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# Maintaining the silence: reflections on long-term RNAi

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Since the demonstration of RNA interference (RNAi) in mammalian cells, considerable research and financial effort has gone towards implementing RNAi as a viable therapeutic platform. RNAi is, without doubt, the most promising strategy for the treatment of human genetic disorders. Because many of the targets proposed for RNAi therapy require chronic treatment, researchers agree that the emphasis must now be placed on the safe and long-term application of RNAi drugs to reap the benefits at last.

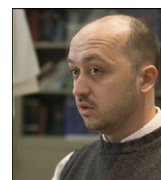
RNA interference (RNAi) represents a powerful and versatile gene silencing process in which double-stranded RNA (dsRNA) triggers the sequence-specific cleavage of mRNA transcripts. An explosion of research that followed its discovery in *Caenorhabditis elegans* in 1998 [1] has recently resulted in RNAi-based protocols for analysis of gene function and therapeutic applications in humans [2,3]. Following pioneering research in plants [4,5] and nematodes [1,6], RNAi was demonstrated in mammalian cells in 2001 by Tuschl and colleagues, who were the first to apply short interfering RNAs (siRNAs) to guide the sequence-specific suppression of gene expression [7,8]. In theory, every gene is amenable to RNAi-based silencing, and therefore these key papers have led to a surge of excitement among researchers in the medical field. For about a decade now, the quest for efficient RNAi therapeutics to treat a wide variety of pathologies has been ongoing and has already made remarkable progress, but we must emphasize that further improvements are still needed to pave the way for RNAi to develop into a viable therapeutic approach.

Obviously, the translation of RNAi into a broadly applicable therapeutic platform needs appropriate pharmaceutical considerations at different levels in clinical drug development (e.g. siRNA design [9,10] and siRNA formulation [11]). In straightforward terms, it can be stated that the most efficient delivery of the most potent siRNA is expected to result in maximal suppression of a target gene.

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Stefaan De Smedt studied pharmacy and received his MS degree in pharmaceutical sciences in 1990 at Ghent University. As a scholar of the Belgian Institute for the



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To extrapolate RNAi from bench to bedside, it is imperative to consider not only the gene knockdown intensity but also the dynamics of gene silencing [12]. On the one hand, precise control over the duration of RNAi gene knockdown can provide new information on complex cellular pathways implying interactions between multiple genes, without having to rely on more elaborate transgene technologies. On the other hand, from a therapeutic point of view, being the main focus of this review, it may be relevant to silence a pathogenic gene for a longer period of time, to improve the clinical outcome. Prolonging the gene silencing effect of a specific siRNA formulation may be beneficial for chronic patient adherence if the administration frequency in the dosing schedule could be significantly scaled down.

Although many *in vivo* studies have already highlighted the enormous therapeutic potential of RNAi therapeutic strategies [13], recent studies have shown unintended off-target effects (OTEs), activation of immune and inflammatory pathways and perturbation of endogenous cellular pathways by small dsRNA drugs. Especially when placing the emphasis on long-term treatment of chronic diseases, toxicity issues become increasingly important and should be dealt with accordingly. At present, few data are available on the long-term toxicity of RNAi triggers but appropriate attention should be paid to these issues in the context of prolonging RNAi silencing.

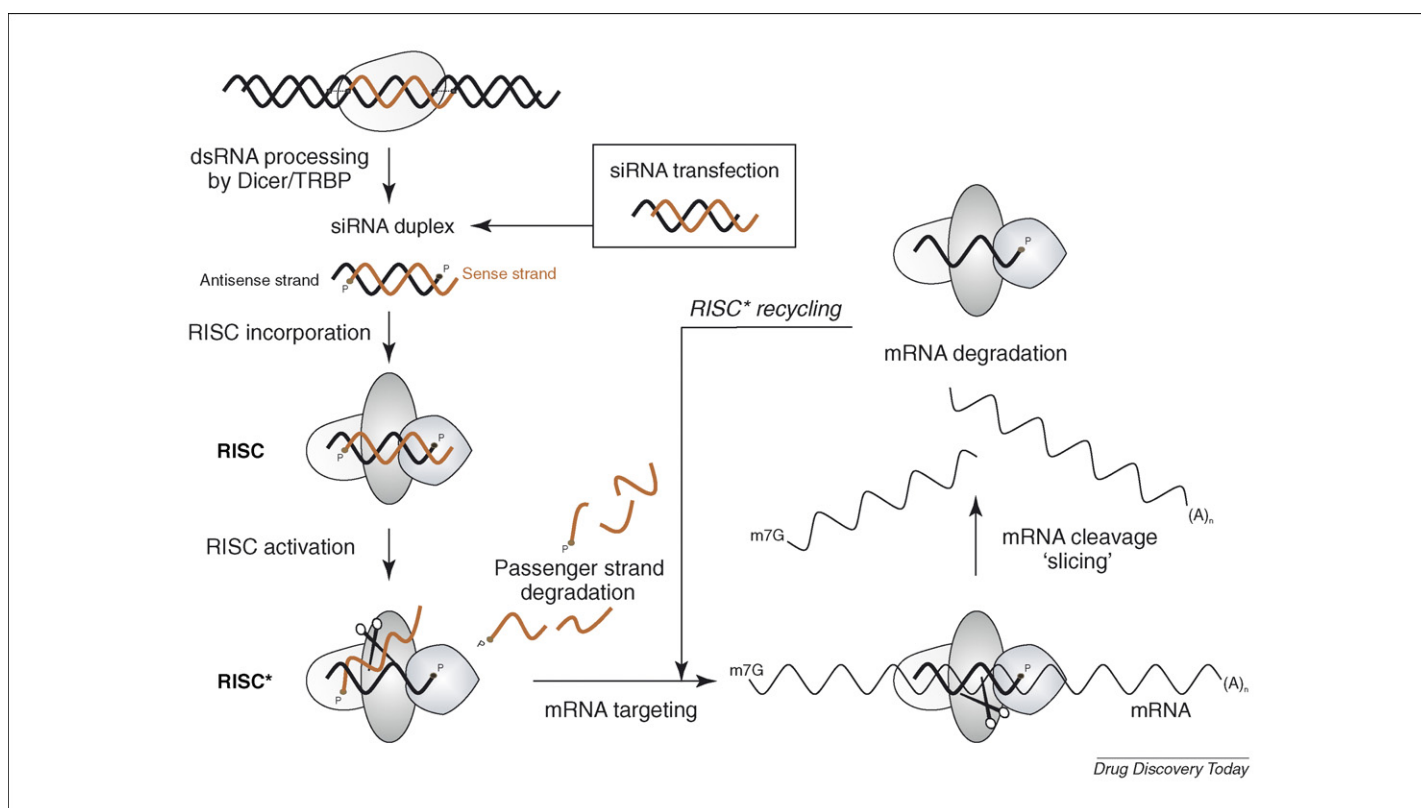
In this review, we will primarily focus on important remarks and strategies in the light of prolonged RNAi gene silencing for ther-

apeutic purposes. In the different sections we attempt to describe several topics from the available literature on RNAi that are of particular relevance to the central theme of this review.

### Intrinsic RNAi gene silencing efficiency and longevity

Since the discovery of RNAi, researchers have revealed the basic steps of the RNAi pathway, but many crucial aspects remain to be unveiled. The RNAi effector molecules, termed siRNAs, are RNA duplexes ~21–25 nucleotides (nt) in length (Figure 1). These small RNA fragments are defined as the products of longer dsRNA processing by an RNase III type enzyme, called Dicer. Once introduced in the cell's cytoplasm, siRNAs are incorporated into a protein complex termed RISC (RNA induced silencing complex) that contains the Argonaute 2 (Ago2) endonuclease [14]. Only one strand of the RNA duplex is retained inside the RISC, which is the antisense strand or guide strand. Subsequently, the activated RISC (RISC\*) uses the guide strand to bind to the complementary region on the target mRNA, followed by Ago2-directed transcript cleavage (also called 'slicing') opposite the phosphate linkage between bases 10 and 11 with respect to the 5' end of the guide strand [15]. The cleavage fragments are then further degraded by cellular RNases.

Our current knowledge of the RNAi pathway is sufficient to recognize the intrinsic gene silencing potential of RNAi. The fact that siRNAs employ endogenous cellular machinery is imperative in this regard. siRNA-activated RISC (RISC\*) is defined as a multi-turnover enzyme that follows Michaelis–Menten kinetics.



**FIGURE 1**

Cellular mechanism of RNA interference (RNAi), mediated by short interfering RNAs (siRNAs). The natural RNAi pathway is initiated by the cleavage of long double-stranded RNA (dsRNA) by the Dicer endoribonuclease into siRNAs. These siRNAs are subsequently incorporated into RISC (RNA induced silencing complex). RISC only maintains one strand (antisense strand or guide strand) while Ago2, the catalytic unit of RISC, cleaves the passenger strand (sense strand). The siRNA guide strand in active RISC\* then recognizes complementary target sites to direct mRNA cleavage, which is again catalyzed by Ago2. Further degradation of the mRNA fragments is carried out by intracellular ribonucleases as a result of the lack of a 5'-cap and a poly(A) tail.

Once the mRNA target is cleaved, RISC\* is recycled in the RNAi pathway to identify and destroy other mRNAs. This implies that a single enzyme complex can bind and cleave multiple mRNA transcripts [16]. Moreover, RISC can provide additional intracellular stability to the siRNA guide strand and protect it against an armada of single-strand-specific RNases. The entrapment of siRNA into RISC and its catalytic action are key parameters explaining the inherent gene silencing efficiency.

When siRNAs are not incorporated into RISC, their double-stranded nature makes them more resistant to intracellular degradation. In contrast to single-stranded RNA, duplex siRNA has the structural advantage of being better protected against ubiquitous single-strand-specific nucleases. This feature makes duplex siRNA significantly more stable intracellularly, which can already be observed over a short time scale [17]. This inherent stability also has to be taken into account especially when comparing the gene silencing efficiency and duration of siRNAs and unmodified antisense oligonucleotides; the latter being rapidly degraded in the cell cytoplasm [18].

Efforts with the aim of improving siRNA potency led to the development of longer siRNAs (25–27mers) that show superior silencing efficiency at certain target sites when compared with the conventional 21mers directed against the same region in the mRNA transcript [10,19,20]. With these types of duplexes, maximal inhibition and longer gene silencing persistence was observed at concentrations lower than those required for conventional 21mer siRNAs [10,19,20]. The improved RNAi silencing is thought to originate from their recognition and cleavage by Dicer (hence the annotation Dicer substrate RNAs or DisRNAs), which could facilitate their incorporation into RISC (Figure 1) because Dicer is believed to participate in the early steps of RISC assembly [21].

Nature holds several examples of convenient RNAi amplification mechanisms, for example in nematodes and plants. One of the known amplification mechanisms is 'systemic spreading' of the RNAi silencing effect. This effect is attributed to a dsRNA receptor, denoted as SID-1 in *C. elegans*, responsible for passive dsRNA translocation over the cell membrane and intercellular dsRNA transport between neighbouring cells [22,23]. The lack of gene silencing when incubating mammalian cells with 'naked' (i.e. unformulated) siRNA makes it likely that, in most mammalian cell types, normal levels of SID-1 homologue expression can be neglected [24,25]. A recent study, however, conducted by Wolfrum *et al.* revealed that SID-1 is at least partially responsible for the *in vitro* hepatocellular uptake of lipophilic siRNAs when they are incorporated in lipoprotein complexes such as HDL and LDL (high and low density lipoprotein, respectively) [26]. Specific RNAi silencing of SID-1 expression in HepG2 cells and blocking of extracellular SID-1 epitopes with SID-1 antibodies seemed to decrease the cellular internalization and subsequent silencing effect of lipophilic siRNAs targeted against apolipoprotein B (*apoB*) [26].

Plants, fungi and worms contain an endogenous RNA dependent RNA polymerase (RdRP) that produces secondary dsRNAs from an mRNA transcript targeted by a primary siRNA. The resulting dsRNA can again be recognized and cleaved by Dicer, increasing the intracellular siRNA concentration. Through this amplification process a small amount of 'initiator' dsRNA can lead to persistent gene silencing [27–29]. As mammalian cells most

probably lack RNA-dependent polymerase activity, one must rely on alternative strategies to achieve prolonged gene silencing [27,30,31].

### Stable RNAi gene silencing

21mer siRNAs, mimicking Dicer cleavage products, can be chemically synthesized and introduced into the target cell (Figure 1), but they can also be produced intracellularly from short hairpin RNA (shRNA) precursors that can be continuously expressed from RNA-polymerase-driven expression cassettes (Figure 2) [32,33]. shRNAs are structurally and functionally related to pre-microRNAs, intermediates in the biogenesis of endogenously encoded microRNAs (miRNAs). miRNAs constitute a highly conserved class of small RNAs that mediate RNAi mainly through translational inhibition [34,35]. As Figure 2 illustrates, the first step in the production of miRNA occurs in the cell nucleus, where long primary transcripts (called pri-miRNA) are expressed from endogenous genetic regions and processed by Drosha (a RNase III enzyme) into ~60–70 nt hairpins with imperfect complementarities in their stems [36]. These precursor miRNAs (pre-miRNAs) are shuttled from the nucleus into the cytoplasm through the Exportin-5/Ran-GTP heterodimer complex [37]. In the cytosol, Dicer processes pre-miRNA into mature ~22 nt miRNA duplexes that interact with RISC to modulate transcriptome expression [38]. Mammalian miRNAs, which tend to contain mismatches with the target mRNA, most often mediate gene silencing through translational suppression rather than transcript slicing, although the latter has also been described. It is believed that the binding of activated miRISC\* to the target mRNA sequesters the latter from the translational machinery through confinement in cytoplasmic foci, called processing bodies (P bodies), thereby preventing protein synthesis [39].

In analogy with miRNA biogenesis, the intracellular processing of shRNAs originates in the cell nucleus, where Exportin-5 is responsible for its nuclear export [40]. In the cytoplasm, these shRNAs are again cleaved by Dicer to yield the active 21mer siRNAs [32]. In correspondence with Dicer substrate RNAs (DisRNAs), plasmid vectors can be designed to produce shRNAs (29 nt stem, 4 nt loop) with higher RNAi potency over smaller hairpin RNAs (19 nt stem, 4 nt loop) owing to improved Dicer recognition and RISC incorporation [41]. However, in a recent report by Li *et al.*, it was shown that, in the context of a 9 nt loop, the shRNA with a 19 nt stem outperformed the longer 29 nt shRNA [42]. The explanation for this discrepancy again lies in the ability of the shRNA to be recognized by Dicer. Indeed, whereas shRNAs with a 19 nt stem and a 4 nt loop bypass Dicer cleavage, increasing the loop length to 9 nt also turns shRNAs with a 19 nt stem into Dicer substrates [42,43]. Building on our increased understanding of miRNA processing, second-generation shRNAs were developed (called shRNA-mir) [44]. In contrast to first-generation shRNA that elicit structural analogy with pre-miRNA, shRNAmir are transcribed as pri-miRNA. Their design is founded on the human miRNA, miR-30, of which the stem is replaced with an RNA sequence of interest. The advantage of shRNAmir over shRNA lies in their recognition and processing by both Drosha (nucleoplasm) and Dicer (cytoplasm), leading to a more efficient intracellular production of mature siRNAs and increased knockdown efficiency [45].

Important in the context of this review is to note that, compared with synthetic siRNAs that induce a transient knockdown, plasmid

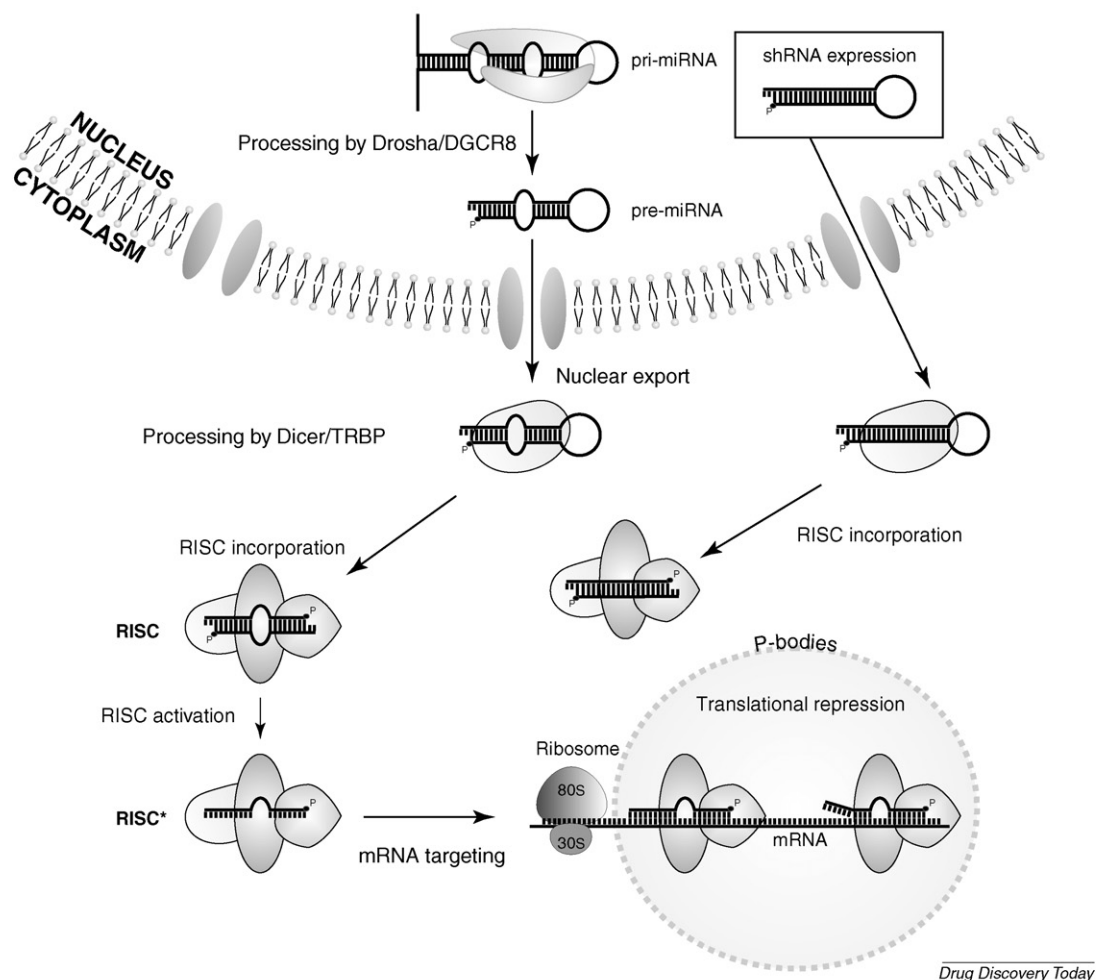


FIGURE 2

Overview of the microRNA (miRNA) pathway, leading to modulation of gene expression by translational repression. Pri-miRNA primary transcripts are processed in the nucleoplasm by the RNase III enzyme Drosha. The resulting pre-miRNA is shuttled to the cytoplasm by Exportin-5/Ran-GTP, where it is recognized and cleaved by Dicer to form mature miRNAs. On their turn, miRNAs are incorporated into RISC, which only retains one strand (most probably with the help of a helicase), thereby allowing the complex to bind to a (partially) complementary region in the mRNA. It is believed that the targeted transcripts are sequestered to cytoplasmic foci (P bodies) that are inaccessible to the translational machinery.

vector based interference shows better potential for long-term gene silencing [46]. To establish stable RNAi gene silencing in cultured cells, researchers mostly appeal to viral expression vectors [32]. Lentiviral vectors allow genomic insertion of the shRNA expressing transgene, while adenoviral and adeno-associated viral vectors (AAVs) show less successful integration and viral DNA remains largely episomal [47]. Genomic integration of shRNA expression cassettes per definition results in stable RNAi-mediated gene silencing because the target gene remains silenced as long as transcription of siRNA precursors proceeds. This RNAi gene therapy approach can be of interest to study loss-of-function phenotypes following prolonged knockdown of a target gene and for long-term treatment of chronic diseases (e.g. viral infections such as hepatitis B [48] and hepatitis C), thereby easing the treatment schedule and improving patient comfort. However, in a clinical setting, the use of synthetic siRNAs for transient RNAi gene silencing may be advantageous over continuous shRNA/siRNA production as the dosing schedule

(and consequently also the resulting intracellular siRNA concentrations) in a therapeutic regimen can be more easily adapted in relation to therapeutic needs [49]. Additionally, transgenes that are stably integrated in the host genome can be silenced rapidly by histone modifications and hypermethylation of CpG islands in the promoter region. Instead of achieving long-term transgene expression, this chromatin silencing will result in a gradual extinction of transgene activity [50].

Recently, Grimm and colleagues disclosed that continuous expression of high intracellular levels of shRNA could result in long-term toxicity in mice [51]. The observed fatal side effects were primarily ascribed to saturation of Exportin-5, leading to interference with nuclear export of miRNA precursors and miRNA function [40].

Possible solutions to overcome these toxicity issues are (a) lowering the initial viral load and (b) implementing vector development with inducible or tissue-specific promoters that allow more feasible control over intracellular shRNA concentration



and may improve the activity/toxicity ratio [51–53]. In a mouse model of hyperbilirubinemia, it was shown that the adenoviral production of shRNAs could silence Abcc2 function (ATP-binding cassette multidrug resistance protein 2), involved in liver bilirubin transport, for up to three weeks. This effect did not seem to correlate with changes in the level of endogenous (precursors of) miRNAs [54].

Nonetheless, it is striking to conclude that the improved RNAi gene silencing of dsRNAs can have a toxic flip side, by taking advantage of the natural miRNA pathway in an earlier stage. In this regard, synthetic 21mer siRNAs that function further downstream in the RNAi pathway are the better and safer choice because they bypass Dicer cleavage and do not require nuclear export for their activity [40,55]. When synthetic siRNAs are employed, however, vigilance is still required, as an intracellular excess of siRNA may possibly interfere with RISC availability, which can also induce competition with cellular miRNAs [55–58]. A recent report, describing potent and specific knockdown of hepatocyte-specific genes in mice and hamsters after systemic delivery of lipid-formulated siRNAs, also demonstrated that the treatment schedule did not influence either miRNA biogenesis or miRNA function [59]. As our knowledge on miRNAs expands, we will be able to better assess any possible cellular disturbances as a result of siRNA/shRNA treatment. Quantitative data on the total number of cellular protein molecules involved in the RNAi pathway and the number of siRNA molecules needed inside a cell for a sufficient silencing effect could be very helpful towards optimization of siRNA therapy without the aforementioned toxicity issues [57].

Another concern that has been raised against the use of (non-integrating) viral vectors is their immunogenicity, especially when long-term use is deemed necessary (e.g. adenoviruses, AAVs) [3,46]. Furthermore, random genomic insertion of a transgene (e.g. with integrating lentiviral vectors) coincides with the risk of hazardous *in vivo* viral recombination and insertional mutagenesis [60,61]. Although at present viruses are still the most efficient gene delivery vectors, the combination of these adverse effects could eventually favour a non-viral approach. Meanwhile, non-viral strategies have also been explored extensively for intracellular shRNA production [46]. siRNA expression plasmids can be introduced in the target cell by complexing with cationic lipids and polymers or by physical methods like electroporation and microinjection. Unfortunately, the nuclear import of the plasmid DNA (pDNA) vector remains the predominant bottleneck in the gene delivery process [62], thereby giving preference to synthetic siRNAs that function mainly in the cell cytoplasm. Non-dividing cells are especially difficult to transfect with non-viral vectors, since insufficient amounts of the pDNA can reach the nucleus in the absence of cell division during which the nuclear barrier is temporarily disassembled [63].

### Perspectives on the delivery of siRNA: biological barriers

Although it is generally said that chemically synthesized siRNAs are promising therapeutic candidates for the treatment of various genetic pathologies, their *in vivo* drug-like properties are regarded as very unfavourable. Before siRNAs can reach the desired intracellular location, many extracellular and intracellular barriers have to be overcome (Figure 3) [24]. Obviously, the efficiency with

which siRNAs can bypass these obstacles will have major implications on the extent and duration of gene silencing and, subsequently, on the determination of a clinically relevant dosing interval. Several concepts and strategies have been proposed to improve the siRNA pharmacokinetics and eventually their therapeutic performance.

### The extracellular compartment: focus on chemical modification and siRNA formulation

It is well known that siRNAs are rapidly degraded in the extracellular environment. When naked siRNA is injected intravenously it is recognized and cleaved by RNase A type nucleases [64], limiting the serum half-life to <30 min. Therefore, much effort has been undertaken to modify siRNA therapeutics chemically, in order to reduce their susceptibility to serum RNases [10,65,66]. Chemical modifications include RNA backbone modifications [e.g. phosphorothioates (PS) and boranophosphates], 2' ribose modifications and terminal 3' and 5' modifications, among others [67]. Initially, some concerns were raised with regard to the possible diminished efficacy of chemically modified siRNAs, owing to interference with RISC incorporation, activation and/or recycling. Since then, however, elaborate research in this field has proved that chemically modified siRNA can also efficiently lower mRNA levels through a sequence-specific RNAi-dependent pathway and that some modifications even show enhanced gene silencing compared with the unmodified counterpart [68,69].

An important consideration to make is whether or not chemical modifications, with the aim of improving the extracellular siRNA stability, are promising in light of prolonging the knockdown of a target gene. One could expect chemically modified siRNAs to have a higher bioavailability because they stay intact for a longer period of time in the blood circulation, thereby also improving the intensity and duration of the RNAi gene silencing effect. Unexpectedly, Layzer *et al.* were not able to demonstrate a stronger silencing or persistence of silencing for stabilized 2' fluoro pyrimidine siRNAs over the unmodified 2' OH siRNAs after hydrodynamic tail vein injection [70]. Most probably, sufficiently high concentrations of both siRNAs can reach the intracellular target site after hydrodynamic injection and can exert an effect before nuclease digestion becomes prominent. In contrast to these findings, a modified siRNA duplex, targeted against hepatitis B virus (HBV) RNA and where all ribose 2' OHs were substituted, was shown to be more efficient in decreasing HBV DNA and HBV surface antigen serum levels when compared with non-modified siRNA [66]. In the same report, hydrodynamic injection was used to co-deliver the siRNA and a replication competent HBV vector. Surprisingly, the difference in activity between modified and unmodified siRNA was only significant at the high-dose level and almost absent at lower doses [66]. When *in vivo* siRNA degradation by nucleases is the main limiting factor, one would expect to observe the opposite trend. This also indicates that factors other than increased nuclease resistance have to be taken into account when working with chemically modified siRNAs.

Besides degradation by RNases, the short *in vivo* half-life of siRNAs is also influenced by their rapid renal clearance. Since siRNAs are highly hydrophilic (~40 negatively charged phosphate groups per siRNA molecule) and have a molecular weight (~14 kDa) far below the cut-off for glomerular filtration

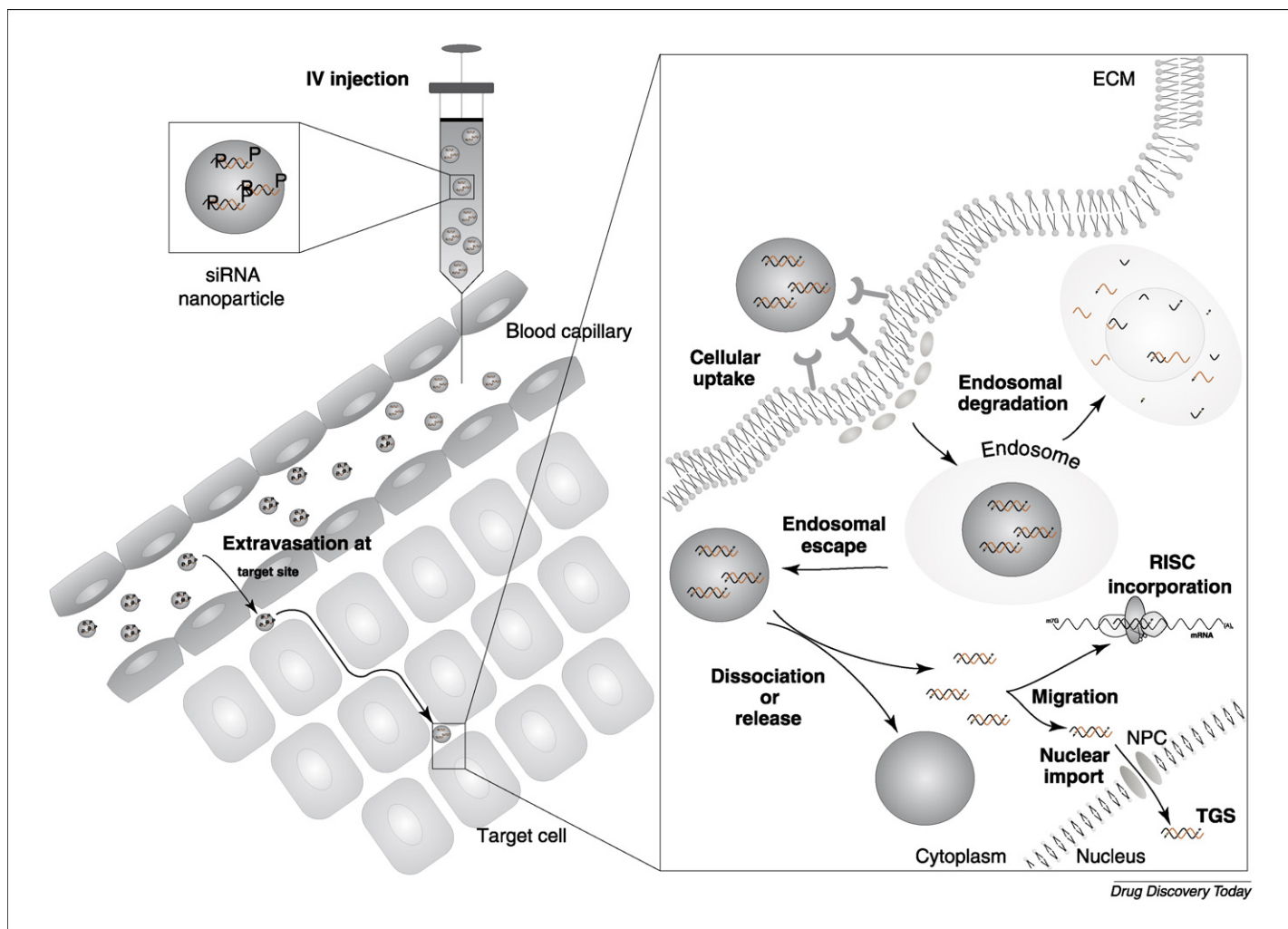


FIGURE 3

Overview of biological barriers for siRNA therapy following intravenous (i.v.) injection. For *in vivo* application, siRNAs can be formulated into nanosized carriers that should fulfil some basic requirements. They should be large enough to circumvent renal clearance, but small enough to be able to cross the capillary endothelium and accumulate in the target tissue. Moreover, the siRNA carrier has to be able to evade uptake by the mononuclear phagocyte system. Once the carrier has reached the target cell, it needs to deliver the siRNA to the cytoplasm. This usually involves cellular uptake through endocytosis, followed by escape from the endosome and carrier disassembly in the cytosol. ECM, extracellular matrix; NPC, nuclear pore complex; TGS, transcriptional gene silencing.

(~60 kDa), one can expect renal clearance to be eminent. Although RNase digestion can be slowed down by chemically modifying the nucleic acid backbone, renal clearance seems to be the rate-limiting factor governing the *in vivo* half-life of siRNAs [57,71].

Some chemical modifications (e.g. phosphorothioate internucleotide linkages or 4' thio ribonucleotides) can increase the circulatory half-life of siRNA by promoting interaction with serum proteins [67]. A more convenient strategy to modulate and improve the siRNA biodistribution is the more dramatic alteration of siRNA structure by conjugation to small molecular weight moieties [67,72,73]. Interesting examples are the coupling of siRNA to heavy-chain antibody Fab fragments [74] or lipidic moieties like cholesterol, bile acids and long-chain fatty acids [26,75]. Lipophilic siRNAs seem to incorporate selectively in lipoprotein particles that are rich in phospholipids and cholesterol (mainly HDL and LDL). These lipophilic siRNA-lipoprotein complexes are able to improve siRNA biodistribution by evading renal clearance and promoting cellular uptake through HDL and LDL

lipoprotein receptors [26]. Chimeric peptide-siRNA complexes have recently been shown to protect mice against infection with Japanese encephalitis virus by delivering their siRNA payload into the brain after intravenous injection [76]. Intriguingly, the majority of the siRNA-treated mice survived for over four weeks, while all animals in the control group died within 10 days. Rozema *et al.* recently introduced a novel polymer based siRNA-conjugate strategy (termed dynamic polyconjugates) for *in vivo* hepatocytic targeting. These conjugates (~10 nm in size) consist of a reversibly shielded membrane-destabilizing polycation, containing a cleavable targeting ligand, to which siRNA is linked through an intracellularly reducible disulfide bond. Effective knockdown of two endogenous hepatic genes *apoB* and peroxisome proliferators activated receptor alpha (*ppara*) was demonstrated in wild-type mice following low pressure i.v. injection [77].

Another convenient way to circumvent renal clearance is by the incorporation of siRNA into gene silencing complexes that are large enough to evade glomerular filtration, thereby improving siRNA pharmacokinetics and biodistribution. Paying proper atten-

tion to the 'packaging' of siRNAs can lead to constructs where the siRNA molecules are shielded from circulating RNases and destabilizing blood components, eliminating the need for chemical modification [78–80]. Many inventive siRNA formulations have already shown to improve the *in vivo* potency of siRNAs dramatically [3,11,57,72,81–83]. A textbook case underlining the importance of siRNA formulation is given by Morrissey *et al.* When administering unformulated backbone stabilized anti-HBV siRNAs through hydrodynamic tail vein injection an effective inhibition of HBV replication could be achieved, albeit at a dramatically high dosing regimen of three daily doses of 30 mg/kg [66]. However, formulating the therapeutic siRNAs into stable nucleic acid lipid particles (SNALPs) of ~140 nm in size, these investigators succeeded in achieving a significant and long-term reduction of HBV activity by three daily injections of 3 mg/kg/d siRNA followed by a weekly administered maintenance dose for up to six weeks [84]. A more recent report, using SNALP technology to deliver anti-ApoB siRNA in cynomolgus monkeys, describes marked reduction in plasma ApoB protein levels for as long as 11 days after a single i.v. injection (2.5 mg/kg) [85]. Mark Davis' group formulated siRNAs in cationic cyclodextrin containing polycations (CDPs) that can be equipped with targeting ligands, for example, transferrin for delivery to tumour cells overexpressing the transferrin receptor. The systemic delivery of these targeted siRNA nanoparticles, twice weekly for >4 weeks resulted in long-term inhibition of tumour cell engraftment and tumour growth in a murine therapeutic model for metastatic Ewing's sarcoma [86]. Interestingly, the long-term inhibition was only observed for the formulation where the surface was modified with transferrin moieties and no positive outcome was demonstrated for the non-targeted formulation. This targeted nanoparticle delivery system also has been evaluated recently in non-human primates [87].

#### *The intracellular compartment: focus on chemical modification and cellular dilution*

Besides improvements at the extracellular level, also the intracellular behaviour of chemically modified siRNAs may play a pivotal part in improving the RNAi gene silencing potency and duration. Some reports ascribe a prolonged gene silencing effect in cell cultures to the use of stabilized siRNA duplexes that show higher resistance towards nuclease attack, suggesting that intracellular degradation of siRNA can indeed be partially responsible for transient RNAi gene silencing [88–90]. An interesting recent report by Takabatake *et al.* demonstrates the dependence of RNAi gene silencing duration on the tissue expression level of *eri-1* (enhanced RNAi-1) nuclease [91]. *eri-1* can be regarded as a type of siRNase, which has been discovered in *C. elegans* mutants [92]. Preliminary experiments in EGFP transgenic rats revealed that a single administration of siEGFP into the kidney resulted in the reduction of EGFP expression for up to two weeks, while the transfection of siEGFP into muscle tissue silenced EGFP for up to 90 days. The authors hypothesize that the higher *eri-1* expression level in the kidney when compared with muscle could be responsible for higher intracellular siRNA degradation and decreased RNAi longevity. They propose that blocking *eri-1* expression or applying *eri-1*-resistant siRNAs can result in more sustained gene silencing [91].

siRNAs modified with 2' fluoro pyrimidines have shown increased persistence in luciferase silencing over unmodified siR-

NAs in cells transiently transfected with a luciferase reporter vector [89]. However, assessment of luciferase silencing with the same type of modified siRNAs, in a cell line stably transfected with the luciferase reporter gene, did not reveal a significant difference in the duration of gene silencing [70]. As explained by Layzer *et al.*, when trying to investigate gene silencing in cell culture on a longer time scale it is preferred to approximate *in vivo* conditions as much as possible by using cell culture models that stably express the transgene of interest [70]. It was questioned recently whether simultaneous transfection of an exogenous gene and the siRNA is suitable to quantify RNAi [93].

Fisher *et al.* investigated the influence of modest altritol (6-carbon sugar) modifications on siRNA stability and gene silencing persistence [94]. Although partially modified siRNAs did not show increased nuclease resistance compared with wild-type siRNA, the authors did observe enhanced duration in MDR-1 gene silencing, resulting in more durable suppression of *p*-glycoprotein expression. These data underscore the fact that, at the cellular level, factors other than mere siRNA stability, like cellular uptake and/or recognition by RNAi machinery, have to be taken into account. Liao and Wang reported on 2'-O-(2,4-dinitrophenyl) modifications that serve to enhance siRNA lipophilicity [69]. These chemical adjustments facilitate the intracellular siRNA penetration and improve the siRNA stability that resulted in a stronger inhibition of insulin-like growth factor receptor expression, relative to unmodified siRNA directed against the same target sequence.

Interestingly, it seems that in fast dividing cell lines gene silencing is limited by intracellular dilution of siRNA (and activated RISC) owing to cell division. As a result, *in vitro* gene silencing usually lasts for only ~4–7 days [49]. In slowly dividing and non-dividing cells the siRNA stability is supposed to be the main limiting factor for prolonged gene silencing as the intracellular siRNA half-life is expected to be shorter than the cell division time [49]. Intracellular persistence of active siRNA has already been shown to maintain target gene inhibition for several weeks in different types of (non-dividing) primary cells [49,95–98]. In these papers it was assumed that the stability of the RNAi effect is representative of the physical stability of the intracellular siRNA trigger. Unfortunately, in most reports this assumption was not experimentally evaluated. Song *et al.*, who did perform a Northern blot analysis for siRNA integrity, suggested that intracellular siRNA survival requires the active production of the target mRNA transcript [95]. Also, Maliyekkel *et al.* reported on more stable gene silencing following transient shRNA induction in growth-arrested non-cycling cells [96]. However, in contrast to other reports on long-lasting RNAi in non-dividing cells [95], the phenotypic RNAi activity did not coincide with the intracellular siRNA decay as analyzed by an RNase protection assay. Two possible explanations for this discrepancy were suggested. Firstly, it is conceivable that the RNAi phenotype is mainly governed by the fraction of siRNA associated with RISC\*. As already mentioned previously, it can be expected that incorporation of the guide strand in the RISC\* complex provides extra protection against degradation. In this case, quantifying total intracellular siRNA does not represent the effective intracellular RNAi potential. Secondly, it was proposed that transcriptional silencing of the target gene could be held responsible for the increased silencing duration (see below).



Recent *in vitro* and *in vivo* data on non-modified and nuclease-stabilized siRNA duplexes have shown that evading nuclease attack by chemically modifying siRNAs does not significantly prolong the duration of gene silencing once the siRNA has reached the cytosol of the target cells [99].

The contradictory findings on the (supposed) advantages of nuclease-stabilized siRNAs described in this section unfortunately hinder drawing clear conclusions in this regard. Nonetheless, although the benefits of nuclease-resistant siRNAs inside the cell cytoplasm still remain questionable, chemical modifications have proved to be a very useful approach to abrogate off-target effects and unwanted stimulation of the mammalian immune system [100–103]. Several native siRNA sequences are known to induce a toll-like receptor (TLR)-mediated immune response through endosomal TLR7/8 recognition [104,105]. Cytoplasmic receptors such as PKR (dsRNA-binding protein kinase receptor), and the RNA helicases RIG-1 (retinoic acid-inducible gene-I) and MDA-5 (melanoma differentiation-associated protein-5), are considered to recognize certain structural RNA characteristics, other than nucleotide sequence [103]. RIG-1, for instance, is activated by uncapped 5' triphosphate RNA and blunt-ended siRNAs [103,106]. Mitigation of immune response is possible by designing siRNAs devoid of immunostimulatory sequence motifs and by paying proper attention to dsRNA physical structure. The most robust approach, however, is the selective incorporation of modified nucleotides (e.g. 2' Ome) to avoid immune receptor activation [103,107]. Kleinman *et al.* showed recently that 21 nt or longer siRNAs inhibited choroidal neovascularization (CNV) in mice in a sequence-independent and target-independent manner. The inhibition resulted from the cell-surface binding of TLR3 by generic siRNAs [108]. Modifying the siRNA duplex to minimize TLR3 activation could reduce any consequential undesired effect and enhance target specificity. Therefore, chemical modifications can certainly help to optimize siRNA design towards minimal *in vivo* toxicity and maximal siRNA tolerance.

### RNAi-induced silencing at the transcriptional level

The majority of reports in literature dealing with mammalian RNAi gene silencing describe siRNA-directed cleavage and destruction of cytoplasmic mRNA transcripts, termed post-transcriptional gene silencing (PTGS). Although it was first believed that siRNA only functions in the cell cytoplasm [109], several reports studied three different siRNA functions in the cell nucleus: RNAi [110]; transcriptional gene silencing (TGS) [111] and antigene RNAs (agRNAs) [112]. To obtain an effect at the transcriptional level, the siRNA molecules need to be taken up by the nucleus. The exact mechanism by which siRNAs are transported through the nuclear pore complex (NPC) still needs to be clarified. In principle, siRNA molecules are small enough to diffuse passively through the NPCs to reach the nucleoplasm.

The RNAi nuclear function was demonstrated by siRNA-mediated degradation of 7SK snRNA [110]—an abundant, well-characterized RNA that has a highly defined structure and specifically localizes in the nucleus [113–115]. Furthermore, it was shown that several siRNAs formed a functional RISC in the nucleus that cleaved the target RNA with high efficiency [110]. Although the cited reports describe RNAi action occurring in the cell nucleus, they still encompass a PTGS process, for which essentially

the same basic rules apply as discussed for cytosolic RNAi (see section above).

TGS was first observed when doubly transformed tobacco plants exhibited a suppressed phenotype of a transgene caused by a methylation process [116]. This RNA-dependent DNA methylation (RdDM) seemed to be induced by RNA sequences identical to genomic promoter regions, leading to TGS [117–121]. While the exact molecular mechanisms of RdDM are unknown, it is likely to involve cytosine methylation, histone modification and chromatin remodelling.

Several recent independent reports also showed the induction of RdDM by siRNAs in mammalian cells, although in this case silencing required the simultaneous use of multiple siRNAs [122–124]. siRNA-mediated TGS in mammalian cells appears to be the result of the siRNA-directed histon H3K9 and H3K27 methylation at the targeted promoter [125], although subsequent DNA methylation has also been observed [126].

To suppress the production of a range of viruses *in vitro* by targeting structural and accessory genes, the duration of the PTGS effect is known to be rather limited (i.e. in HIV-1 varying from four to seven days [127]). Prolonging HIV-1 silencing up to 14–25 days was achieved using adeno-associated or lentiviral vectors; however, the efficacy of HIV-1 treatment based on a PTGS approach is potentially further limited because HIV-1 is known to adapt to environmental pressure, and rapid selection of siRNA escape mutants has been described *in vitro* [128]. For this reason, Suzuki *et al.* made use of TGS as another approach to prolong the suppressive effect of siRNA that would be less susceptible to the adaptability of HIV-1 [129]. A prolonged effect can be expected because it has been shown that, regardless of the exact mechanisms, RdDM is long lasting and can be passed on across generations in plant systems [130–132] and in *C. elegans* in the absence of the original RNAi trigger [133]. Therefore, epigenetic modifications through the use of promoter-specific siRNAs could be an interesting method of achieving a more robust gene silencing effect, although it has been suggested that this probably needs a constitutive expression of promoter-directed siRNA/shRNA.

A third example of nuclear siRNA function implies the inhibition of gene expression by antigene RNAs (agRNAs), complementary to transcription start sites within human chromosomal DNA [112]. In contrast to TGS, no methylation of DNA was required, but the silencing was accompanied by dimethylation of Lys9 in histone H3 (H3K9) [134,135]. AgRNA treatment was further shown to be dependent on Argonaute-1 (AGO1) and Argonaute-2 (AGO2) activity [136]. Potent inhibition of multiple genes suggests that agRNAs may represent a natural mechanism for controlling transcription, which will be valuable for applications of silencing gene expression. The relation of this silencing process to prolonged gene silencing still remains unclear. In addition, two papers recently demonstrated that synthetic agRNAs could also potentially activate gene expression in human cancer cell lines by 10 to 20-fold [137,138].

Presently there is sufficient evidence to suggest that TGS could be applied in future therapeutic protocols where prolonged gene silencing is desired. The exact mechanism of TGS should first be unravelled in more detail to be able to translate this gene silencing strategy into a viable clinical application.

## Mathematical modelling to describe RNAi gene silencing kinetics

A broad spectrum of variable parameters can be defined that may influence the outcome of RNAi gene silencing and the knockdown duration. These parameters are situated on three distinct levels: (i) the siRNA delivery formulation; (ii) the intracellular RNAi pathway and (iii) the envisioned target.

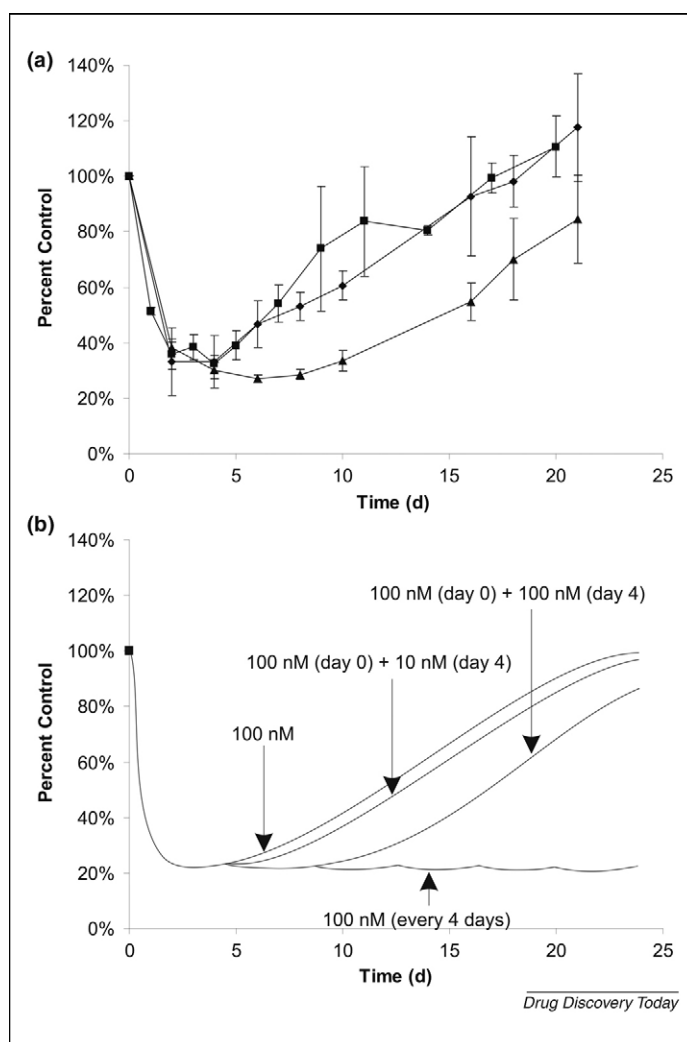
At the level of siRNA delivery one must keep in mind that *in vivo* the tissue distribution and pharmacokinetics of the siRNA formulation (e.g. siRNA containing nanoparticles) will have a major impact on the RNAi effect in the target cells, as discussed earlier. Because the siRNA targets are located intracellularly, not only the biodistribution in the extracellular space but also the different steps in intracellular trafficking have to be considered. Important processes like the cellular uptake mechanism, the confinement of siRNA carrier to cytosolic vesicles and siRNA release from its carrier will influence the RNAi effect (Figure 3). For example, several studies have shown that the ability of siRNA or siRNA carrier complexes to escape from the endosomal compartment can be a limiting step in their gene silencing efficiency [139,140].

Obviously, RNAi-specific parameters such as intracellular siRNA stability, RISC activation and siRNA-RISC-mRNA complex kinetics have to be accounted for. The importance of RISC\* recycling in the RNAi pathway towards siRNA gene silencing potency and the potential influence of siRNA chemical modification was emphasized earlier.

Last but not least, the turnover of the intracellular mRNA transcript and target protein stability should be carefully considered. If the protein half-life extends over several days, even efficient knockdown of intracellular mRNA levels after a single siRNA dose may fail in altering the cellular phenotype owing to residual amounts of the stable protein. The rate of cell division is imperative for the duration of gene silencing in a transient RNAi approach because it determines how transient the RNAi effect really is. In this regard, the targeted cell type and the envisioned therapeutic outcome are of great importance. If a single administration of siRNA is effective in blocking tumour cell growth, this inherently implies a prolonged effect since the intracellular siRNA dilution due to cell division can be disregarded [141].

A detailed knowledge of the impact of all the parameters listed above, in relation to RNAi gene silencing kinetics, could expedite the design of therapeutic siRNA strategies. Predictive mathematical models, illuminating the key factors that govern the duration of gene silencing, could be helpful instruments in the setup of siRNA treatment regimens. Several reports that describe mathematical equations to gain more insight into the RNAi silencing pathway are available in the literature [142,143]. Raab and Stephanopoulos studied the impact of the siRNA concentration and the time of siRNA transfection relative to reporter plasmid co-transfection in mouse hepatoma cells [12]. Their model could be used to select the appropriate time of siRNA transfection in function of gene induction. Moment analysis was used by Takahashi *et al.* to evaluate the time course of endogenous protein expression in function of siRNA concentration [144]. Likewise, an interesting report by Arciero *et al.* describes mathematical modelling as a tool to predict tumour-immune evasion and to study the impact of siRNA administration directed against the immune suppressive cytokine TGF- $\beta$  on tumour growth [145].

Bartlett and Davis were the first to construct a mathematical model incorporating parameters that govern the *in vivo* siRNA delivery process, such as biodistribution of the siRNA carrier and intracellular trafficking (vector unpackaging and endosomal escape), to study the kinetics of siRNA-mediated gene silencing [49]. Model calculations were also applied to define a dosing schedule, consisting of repeated siRNA injections, which results in persistent gene silencing, dependent on the half-life of the target protein and the target cell division rate. An illustrative example for non-dividing fibroblast is given in Figure 4. The same authors further employed their mathematical model in several *in vitro* and *in vivo* gene silencing studies performed with targeted siRNA nanoparticles based on cationized cyclodextrin, to provide information that could aid in designing more effective siRNA delivery strategies [141,146].



**FIGURE 4**

Effect of siRNA dose frequency on the duration of luciferase knockdown by siRNA in non-dividing fibroblasts that stably express the luciferase gene. (a) Experimental results obtained with Oligofectamine formulated siRNA, directed against pGL3 luciferase. Squares, 100 nM (day 0); diamonds, 100 nM (day 0) + 10 nM (day 4); triangles, 100 nM (day 0) + 100 nM (day 4). (b) Luciferase knockdown following siRNA transfection, as predicted by mathematical modelling. Reprinted and modified with permission from Bartlett and Davis [49]. © Oxford University Press 2006.

When considering different siRNA delivery strategies, also note that it could be of interest to incorporate the delivery kinetics of siRNA from its formulation into predictive mathematical models, as the amount of siRNA delivered into the cytoplasm in function of time can be a determining factor towards the eventual therapeutic outcome and the intracellular toxicity.

### Time controlled intracellular release of siRNA

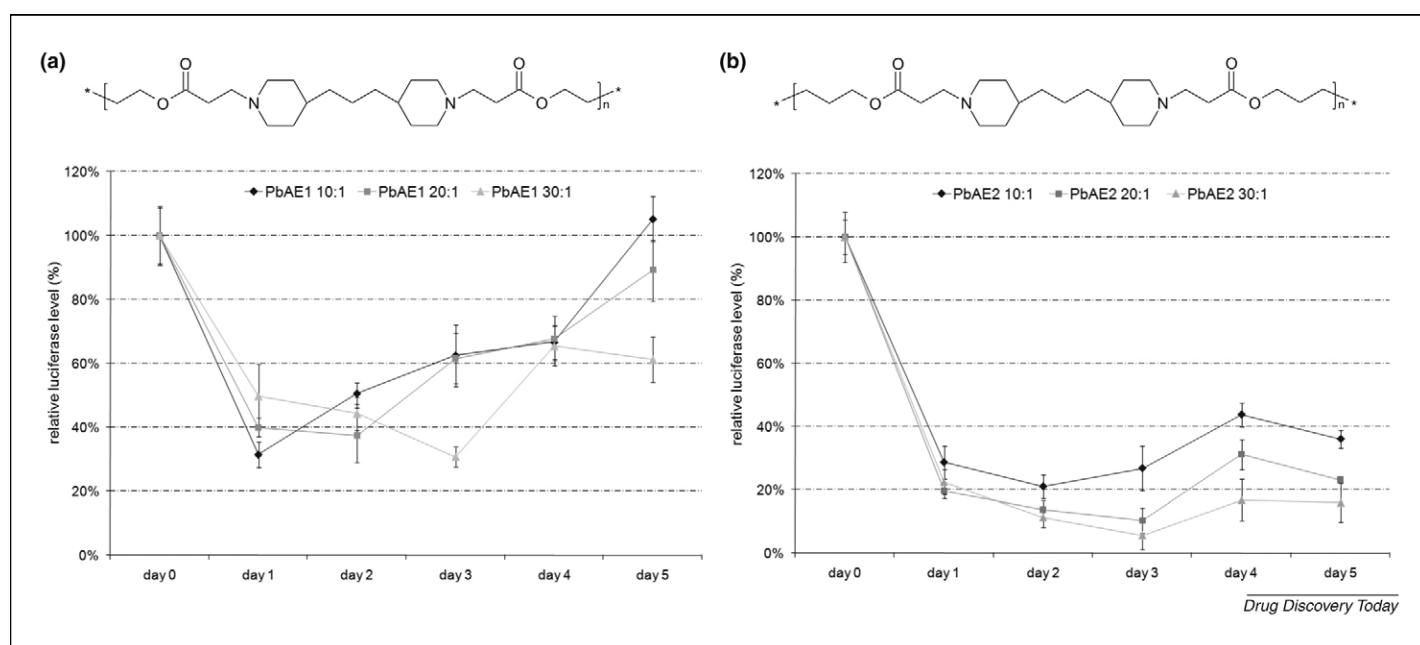
'More may not always be better', it was sharply put by Marsden in a recent edition of *New England Journal of Medicine* [147], and can be regarded as a basic toxicological paradigm. In theory every substance is potentially harmful when exceeding a certain concentration level or exposure time. This is also true for siRNA therapeutics, judging by the concentration dependency of adverse effects such as off-target silencing, induction of immune response and saturation of the endogenous RNAi pathway [58,103]. This obviously stresses that it is imperative to work with the most potent siRNA designs at the lowest concentration possible. Keeping this in mind, one cannot overemphasize the need for rigorous control over the intracellular siRNA/shRNA concentrations.

To regulate the number of active siRNA molecules in the cell cytosol, one could consider the use of conditional shRNA expression from plasmid expression cassettes delivered in the target cells by viral or non-viral carriers. However, the regulation of intracellular siRNA concentrations becomes more complex in the case of (non-viral) synthetic siRNA delivery where mostly (electrostatic) complexes between siRNA and (cationic) polymers (polyplexes) or (cationic) lipids (lipoplexes) are applied to the cells. With these polyplexes and lipoplexes a transient RNAi effect lasting for less than one week is generally observed. For

many of these formulations, one can expect that upon escape from the endosome, they provide a burst release of siRNA in the cytoplasm. The released siRNA is subsequently prone to dilution through cell division and (possibly) intracellular degradation, leading to a transient gene silencing. In theory, time-controlled release of siRNA in the cell cytoplasm could be interesting to maintain intracellular siRNA concentrations for a longer period of time above the minimal threshold required for efficient gene silencing, without flooding the cell cytoplasm with uncomplexed (naked) siRNAs. Controlling the amount of siRNA that is released in the cytosol could therefore result in a prolonged RNAi effect.

To achieve this goal, there is a need for delivery vehicles that exhibit tailored time-controlled siRNA delivery. Our group recently published on cationic biodegradable poly- $\beta$ -amino esters (PbAEs) that are able to form nanosized electrostatic complexes with the negatively charged siRNA (Figure 5). Following endocytosis of the polyplexes, the hydrolytic degradation of the cationic polymer is believed to increase the osmotic pressure inside the endosomal vesicles owing to an accumulation of its degradation products [148]. When exceeding a crucial pressure threshold, this eventually leads to endosomal rupture. It is hypothesized that the polymer degradation rate and the number of polyplexes residing in a single endosome will govern the kinetics of the rise in osmotic pressure over the endosomal membrane. Therefore, not all endosomes are expected to rupture at the same time. In this way a more gradual siRNA release into the cell cytoplasm is expected in function of polymer degradation, thereby altering gene silencing kinetics (Figure 5).

In a comparable approach, our group is also involved in the design of biodegradable siRNA impregnated gel beads (microgels),



**FIGURE 5**

Long-term gene silencing with biodegradable poly- $\beta$ -amino esters (PbAEs) in HUH-7 hepatoma cells that stably express the luciferase gene. The cells are transfected with (a) PbAE1:siRNA and (b) PbAE2:siRNA complexes containing siRNA targeting the pGL3 luciferase gene in different molar ratios. The chemical structure of both cationic polymers is depicted. It is hypothesized that the slower degradation rate of PbAE2 results in a more sustained gene silencing effect when compared with the more labile PbAE1 polymer. Adapted with permission from reference [148]. Copyright John Wiley & Sons, Ltd 2008.

which elicit time-controlled release of the incorporated siRNA governed by the hydrolysis of the hydrogel crosslinks [149]. Depending on the hydrogel characteristics, the siRNA release time can be tailored from hours over days to several weeks. As such gels can be taken up by target cells they could serve as intracellular siRNA depots slowly disintegrating and releasing the entrapped siRNA.

Owing to the high gene silencing potential of siRNAs, only a small number of active siRNAs per cell (~several hundreds) are needed for activity. Veldhoen *et al.* provided a detailed quantitative analysis of cellularly internalized siRNA through a liquid hybridization protocol [150]. Combining these data with the observed RNAi effect revealed that, for a half maximal silencing,  $\sim 10^4$  siRNA molecules were necessary in case of a cell penetrating peptide (CPP) assisted transfection and only  $\sim 300$  were required in the case of transfection with lipofectamine<sup>TM</sup> 2000 [150]. This indicates that the amount of siRNA molecules that become available for biological activity may depend strongly on the delivery agent. Data on the minimal number of siRNA molecules needed to trigger a sufficient RNAi effect and knowledge on the kinetics of the RNAi effect in relation to the intracellular siRNA concentration are very important when attempting to achieve a long-term maximal inhibitory effect.

We emphasized that a number of crucial parameters need to be considered for an understanding of the RNAi gene silencing kinetics. Similarly, an optimal controlled siRNA release depends on the cell type and the target gene. For instance, it should be clear that the optimal siRNA release profile to achieve a more sustained gene silencing will differ significantly for slowly dividing, fast dividing and non-dividing cells. The expression level of the target gene will also greatly influence the amount of siRNA needed inside the cell cytoplasm for maximal gene silencing.

### Combinatorial approach in RNAi therapy

Because siRNAs/shRNAs can be developed to target viral transcripts in a sequence-specific manner, RNAi could prove to be the next best thing to block viral replication [151]. Unfortunately, many reports have mentioned drug resistance through induced viral mutagenesis under pressure of RNAi monotherapy [128,151–153]. The perplexing viral genetic flexibility could also entail the emergence of genetically encoded viral RNAi suppressors [154]. Despite the great potential of RNAi for antiviral therapy, the issues raised above complicate the achievement of a persistent viral suppression using RNAi monotherapies. Even highly active anti-retroviral therapy (HAART), where a cocktail of three or more antiretroviral drugs is used, cannot eradicate viral replication and only delays disease progression. Moreover, HAART is notorious for the induction of severe toxicity, putting up an extra barrier for efficient therapy [155].

In correlation with HAART, a novel RNAi approach was put forward, called combinatorial RNAi or coRNAi [153]. This multiplex approach involves the concerted action of different siRNA/shRNA effectors, directed against multiple viral sequences. Although the majority of antiviral RNAi studies focus on the targeting of viral transcripts, some studies have also been devoted to suppressing the expression of cellular genes that are of key importance in the viral life cycle.

The primary goal of coRNAi is therefore to minimize the emergence of viral escape mutants in order to maintain a long-term antiviral effect. To give an example, it was shown for HIV-1 that, in response to a single shRNA inhibitor, mutations inside and even outside the RNAi target sequence provided an escape route for the virus eventually nullifying the antiviral effect [156]. A combinatorial strategy applying multiple shRNAs, expressed from a single lentiviral vector and targeting a different region in the HIV-1 genome, increased the viral inhibitory activity. It seemed that a double expression vector also provided a more durable viral inhibition. Comparable observations are presented in literature for Coxsackievirus B3 [157] and SARS-associated coronavirus, where a clear synergistic antiviral effect was obtained through combination of siRNAs targeting different functional genes in the coronavirus genome [158]. Currently, the concept of coRNAi also includes combining RNAi triggers with non-RNAi-based suppressors of gene expression [159] or even anti-viral proteins [160]. For instance, a multiplex strategy resulting in long-term HIV-1 inhibition in primary cells was reported using a triple combination of an anti-HIV shRNA, a hammerhead ribozyme targeted against the HIV co-receptor chemokine receptor 5 (CCR5) and a RNA decoy of HIV-TAR [159].

It would be beyond the scope of this review to discuss the plethora of reports available in the literature with regard to combinatorial RNAi. Recently, an excellent review solely devoted to this topic has been published by Kay and Grimm [153]. We would like to refer the reader to this review (and references herein) for a comprehensive overview of the different strategies already explored for coRNAi therapy.

In the latter paper [153], the authors focus mainly on the antiviral potential of coRNAi. Nevertheless, RNAi can be regarded as a broad therapeutic platform that can be additionally deployed in other challenging-to-treat human pathologies, such as metabolic disorders and cancer, where a combinatorial approach could be a therapeutic asset in the context of achieving long-term silencing. Recent reports established a synergistic effect of an RNAi trigger especially when combined with conventional low molecular weight chemotherapeutics. Takei *et al.* employed an effective siRNA against midkine (MK) together with low (and essentially non-toxic) doses of paclitaxel in human prostate cancer xenografts and found that paclitaxel significantly augmented the antitumour effect of MK-siRNA [161]. In this way, tumour growth inhibition could be maintained for several weeks without the need to switch to higher (and most probably toxic) doses of paclitaxel. Unfortunately, the *in vivo* administration of the siRNA-atelocollagen formulation used in that report was restricted to intratumoural injection. The same synergistic phenomenon, however, was found for an intravenously administered targeted nanoparticle formulation of anti-EGFR siRNA, in combination with intraperitoneal cisplatin, in a human lung cancer xenograft model. In contrast to monotherapy with targeted nanoparticles containing anti-EGFR siRNA, which only partially reduced tumour growth, the combination of the targeted siRNA-nanoparticles with cisplatin completely inhibited tumour proliferation for  $\sim 1$  week [162]. A third and earlier report describes the packaging of siRNA into neutral liposomal vesicles for the targeting of the tyrosine kinase receptor EphA2 oncogene, which is overexpressed in ovarian cancer. When liposomal anti-EphA2 siRNA was administered in addition to paclitaxel, a significant reduction in tumour



growth was observed when compared with paclitaxel in combination with non-silencing siRNA [163]. The same neutral liposomal formulation was applied to target focal adhesion kinase (FAK) and, again, a synergistic effect could be obtained when combining therapeutic siRNA with conventional chemotherapeutics such as docetaxel and cisplatin [164].

Altogether, the studies cited in this section exemplify the tremendous potential of coRNAi in the battle against various challenging therapeutic targets in human disease. A particular advantage when combining different RNAi effectors (with or without alternative therapeutics) is the maximization of the silencing effect, both acute and long-term. Needless to say that the safety concerns that have been raised for RNAi monotherapy also hold true for coRNAi. Multiplexing several siRNA/shRNA triggers could even augment the risks inherently linked to RNAi, such as off-targeting, immunostimulation and competition with endogenous miRNAs. Following co-administration of different types of RNAi drugs, they can even compete with each other for the (limited) amount of cellular RNAi proteins, thereby diminishing each other's efficacy [55,165]. By contrast, combining RNAi effectors with other non-RNAi-based therapeutics may allow lowering the dosing of both drugs. In this way, coRNAi can still profit from the synergistic effect along with the benefit of reduced toxicity.

## Conclusions

Advances in biotechnology have led to the emergence of many macromolecular drugs, such as peptides, proteins and nucleic acids. Researchers have been trying ever since to improve the therapeutic outcome of this new spectrum of drugs using different strategies related to the drug itself or the formulation needed to deliver the drug *in vivo*. RNA interference has the advantage of being able to profit from lessons learned during preclinical development of antisense oligonucleotides (AONs) and ribozymes [166]. After several decades of research on AONs, leading experts of the industrial and academic laboratories still have to acknowledge that inventive engineering strategies to improve the design of siRNAs/shRNAs, and to optimize their *in vivo* delivery, are still needed to turn the therapeutic promise of RNAi into clinical reality.

The clinical application of siRNA will call for repeated (often intravenous) administration, so it is desirable to aim for a durable effect to lengthen treatment intervals. Direct application of synthetic siRNAs has the disadvantage that the RNAi effect is transient, mainly owing to intracellular dilution of active siRNA dependent on the cell division rate. To maintain a sufficient silencing of the target gene expression over a prolonged period of time, different strategies have been proposed. Paying proper attention to their practical implication should eventually lead to a maximal RNAi effect, both in terms of magnitude and duration. siRNA duplexes can be designed and modified to optimally exploit the endogenous RNAi pathway in order to increase their gene silencing potency. Pursuing optimal siRNA design will lead to siRNA drug candidates with lower  $IC_{50}$  values that should enable effective medical treatment at lower doses. Incorporation of chemically modified nucleotides into the siRNA sequence has proven to enhance their half-life in the bloodstream by protecting them against nuclease activity. It is, however, conceivable that the

### BOX 1

[www.clinicaltrials.gov](http://www.clinicaltrials.gov)

This website provides an overview of clinical trials, already completed or in preparation with siRNAs. More specific information and background on the pathology and the gene target can be found on the following websites:

[www.sirna.com](http://www.sirna.com)

[www.alnylam.com](http://www.alnylam.com)

[www.quarkpharma.com](http://www.quarkpharma.com)

[www.opko.com](http://www.opko.com)

[www.allergan.com](http://www.allergan.com)

[www.allerganclinicaltrials.com](http://www.allerganclinicaltrials.com)

[www.calandopharma.com](http://www.calandopharma.com)

[www.merck.com](http://www.merck.com)

For other interesting URLs on siRNA delivery and clinical application, we would like to refer the reader to Behlke [13].

application of naked (i.e. unformulated or non-conjugated) siRNAs will be mainly limited to confined target sites after local administration, such as the eye or the respiratory tract [165,167,168].

Advances in materials science increased chances of designing new nucleic acid delivery concepts. The nano revolution led to the development of intelligent nanodevices, which should enable extrapolating drug delivery from the laboratory to a real *in vivo* situation. Multifunctional delivery vehicles and siRNA conjugation strategies offer many advantages for the systemic application of siRNA because they are usually equipped with targeting ligands and carry stabilizing hydrophilic polymers to avoid aggregation in the bloodstream and prevent non-specific uptake by the reticulo-endothelial cells [169]. Moreover, delivery vehicles are often modified to improve the intracellular trafficking (e.g. endosomal escape) that can lead to increased intracellular bioavailability. Including all these factors in carrier design should enhance the percentage of the administered siRNA dose that reaches the target site after systemic delivery. The ultimate goal is to define the appropriate dosing schedule for a given siRNA formulation to maintain the desired therapeutic outcome. Model predictions can aid in recognizing the main factors that govern the duration of the siRNA effect in order to adjust the siRNA dose and frequency of administration accordingly. This can provide researchers with general meaningful insights on systemic siRNA delivery that may apply to a variety of siRNA carriers.

Depending on the pathological target, a more sustained gene silencing than readily achievable with synthetic siRNA may be advisable. For this purpose researchers seek solace in the intracellular shRNA production from plasmid or viral vectors. Although much progress has been made in the development of viral gene therapy vectors, there are still important safety concerns that remain troublesome [170]. Moreover, little information is available on the adverse effects that are linked to sustained shRNA expression *in vivo*.

Most probably, a safe and long-term application of RNAi drugs will require rigorous spatiotemporal control over the intracellular siRNA/shRNA concentrations. Smart nanodevices for controlled intracellular siRNA delivery or smart plasmids for controllable shRNA expression may fulfil some of the necessary requirements. Extra information is detailed in Box 1.

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