro* can be solved by carefully optimizing the pre-incubation conditions with the uptake enhancer, the cellular uptake of siRNA *in vivo* still represents a real challenge.

## siRNA FOR *IN VIVO* STUDIES

Given the impressive RNAi results *in vitro*, it took only a year before the first mammalian RNAi *in vivo* data were available. In 2002, McCaffrey et al. [48] published the first proof-of-concept study for gene silencing by siRNAs and shRNAs *in vivo*. A luciferase-expressing plasmid was co-injected intravenously with a synthetically synthesized siRNA or an shRNA-expressing plasmid into mice. Luciferase activity in livers was monitored by quantitative whole-body imaging. Mice receiving luciferase-targeting RNAi treatment displayed a more than 80 % decrease in hepatic luciferase expression compared with controls.

A year later, the first RNAi *in vivo* study with a therapeutic intention was reported by Song et al. [56] in a mouse model of fulminant hepatitis. Acute liver failure was induced by injection of either Concanavalin A or Fas-activating antibody. Fas gene silencing mediated by administration of Fas gene-targeting siRNAs by tail vein injection virtually eliminated the liver-damaging effects of hepatotoxic agents. Impressively, all mock-treated mice challenged with Fas-activating antibody died 3 days after treatment due to acute liver failure, whereas 80 % of Fas siRNA-treated mice survived for 10 days.

As promising as these studies are, both had to deal with a severe drawback of systemic siRNA application. The administration of siRNAs was performed by hydrodynamic high-pressure tail vein injection. In doing so, a large volume (approx. 1 ml of siRNA solution) is rapidly injected into the tail vein frequently causing acute cardiac failure in mice. Given an average mouse weight of 20 g, this application would equal a rapid high-pressure bolus injection of 3.5 litres into a 70 kg patient, an administration obviously not applicable in man.

The reason for using the hydrodynamic injection of siRNA in these studies is due to the pharmacokinetics of siRNA. In terms of pharmacokinetic properties,

siRNAs are similar to ASOs. Systemically administered siRNA readily accumulate in liver, spleen and kidneys, whereas other tissues, including brain, show only moderate-to-low uptake [57]. In plasma, single-stranded RNA molecules are degraded by nucleases within seconds, whereas dsRNA molecules, such as siRNAs, have a somewhat longer half-life [58]. For example, in rats, systemically administered unmodified siRNA has a half-life of approx. 6 min [59], which is probably still well below the stability requirements needed to become effective by standard (non-high pressure) systemic administration.

In order to circumvent the challenge of systemic siRNA delivery, local administration of siRNAs was tested in a variety of animal studies. For instance, mice were pre-treated with anti-(TNF-$\alpha$) (tumour necrosis factor-$\alpha$) siRNA intraperitoneally before injection of LPS (lipopolysaccharide) intraperitoneally to induce a septic-shock-like syndrome [60]. Coinciding with a decrease in TNF-$\alpha$ in peritoneal lavage, siRNA treatment protected the animals from septic-shock-induced death. In another *in vivo* study repeated intratumoral administration of siRNA targeting CSF-1 (colony-stimulating factor-1) in a mammary carcinoma xenograft model led to selective down-regulated target protein expression in tumour lysates and suppressed tumour growth in mice [61].

The airway epithelium seems to be particularly well suited for the uptake of RNAi-based drugs. The respiratory mucosa offers a large easily accessible and well-absorbing area for siRNA uptake by inhalation, also conferring the advantage of bypassing extensive trapping, metabolism and excretion by liver and kidneys. Since RNAi has evolved, among its other functions, as a kind of viral defence, viral airway infection may be well suited for RNAi treatment. In a study published last year, Bitko and co-workers [42] evaluated a siRNA strategy targeting RSV (respiratory syncytial virus) and PIV (parainfluenza virus), two major pathogens causing croup, pneumonia and bronchiolitis [62]. Chemically synthesized siRNAs targeting RSV or PIV were administered intranasally to infected mice without or with the addition of an uptake-enhancing reagent. This study revealed that intranasally administered siRNA almost completely prevented viral replication of RSV and PIV by lowering viral titres by 99.98% and 99% respectively. When assessed for viral-induced pathology, lungs of animals treated by siRNA were virtually protected from tissue damage with a pulmonary pathology score similar to uninfected animals. Even when treatment was delayed for days, intranasal siRNA improved pulmonary pathology remarkably and ameliorated clinical symptoms such as respiratory distress. Importantly, this success was a direct consequence of RNAi and was apparently not mediated by triggering a non-specific IFN (interferon) response [42], as was shown for other formulations of siRNAs [63].

These preclinical examples support the notion that local administration of siRNA could be more favourable for clinical development at the moment than systemic approaches.

For systemic delivery of siRNA by non-hydrodynamic methods, the pharmacokinetic profile of siRNA needs substantial improvement. The challenge is to increase nuclease resistance and improve cellular uptake while maintaining gene-silencing properties. Perhaps the most widespread approach is the chemical modification of the siRNA itself. With regard to this point, a great deal of experience has been gained from the clinical development of ASOs. Unmodified ASOs and siRNA share the problem of limited stability and rapid degradation by serum nucleases; however, chemically modified ASOs are reasonably stable in human plasma without compromising their gene-silencing properties [64]. This improved serum stability paved the way for systemic administration of ASOs for therapeutic purposes. Today, there are several ASO compounds chemically stabilized using different chemistries in Phase 1–3 clinical evaluation (for further information on ASOs, see Gleave and Monia [65] or Opalinska and Gewirtz [10]).

This valuable know-how regarding serum stability has been transferred from ASO to siRNA development. Modifications of the phosphate backbone (for example, phosphorothioates [66]), the riboses (for example, locked nucleic acids [67], 2′-deoxy-2′-fluorouridine [67] and 2′-O-methyl [68]) or base (for example, 2′-fluoropyrimidines [69]) have been engineered to provide siRNAs with increased nuclease resistance while retaining induction of RNAi. In order to enhance the cellular uptake of oligonucleotides, siRNA conjugated with lipophilic derivates of cholesterol, lithocholic acid or lauric acid have been synthesized [70]. This approach is reminiscent of lipid complex formulations of siRNA with the advantage that resulting molecules are smaller and chemically well defined. Notably, in contrast with conjugated siRNA, alterations in pre-complexing conditions (including transport and storage) used to assemble siRNA–lipid complexes can significantly alter the chemical and biological properties of the siRNA formulation [71]. Cholesterol conjugated to the 3′-end of the siRNA sense strand by means of a pyrrolidine linker (chol–siRNA) has dramatically prolonged elimination *in vivo* half-life approaching approx. 95 min [59].

In a hallmark study using chol–siRNAs, Soutschek et al. [59] demonstrated, for the first time, that systemically administered siRNAs induced RNAi-mediated silencing of an endogenous gene after non-hydrodynamic intravenous injection in mice. ApoB (apolipoprotein B) was selected as the target, which is expressed in large quantities in hepatocytes and is essential for the formation and metabolism of LDLs (low-density lipoproteins). Serum levels of apoB, LDL and cholesterol correlate significantly with increased risk of atherosclerosis [72],

and lowering serum cholesterol and LDL levels is reported for prevention and management of coronary artery disease and stroke [73]. Down-regulation of apoB is a potential strategy to lower circulating LDL, as previous gene-knockout studies in mice have shown that heterozygous knockout of apoB leads to a decrease in blood cholesterol levels and to resistance to diet-induced hypercholesterolaemia [74]. Indeed, targeting endogenous apoB by siRNAs led to diminished apoB protein levels and, subsequently, lowered blood cholesterol [59]. Notably, silencing apoB was not limited to liver and serum as chol–siRNA also reduced apoB levels significantly in the jejunum.

Besides modification of siRNA nucleotides, lipid-based formulations of siRNAs might also prove to be useful for *in vivo* use analogous with non-viral transfection strategies. Liposomal formulations improved the pharmacokinetics of drugs, such as amphotericin B [75] or doxorubicin [76], and are approved for clinical use, raising hopes that similar liposomal formulations might be applicable to siRNA as well. In a study published recently [77], systemic administration of synthetic siRNAs complexed with low-molecular-mass polyethylenimine resulted in delivery of siRNA into subcutaneous tumour xenografts and Her-2 target down-regulation in mice. Nevertheless, lipid-based formulations have occasionally been associated with higher toxicity in the past [78]. To minimize toxicity, modifications of naturally occurring lipids (e.g. cardiolipin by NeoPharm) are being developed, which might display an acceptable toxicity profile *in vivo*. Interestingly, lipid-encapsulated siRNA can induce a profound IFN response *in vitro* and *in vivo*, whereas independent administration of single agents does not elicit detectable activation of murine defence [63]. Functional non-immunostimulatory siRNA–lipid complexes can be designed [63], but careful evaluation of formulations is required.

In addition to improved pharmacokinetics, lipid carriers can be modified with cell-type-specific ligands that render them amenable to cellular binding and cell-targeted uptake of siRNA. Recently, formulation of siRNA into CDP (cyclodextrin polymer)-based vehicles modified with transferrin as the ligand has been reported in a Ewing sarcoma model [79]. In approx. 90 % of all Ewing's sarcoma family tumours, chromosomal translocations lead to the expression of the EWS–FLI1 fusion oncogene product [80], which is thought to be involved in malignant transformation of cells. The siRNA was condensed into CDP nanoparticles and coupled with transferrin ligands to allow receptor-mediated endocytosis of the complex via the transferrin receptor, which is typically up-regulated on the surface of tumour cells. Systemic administration of EWS–FLI1-targeting CDP–transferrin-formulated siRNA markedly inhibited tumour growth in a murine model of disseminated Ewing's sarcoma family tumour disease [79], whereas

CDP-formulated siRNA without transferrin did not. These data clearly underline the relevance for lipid-formulation-mediated and -targeted delivery of siRNA *in vivo*.

Other than transient transfection by siRNA, stably transfected shRNA-producing plasmids also offer *in vivo* advantages in terms of longevity and potency of the RNAi process. For transfection of target cells by shRNA-producing plasmids, several expression strategies were adopted from the field of gene therapy, because both approaches are directed to achieve a constant long-lived and well-controlled RNA expression in the targeted cells.

The most efficient way for gene delivery is the use of viral vectors. For RNAi, the successful application of retroviral [81], adenoviral [82] and AAV (adeno-associated viral) [43] strategies has been demonstrated *in vivo*. Among the retroviral vectors, most studies have been done on lentiviruses as they are particularly attractive due to their ability to also transduce terminally differentiated quiescent cells, including neurons. Very recently, shRNA-producing plasmids were successfully delivered to neurons using a lentiviral vector in a transgenic mouse model of familial ALS [83]. Intraspinal injection of a lentiviral vector inducing RNAi-mediated silencing of mutated SOD1 (superoxide dismutase 1) substantially retarded both the onset and progression rate of the disease in mice. However, a shortcoming for their potential clinical use is that retroviruses insert into the host genome with poor control of the insertion site, which can trigger oncogenic transformation, such as in the SCID (severe combined immunodeficiency) clinical trial [84,85].

rAAV (recombinant AAV) vectors appear to be a good compromise in terms of efficacy and safety for RNAi transfection *in vivo*. AAV vectors commonly inhabit hosts without causing detectable pathology [86]. Since they require a helper virus for replication and do not spread once inside a cell, they do not cause an inflammatory response and show higher transfection efficiency than lentiviruses, including in quiescent cells such as neurons. Indeed, clinical trials using rAAV vectors for gene therapy are under way [87]. RNAi using rAAV vectors was achieved in a rodent model of spinocerebellar ataxia [43]. Transgenic mice harbouring mutant human ataxin-1 had a significant improvement in cerebellar pathology and motor performance after intracranial delivery of rAAV shRNA targeting the disease-causing ataxin-1. Although rAAV vectors do not usually integrate into the host genome, insertions in up to 10 % of transfected cells have been reported [88], probably by fusing pre-existing chromosomal breaks [89]. Therefore oncogenic mutagenesis is less likely than with lentiviral vectors, but cannot be excluded entirely. Moreover, although RNAi delivered by viral vectors provides many advantages over administration

of synthetically synthesized siRNA with respect to transfection efficacy, potency and longevity of RNAi silencing *in vivo*, the need for laborious and expensive genetic engineering will probably prolong their time to approval for human therapy substantially.

Not all of the impressive effects of RNAi observed *in vivo* are likely to be due solely to target-specific silencing. On top of the off-target effects discussed above, the *in vivo* use of RNAi has to deal, in particular, with the unspecific effects caused by activation of two components of the innate immune system: the IFN response and signalling mediated by TLRs (Toll-like receptors) [90].

The IFN response is a core component of the intracellular defence against viral infection. Among many other signalling pathways, the RNA-dependent PKR (dsRNA-dependent protein kinase) becomes activated upon intracellular detection of relatively long (approx. > 50 nt) dsRNA oligonucleotides which are common intermediates in viral replication. Activated PKR phosphorylates and inactivates eIF2A (eukaryotic translation initiation factor 2A), thereby globally halting translation of proteins [91]. Reports have been accumulating showing the absence or presence of an IFN response for RNAi *in vivo* [42,63,92,93]. In essence, activation of the IFN response by siRNA can never be ruled out entirely *a priori* and therefore has to be excluded for every compound or setting individually.

Furthermore, siRNAs are able to activate the innate immune system via TLRs. TLRs are a class of pattern-recognition receptors expressed on immune cells, including macrophages, monocytes, dendritic cells and B-cells. Host defence uses TLRs to distinguish pathogen-associated molecular structures from self [94]. So far, eleven TLRs have been identified in mammals that recognize pathogen-associated structures such as bacterial cell wall materials or bacterial and viral genomic DNA/RNA [95]. TLRs 3, 7 and 8 recognize viral and synthetic single-stranded RNAs [96] and dsRNAs [97], including siRNA [98]. Once activated, the TLR-induced immune activation results in a plethora of changes in gene expression. Further studies are warranted to elucidate in detail the immune activation by siRNA.

## FIRST siRNA CLINICAL TRIALS

As for other previous nucleotide-based therapeutic approaches, the successful demonstration of siRNA *in vivo* caused an outbreak of enthusiasm for the development of RNAi-based therapeutics. Currently, there are several biotechnology companies developing RNAi-based drugs for clinical use. As learnt from ASO drug development, local administration of siRNA was chosen as the probably safest bet to begin with.

The first ever clinical trial in man administering an siRNA-based therapeutic was initiated in autumn 2004 by Acuity Pharmaceuticals in patients with AMD (age-related macular degeneration). An siRNA targeting the growth factor VEGF (vascular endothelial growth factor), which is believed to be a primary cause of overgrowth of blood vessels in the 'wet' form of AMD, was administered by intravitreal injection. The primary goal of this Phase 1 study was to evaluate the safety of the siRNA and it is expected to be completed in summer 2005. Independently, Sirna Therapeutics developed an siRNA targeting VEGF receptor 1 for AMD (Sirna-027) and started a clinical trial a few months later. Only recently, interim data from this ongoing Phase 1 trial were presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO, 1–5 May 2005, Fort Lauderdale, FL, U.S.A.). A single intravitreal dose of Sirna-027, ranging from 100–800 $\mu$g, appears to be safe and well tolerated with no systemic or local adverse events related to the drug. So far, according to the clinical investigators, all patients enrolled in this trial have experienced visual acuity stabilization during their trial participation. Since no dose-limiting toxicity has been observed, dose escalation will continue with the objective of defining a maximum tolerated dose. These first preliminary data are encouraging in terms of tolerability of siRNA compounds and further results regarding the clinical activity of both siRNA compounds are eagerly awaited.

Further clinical trials for siRNA-based compounds are intended, and the diseases targeted cover a broad range spanning from ocular diseases, viral infections and cancer to metabolic disorders (Table 3). Alnylam, an siRNA company probably holding the biggest share of intellectual property regarding RNAi technology, is setting up to initiate clinical trials for targeting RSV. To date, no effective therapy for established RSV-induced disease or prophylactic vaccines preventing full-blown infection are available, despite intensive research efforts [99]. A clinical trial investigating local administration of an siRNA targeting RSV mRNA by inhalation is expected to start by the first half of 2006. For treatment of HIV, Benitec Ltd pursues a strategy of harvesting leucocytes from the peripheral blood, treating them with an anti-HIV shRNA-producing DNA construct and subsequently re-infusing the transfected cells. This gene-therapy-based approach attacking multiple targets of the HIV virus simultaneously is expected to enter clinical trials in 2005.

Reflecting the seemingly unlimited potential of RNAi to treat human disease, companies such as ToleroTech, Atugen or Genesis R&D have announced plans to start clinical trials with siRNA-based therapeutics for transplant rejection, diabetes and asthma respectively.

## OUTLOOK FOR siRNAs AS THERAPEUTICS

There are clearly many good reasons qualifying siRNA compounds for development as therapeutic drugs. As

**Table 3   RNAi-based therapies in late preclinical/early clinical development**
IND, investigational new drug.

| Company | Product | Disease/condition | Progress |
|---|---|---|---|
| Acuity Pharmaceuticals (www.acuitypharma.com) | Cand5 | AMD | Phase I initiated October 2004 |
| Sirna Therapeutics (www.sirna.com) | Sirna-027 | AMD | Phase I initiated November 2004 |
| Alnylam (www.alnylam.com) | Ocular siRNA Tx | AMD | Phase I due 2005 |
|  | RSV siRNA Tx | RSV infection | Phase I due 2006 |
| Benitec (www.benitec.com) | BLT-HCV | Hepatitis C | Phase I due 2005 |
|  | RNAi HIV Tx | HIV and AIDS | Phase I due 2005 |
| Genesis R&D (www.genesis.co.nz) | RNAi | Asthma | Phase I due 2006 |
| ToleroTech (www.tolerotech.com) | ToleroVax | Transplant rejection | IND application filing due 2005 |
| Atugen (www.atugen.com) | atuRNAi | Diabetes | IND application filing due 2005/2006 |

for other mRNA-targeting strategies, one of the main advantages of siRNA technology for drug development is the option to design a specific siRNA compound for any target gene of interest within short periods of time and at comparatively low development costs. This flexibility provides a clear advantage compared with small-molecule-based drug development. However, even the most careful siRNA drug design does not guarantee exclusive target specificity. Off-target effects, including unwanted miRNA mimicking by siRNA-based drugs, might pose a major challenge for the intended selective silencing of the target gene.

Besides optimizing the siRNA drug design, innovative trial designs with alternative end points have the potential to improve the efficiency of clinical testing of siRNAs [100]. Since the intended mode of action of siRNA compounds is well defined, evaluating target gene expression knockdown for guiding dose escalation in early phases of clinical trials, rather than relying solely on the concept of maximum tolerated dose, provides the opportunity for obtaining siRNA drug levels with optimal biological activity. In addition to traditional clinical criteria for Phase 1 dose-escalation studies, measurement of protein or mRNA levels in target or surrogate patient specimens (e.g. PBMCs) can be performed to determine the pharmacokinetic/pharmacodynamic relationship of an siRNA drug. According to the concept of 'optimal biological dose', the lowest dose level with the maximum of intended target down-regulation will be regarded as the optimal biological dose and recommended for further Phase 2 testing. This would not only allow establishment of the 'proof of concept' of RNAi-based therapeutics in man, but might also help to avoid expensive and potentially harmful overdosing of siRNA compounds in man. However, there might also be effects by siRNA beyond the intended specific target down-regulation. If such off-target effects elicited by siRNA result in alterations of gene expression adding net clinical benefit, they would be clearly welcome even though not intended. For example, as learnt from the clinical development of ASOs, immune stimulation by sequence

motifs in oligonucleotides (i.e. CpG) causing activation of the innate immune system might be favourable in particular for nucleotide-based cancer therapeutics in addition to gene silencing for the overall antitumour activity [10].

Therefore it needs to be carefully considered in early phases of drug development how dose finding for siRNA will be performed. Potentially, it will be necessary to study multiple biomarkers to elucidate the entire activity spectrum of siRNA drugs. From this background it becomes obvious that for a rational decision about clinical trial design it is essential to have a comprehensive understanding of all of the preclinical *in vivo* activity of an siRNA compound, including a detailed pharmacokinetic/pharmacodynamic relationship. Furthermore, any biomarker designated for clinical testing should be thoroughly validated *in vivo* prior to clinical use.

## CONCLUSIONS

RNAi is a still developing field of biology, which could prove to be of enormous importance for the therapy of various human diseases. So far, current drug therapy is based on only approx. 500 molecular targets which can be influenced by common therapeutics such as small molecules [101]. With siRNA, virtually every gene in the human genome would become amenable to regulation, thus opening unprecedented opportunities in the field of drug discovery. Whether siRNA will make it as novel chemical entity from 'bench to bedside' will probably depend largely on overcoming pharmacokinetic obstacles such as plasma stability and, especially, cellular uptake. In the short term, local administration of siRNA seems to be more likely than systemic application. Accordingly, Phase 1 clinical trials initiated so far are limited to achieving knockdown of disease-causing genes by local delivery of siRNA. As the field of oligonucleotide therapeutics has matured after the initial enthusiasm, experience gathered from years of intensive

research for systemic administration of nucleic-acid-based compounds may help to solve the challenging pharmacokinetic issues for siRNA therapeutics. In the long term, chemical modification as well as lipid-based formulation in combination with specific ligands for cell-targeted uptake of siRNA might pave the way for successful systemic delivery of siRNA-based therapeutics. However, irrespective of its therapeutic potential, RNAi has definitely become a well-established tool in the field of basic research and, thereby, it will continue to have a major impact on medical science.

## ACKNOWLEDGMENTS

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