The era of RNA interference medicines: the clinical landscape of synthetic gene silencing drugs

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ABSTRACT: Therapeutic oligonucleotides, such as small interfering RNAs (siRNAs), provide a simple and effective tool to modulate expression of any gene. siRNAs harness the RNA interference (RNAi) pathway to degrade disease-associated messenger RNAs (mRNAs). The inherent sequence specificity and potency of siRNAs makes them ideal drug candidates that are expected to transform drug development and our approach to human health. However, the first wave of clinical trials were not immediately successful and temporarily dampened the excitement over this newly discovered technology. Most studies did not meet desired efficacy and failed to achieve clinically--relevant endpoints. Poor chemical design, lack of enzymatic stability and inadequate delivery strategies were found to be the main issues stifling success.

Recent advancements in RNA chemistry, biology, and mechanistic understanding of factors that define oligonucleotide pharmacokinetic/pharmacodynamic behavior have resulted in a fundamental shift in the clinical landscape of this novel class of therapeutic modalities . As a result, there has been a dramatic increase in both the numbers of clinical trials and, more importantly, the level of observed clinical efficacy. In 2018, we witnessed a major landmark for the field with the first formulation-based RNAi therapeutic, Patisiran (Onpattro™), being approved by the Food and Drug Administration and the European Medicines Agency. Approximately a year later, another breakthrough was achieved with the approval of Givosiran (GIVLAARI™), the first siRNA using the conjugate-mediated approach for targeted delivery to hepatocytes. Both these approvals brought revitalized hope and enthusiasm to the field, and have restored the interest in RNAi, as a powerful disease-modifying therapeutic strategy for a variety of genetically-defined disorders.

This review gives an overview of the clinical landscape of synthetic RNAi drugs, contextualizing how advances in RNAi chemistry and formulation strategies have helped define the clinical utility of this promising class of drugs.

Keywords: RNA interference; siRNA; miRNA; Therapeutic oligonucleotides; Gene knockdown; Drug development.

A era dos medicamentos de ARN interferência: o panorama clínico dos fármacos para silenciamento génico

RESUMO: Oligonucleotídeos sintéticos, como os small interfering RNAs (siRNAs), providenciam uma forma simples e eficiente de modular a expressão de qualquer gene. siRNAs utilizam um mecanismo endógeno, chamado ARN interferência, para degradar ARN mensageiros que estejam associados a condições patológicas. A capacidade de silenciar genes com elevada especificidade e potência faz dos siRNAs fármacos ideais com um elevado potencial para transformar o ramo da medicina e a forma como se faz desenvolvimento farmacêutico. Contudo, os primeiros ensaios clínicos com esta tecnologia não tiveram sucesso imediato, o que reduziu temporariamente o entusiasmo da comunidade científica. A maioria destes estudos iniciais não atingiram a eficácia clínica desejada. A introdução prematura de fármacos que não se encontravam devidamente estabilizados e a utilização de estratégias de administração inadequadas foram as principais causas dos primeiros fracassos.

Avanços recentes na síntese química de oligonucleotídeos, como a melhor compreensão dos processos biológicos que definem a farmacocinética/farmacodinâmica destes fármacos, resultou

numa mudança drástica no panorama clínico desta nova modalidade terapêutica. O número de ensaios clínicos tem aumentado significativamente ao longo dos últimos anos, em paralelo com o aumento da eficácia terapêutica destes medicamentos. Em 2018 foi testemunhado um marco importante para o ramo de desenvolvimento destes fármacos, com a aprovação do primeiro produto baseado em liposomas, Patisiran (Onpattro™), pela *Food and Drug Administration* e pela *European Medicines Agency*. Aproximadamente um ano mais tarde foi aprovado o primeiro fármaco que utiliza a estratégia de conjugados que possibilita a internalização específica em hepatócitos, Givosiran (GIVLAARI™). A recente aprovação destes dois fármacos trouxe uma esperança renovada ao ramo de ARN interferência, alimentando o interesse nesta estratégia como poderosa ferramenta terapêutica para doenças do foro genético.

Este artigo de revisão pretende providenciar uma perspetiva geral sobre o panorama clínico dos fármacos para silenciamento génico, contextualizando o papel dos avanços tecnológicos que permitiram a definição desta modalidade como uma nova classe farmacêutica.

Palavras-chave: ARN interferência; siRNA; miRNA; Oligonucleotídios para terapêutica; Desenvolvimento de novos fármacos.

Introduction

Gene silencing oligonucleotides hold great promise as disease-modifying therapies for genetically-defined disorders by inhibiting the expression of toxic gene products. The inherent sequence-specificity of these approaches enables gene silencing through messenger RNA (mRNA) degradation, providing an attractive therapeutic alternative for molecular targets that have been deemed 'undruggable' by small molecules. In this context, post-transcriptional gene silencing has been achieved using several classes of oligonucleotides, such as ribozymes, antisense oligonucleotides (ASOs) and RNA interference (RNAi)-based oligonucleotides. Among these, RNAi-based oligonucleotides have gained remarkable attention due to their potency and long-lasting gene knockdown effects. Indeed, since the first observation more than 25 years ago in petunias[1], to the identification of the RNAi pathway in the nematode *C. elegans*[2] and translation to mammalian systems[3], the applications of this young technology have grown considerably. RNAi has not only become an invaluable research tool to study gene function and dissect complex biochemical pathways, but has also emerged as a viable therapy, with the first product (Patisiran, Onpattro™) being recently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA)[4].

Although several approaches have been used to hijack the RNAi pathway experimentally, synthetic small interfering RNAs (siRNAs) have dominated the clinical landscape of RNAi--based drugs. Capitalizing on lessons learned from the clinical development of ASOs and other oligonucleotides[5], synthetic RNAi-based drugs exhibited early and rapid progress to the clinic, with the first two drug candidates beginning clinical trials in 2004. These studies were mainly initiated by small biotechnology companies, but the following years were characterized by enormous investment from big pharmaceutical companies that propelled several other drug candidates to the clinic[6]. The lack of clinically-relevant efficacy in many of the initial trials caused the scientific community to re-think rational approaches for tackling challenges such as poor stability and acute immunostimulatory effects upon administration. In factHowever, recent advancements in RNA chemistry, biology, and the mechanistic understanding of factors defining oligonucleotide pharmacokinetic (PK)/ pharmacodynamic (PD) behavior[7], have resulted in a fundamental shift in clinical efficacy with several candidates likely to join Patisiran as approved products in the near future.

This review will provide a basic overview of the endogenous RNAi pathway, and the approaches used to harness gene silencing machinery for therapeutic applications. Particular attention will be paid to aspects of siRNA design and delivery. Finally, this review will provide a historical journey of the clinical landscape of synthetic RNAi drugs, examining how rational design of candidates and delivery strategies have shaped 15 years of clinical development.

The RNA interference pathway

The RNAi pathway is used by eukaryotic cells to regulate gene expression (Figure 1). It is thought to have evolved from viral defense mechanisms[8], and starts with a long double-stranded RNA (dsRNA) that is processed into 21-23 nucleotide-long siRNAs by a cytosolic RNase called Dicer[9]. The canonical siRNA duplex consists of a guide strand and a passenger strand that are fully complementary in their central region (19-nt) and contain unpaired 2-nt overhangs at the 3'-ends of each strand. The guide strand is selected based on its thermodynamic instability at the 5'-end, and is loaded into the RNA-Induced Silencing Complex (RISC), while the passenger strand is cleaved and discarded by Argonaute 2 (Ago2)[9]. Thereafter, RISC actively searches for the mRNA target, binds via Watson-Crick base pairing, and silences it by Ago2-mediated cleavage. After cleaving the target, RISC is free to search the transcriptome again and degrade other complementary mRNAs.

A class of endogenous non-coding RNAs, termed micro RNAs (miRNAs), also use RNAi machinery to modulate various cellular pathways[9]. miRNAs are encoded within the host genome as stand-alone individual genes, gene clusters,

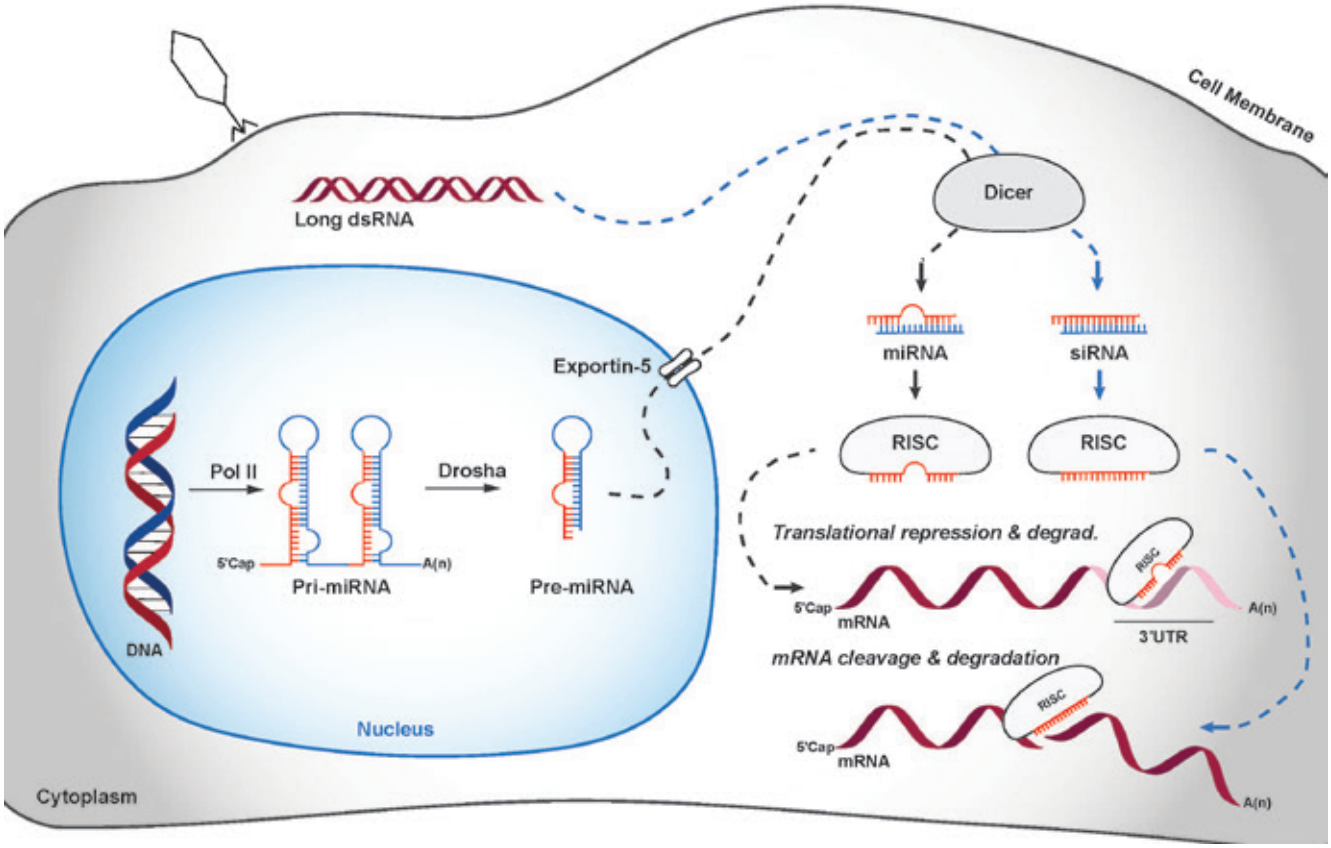

Figure 1. The mechanism of RNA interference.
The RNA interference (RNAi) pathway is as an endogenous regulatory pathway that enables modulation of gene expression. The biogenesis of micro RNAs (miRNAs) (A) and small interfering RNAs (siRNAs) (B) are depicted above.

or introns, and are transcribed by RNA polymerase II[9]. The initial transcript is called primary (Pri)-miRNA and consists of a single strand RNA molecule that self-hybridizes forming an imperfectly-matched duplex containing terminal hairpin loops (Figure 1). Pri-miRNA is processed by Drosha to form a 20-25 nt-long precursor termed (Pre)-miRNA, which is then exported from the nucleus to the cytoplasm by exportin 5[9]. In the cytoplasm, Dicer further processes this small RNA duplex to remove the stem loop structure, and the final miRNA product is loaded into RISC. However, unlike siRNAs, miRNAs do not require perfect complementarity to their target to exert an effect. miRNA-programmed RISC complexes can modulate multiple mRNA targets by binding the 3'- untranslated region (UTR), causing both mRNA relocalization/degradation and ribosomal translational arrest[9]. miRNA dysregulation may detrimentally affect transcriptome homeostasis and result in disease (e.g. cancer, cardiovascular diseases, neurodegenerative disease)[10]. Thus, in addition to their applications as therapeutic intervention, miRNAs can serve as biomarkers to characterize or diagnose certain disease pathologies.

The RNAi pathway holds great potential for therapeutic modulation of gene expression. The multiple turnover activity of RISC allows for potent and sustained gene silencing of virtually any given mRNA target. This particular feature spurred great interest in the scientific community that rapidly developed strategies to harness this endogenous pathway for therapeutic gene silencing. The next section reviews these approaches.

Hijacking the RNA interference pathway for therapeutic gene silencing

Approaches to artificially hijack RNAi for therapeutic purposes can be broadly classified as: (i) Viral based, i.e. delivering an expression vector or virus encoding a short-harpin RNA (shRNA) or miRNA; and (ii) Non-viral, i.e. delivering synthetic siRNA or miRNA.

Viral-based RNAi delivery has numerous advantages owing to the variety of recombinant viruses that have been engineered for research and therapeutics[11-12]. Depending on the application, recombinant viruses are selected based on their tropism for a specific cell-type or organ-system, and in their capacity to integrate into the host genome. RNAi delivery through a virus allows for continuous expression of the

shRNA or miRNA over extended periods of time, resulting in long-lasting gene silencing effects after a single administration[12]. Although vector-induced immunological responses were associated with fatalities in the clinic during the early 2000's[11,13], the safety of this approach has improved considerably thanks to advancements in vector engineering. In fact, several gene therapy products for gene substitution have been approved in recent years, including Glybera® (Alipogene tiparvovec) for lipoprotein lipase deficiency, IMLYGIC® (talimogene laherparepvec) for melanoma, and Zolgensma (onasemnogene abeparvovec-xioi) for spinal muscular atrophy[14]. However, only a handful of trials have been initiated to test the utility of viral-based RNAi for therapeutic gene modulation, mostly focusing on *ex vivo* approaches. An overview of the clinical landscape of viral-based RNAi is out of the scope of this paper, but the potential of this approach for *in vivo* therapeutic gene silencing has been recently reviewed elsewhere[12,15].

By far, non-viral delivery of synthetic siRNA or miRNA is more commonly used to induce therapeutic RNAi in the clinic. Oligonucleotides and transfection reagents can be synthesized in the lab in a controlled fashion, and can be easily scaled-up for production. However, non-viral delivery of synthetic RNAi drugs have faced its own set of challenges over the years, including: poor stability in serum, rapid renal clearance, immunogenicity, off-target effects, limited tissue distribution, reduced or non-specific cellular uptake, and inadequate subcellular localization[7]. The next section will discuss the design and delivery considerations for enhancing the therapeutic utility of synthetic RNAi drugs.

Design and delivery considerations for synthetic RNAi drugs

Several algorithms have been developed and are available online for the design of potent siRNAs against any given mRNA target[16-19]. These algorithms avoid regions/sequences that share complementarity with other transcripts to reduce possible off-target effects; but also, avoid regions within the target mRNA with complex secondary structures, since these are inaccessible to the loaded RISC. Mismatches in the seed region (nt 2-7 of the guide strand) are not well tolerated and will drastically reduce gene silencing activity; and thus, should also be avoided. The remainder of the sequences are generally sorted based on established criteria, including: specificity, base preference composition, seed complement frequency/low seed frequency, and thermodynamic bias (e.g. GC content)[18-19]. Strand bias, achieved by generating duplexes with low internal stability at the 3'-end of the passenger strand, is particularly important since it determines loading of the guide strand into RISC, greatly reducing off-target effects[18]. After sequence design, lead compounds are identified based on large *in vitro* screens in relevant cell cultures. The top compounds are then selected based on potency (EC50) for *in vivo* evaluations.

Delivery of nucleic acids *in vivo* to target tissues of therapeutic interest has been a major challenge in the field. siRNAs and miRNAs have poor drug-like properties: they are fairly large (12-14 kDa) and highly hydrophilic, with a negatively-charged phosphate backbone (~40 negative charges)[7]. These unfavorable PK characteristics result in poor tissue distribution and cellular internalization, and rapid clearance by glomerular filtration after systemic administration[20]. Moreover, unmodified siRNAs are prone to fast enzymatic degradation by endo- and exonucleases, which further reduces their half-life in both blood and tissue, and negatively impacts their efficacy[7]. Finally, unmodified siRNAs can induce unexpected immunoinflammatory responses through toll-like receptors (TLR), such as TLR3 and TLR7[21-22], and activation of Retinoic acid-inducible gene (RIG-I) and dsRNA-dependent protein kinase (PKR)[23-24]. Chemically modifying synthetic RNAi drugs has been explored as a strategy to abrogate immunostimulatory effects and improve stability and tissue accumulation. The next section reviews the most common modifications employed in current RNAi-based therapeutics.

Chemical modification of synthetic RNAi drugs

Synthetic siRNAs and miRNAs may be modified at several sites within their structure, including the phosphate backbone, sugar moieties or nitrous bases[5,25]. Substituting one of the non-bridging oxygens on the phosphate backbone with a sulfur—a phosphorothioate (PS) backbone modification—confers substantial resistance to nuclease degradation, and facilitates binding to plasma proteins for enhanced circulation time in the blood. The most common 2' sugar modifications, 2'fluoro (2'F) and 2'-methoxy (2'-O-methyl), confer nuclease resistance and reduce recognition by the imune system[25]. Larger chemical groups may be used for 2'-ribose modifications, provided that the steric constraints of siRNA loading into Ago2 are properly considered. Synthetic phosphorylation of the 5'-end of the guide strand has been successfully employed as a rational strategy to facilitate its loading into RISC; and stabilization of this moiety using phosphate analogs improves metabolic resistance and substantially enhances the duration of effect[26]. Several other modifications (reviewed elsewhere[25]), such as base modifications, have been tested in the lab, but have not yet progressed to the clinic.

Chemical modifications can be used to enhance stability and reduce immunological responses, but additional strategies are required to aid tissue distribution and cellular internalization. For *in vivo* applications, two main strategies are used: (i) *Formulation*, which can be employed with or without chemical modifications, and (ii) *Conjugation*, which relies heavily on chemical modifications to protect the oligonucleotide from nucleases. The next section discusses the advances in formulation- and conjugation-based delivery strategies.

Delivery strategies for RNAi drugs

Formulation

A wide variety of biomaterials have been used in the clinic to enhance distribution and promote cellular uptake of drugs, including nucleic acids[27-28]. Examples of such are cationic lipids (e.g. D-Lin-MC3-DMA), polymers (e.g. cyclo-

dextrin-based polymers and biocollagen), polypetides, and exosomes. Generally, these materials interact with nucleic acids and self-assemble into nanoparticles that protect the cargo from enzymatic degradation. Cationic non-viral vectors confer a positive surface charge to the complex that facilitates interaction with negatively-charged cellular membranes and endocytosis[27-28]. Nanosystems may be engineered to promote release from the endosomal compartment by including endosomolytic moieties (e.g. melittin-like peptides) or fusogenic lipids (e.g. DOPE) that enable immediate release of siRNAs to the cytoplasm[27-28]. Thus, nanocarriers can drastically reduce dose requirements by improving the PK and distribution profiles of siRNAs *in vivo* after intravenous administrations. However, some nanosystems they have been associated with toxicities, mostly caused by aggregation and subsequent deposition in small capillary beds, such as alveolar capillaries in the lung. To limit this issue, advanced delivery systems include a 'stealth' layer, usually consisting of a polyethylenoglycol (PEG)-lipid, that masks the positive charge and considerably reduces aggregation *in vivo*[27-28]. The significant advancements in nanoparticle design were fundamental for the approval of the first RNAi-based drug.

Conjugation

Direct conjugation of ligands to improve bioavailability of RNAi drugs has gained significant traction as an effective alternative to formulation, especially since RISC-compatible chemical modification patterns have been identified. Ligands have been successfully attached to the 5'- and/or 3'-end of the sense strand without affecting RISC loading[20]. In the lab, a very diverse list of ligands, including lipids, carbohydrates and peptides have been investigated (e.g. cholesterol, transferrin, tat peptide, GLP1, folate, vitamin E and N-acetylgalactosamine (GalNAc)[20]. Lipid bioconjugates, such as cholesterol and docosahexanoic acid (DHA), promote broad functional delivery to many different tissues, and possess distinct distribution profiles. Thus, the choice of conjugated modality is determined by the desired target tissue[20]. The distinctive distribution profiles of different conjugates is believed to be, at least in part, a result of differential partitioning to serum proteins[20,29]. On the other hand, ligands, such as GalNAc, enable targeted delivery to specific cell-types by hijacking receptor systems[30]. In this case, GalNAc-siRNAs bind to the asialoglycoprotein receptor (ASGPR), which is highly expressed by hepatocytes, allowing for targeted delivery to the liver. Currently, this is the most impactful targeted-delivery platform in the clinical setting[31]. Many other ligands and oligonucleotide scaffolds are now being considered in the lab, and are expected to enter clinical development in the next few years.

The advances in chemical modification, formulation, and conjugation have shaped the clinical landscape of synthetic RNAi drugs over the last few decades. The next section will recount the evolution of RNAi drug candidates that entered clinical development, discussing failures, as well as rising trends and strategies in the field.

Historical perspective on the clinical landscape of synthetic RNAi drugs

Synthetic RNAi-based compounds made their debut on the clinical stage in 2004. Since then, more than 60 drug candidates have been or are currently being evaluated (Figure 2 and Table 1), with 144 clinical trials (enrolling an estimated total of ~30,000 patients) either carried out or in progress (Figure 2). Most trials conducted in the early years of this technology did not progress beyond Phases I and II, which test safety, PK/PD, and initial therapeutic efficacy. After significant improvements in stability and delivery strategies a steady and considerable increase in the number of Phase III trials was observed from 2013 (Figure 2a). In August 2018, a major landmark in the field was achieved, with the first RNAi drug (Patisiran, Onpattro™) being approved by the FDA[4].

The early years: challenges with partially-modified siRNAs, and the emergence of formulation

With the safety of RNAi-based drugs being unknown, it was critical for initial clinical trials to focus on local administration, carefully consider administration routes, and limit drug effects to a localized site before testing systemic administration. The first clinical trials focused on ocular delivery. The eye was a rational first choice for inaugural studies in the clinic because it is an immune-privileged organ and intravitreal injections can be performed routinely as an out-patient procedure. In these trials, minimally-modified siRNAs, Bevasiranib or AGN211745, were used in the eye against the vascular endothelial growth factor (VEGF) or VEGF Receptor, respectively (Figure 2b-f, Table 1). These siRNAs were locally injected in the eyes of patients with age-related macular degeneration to block the angiogenic effects of VEGF, reduce macular edema, and improve visual acuity[32-33]. Around the same time, other local routes of administration were explored, including intranasal (ALN-RSV01), intratumoral (ATN-RNA) and intradermal (TD101), to treat different conditions (Table 1). Among the first systemically-injected siRNAs was the unformulated, partially-modified Teprasiran, which targeted p53 for the treatment of acute kidney injury[34]. Despite encouraging safety and tolerability results in the aforementioned trials, most programs were terminated because primary clinical outcomes were unlikely to be met, or serious adverse events occurred. Unmodified or partially modified siRNAs employed in these trials were likely to have been readily degraded *in vivo* upon injection, and were unable to achieve meaningful therapeutic effect. Furthermore, the higher doses requirements likely caused toxicities, immunostimulatory effects elicited through TLRs, PKR and RIG-I[24]. Together these results led to a wave of disappointment in the field.

To overcome some of the difficulties encountered in these first trials, the community turned to formulation approaches. A study sponsored by the University of Duisburg-Essen (Germany) was the first to use an anionic liposomal system to deliver siRNAs against bcr-abl proto-oncogene in a patient with Chronic Myeloid Leukemia[35]. The liposome-formulated drug was administered intravenously and

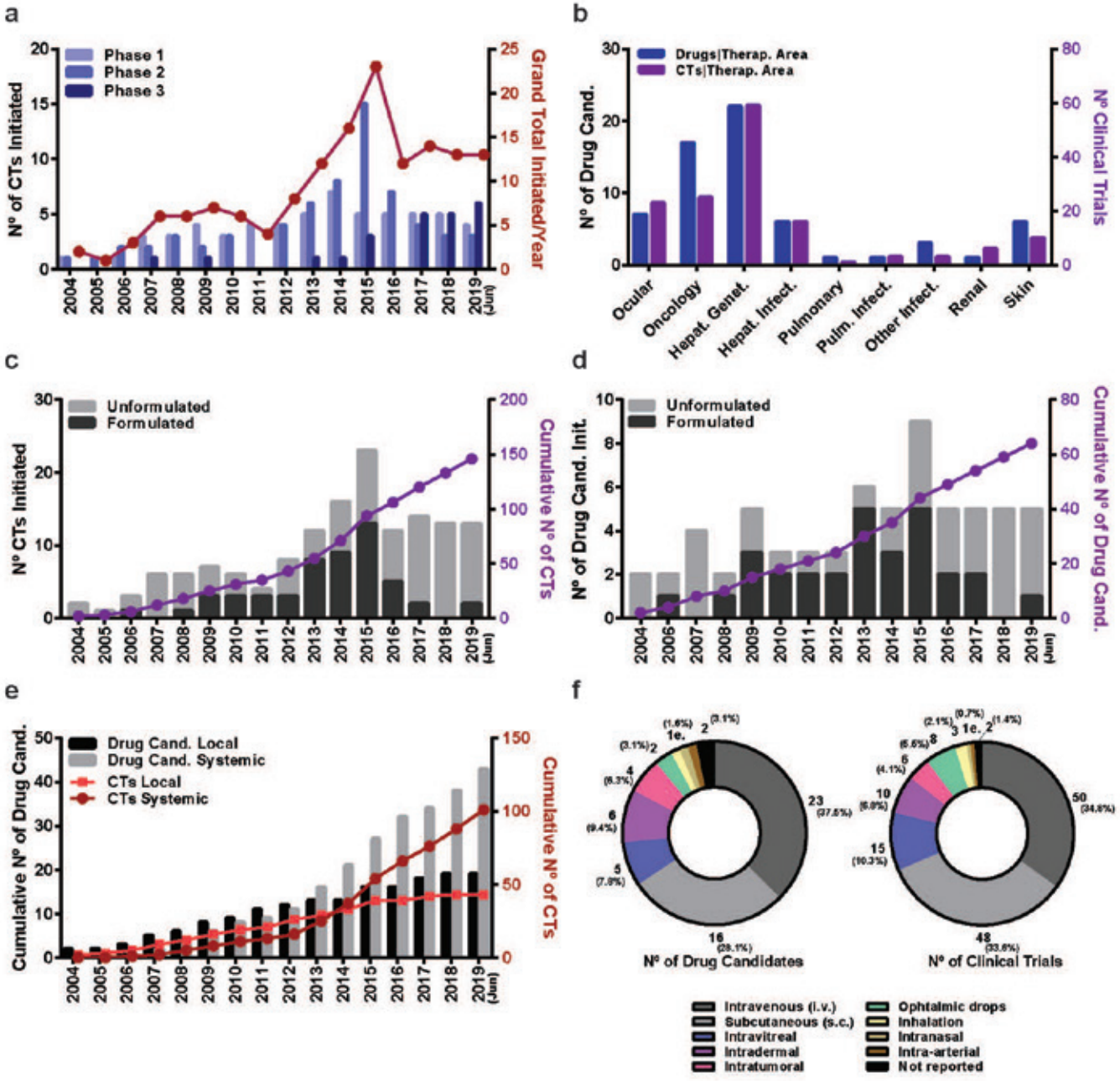

Figure 2. The evolution of the clinical landscape of synthetic RNAi drugs.

Clinical trial registry databases, including clinicaltrials.gov, EudraCT, ANZCTR, Japan UMIN-CTR, ChiCTR and ICTRP-WHO, were searched for the following terms: RNA interference, RNAi, small interfering ribonucleic acid, small interfering RNA, and siRNA. Registries were also searched for specific RNAi industries (e.g. Alnylam pharmaceuticals, Arrowhead pharmaceuticals, etc). All searches were performed by June 2019. (a) Bars indicate total number of clinical trials (CTs) per year, categorized by Phase. Red line denotes the grand total of CTs per year. (b) Total number of RNAi drug candidates (blue) and CTs (purple) per therapeutic area. (c) Bars represent the number of RNAi drug candidates entering clinical development per year, categorized by delivery strategy. Purple line symbolizes the cumulative number of drug candidates entering clinic during the 15 years of development. (d) Bars represent the number of CTs initiated per year, categorized by delivery strategy. Purple line symbolizes the cumulative number of CTs initiated during the 15 years of development. (e) Bars indicate the cumulative number of RNAi drug candidates comparing local vs. systemic approaches. Red line denotes the cumulative number of CTs per local vs. systemic strategies. Information was not available for 2 trials, which were excluded from the graph. (f) Pie charts represent the number of RNAi drug candidates (left) and number of CTs (right), and respective percentages, per route of administration used.

produced remarkable downregulation of the target without any reported adverse events[35]. The first large Phase I study was conducted by Calando Pharmaceuticals with a cyclodextrin (CD)-based polymer as a delivery vehicle for unmodified siRNAs targeting RRM2 (CALAA-01)[36]. In the clinic, this was the first example of a targeted delivery system for siRNAs that was decorated with transferrin ligands, which enabled cellular uptake in solid tumors after intravenous injection[36]. First-generation lipid-based nanoparticles were introduced in subsequent years mostly for the treatment of various types of cancers (e.g. Atu027), but also for the treatment of liver diseases (e.g. ALN-TTR01, PRO-040201). In contrast to unformulated siRNAs, their formulated counterparts were mostly administered systemically. These systems allowed for substantial reductions in dose, but unfortunately many were associated with significant immunological responses (e.g. cytokine storm) against their biomaterial excipient. Thus, the progress of drug candidates relying on first-generation delivery systems was halted based on safety concerns.

After the discouraging outcomes of initial trials using unmodified (or minimally modified, <50% of nucleotides modified) siRNAs and first-generation nanoparticles, there was a noticeable slowdown in the number of clinical trials being initiated during 2010 and 2011 (Figure 2a, 2c, 2d). Big pharmaceutical companies, such as Novartis, Pfizer, Merck, Medtronic, Roche and Abbott, pulled out their investments or shut-down their in-house programs, aggravating the loss of confidence in this technology[6]. Delivery, stability and anti-immunostimulatory strategies needed substantial improvement to regain credibility with the investing community and the market.

Advances in formulation and biomaterial design renew hope in RNAi-based drugs

Significant efforts and resources were invested in biomaterial design to accelerate drug leads into clinical programs. Alnylam Pharmaceuticals partnered with Arbutus to test subsequent 2nd and 3rd generations of stable nucleic acid lipid nanoparticles (SNALP), opening their first program for familial hypercholesterolemia (ALN-PCS02) and second program for transthyretin (TTR)-mediated amyloidosis (Patisiran/ALN-TTR02). The most advanced SNALP generation employs a novel D-Lin-MC3-DMA cationic lipid which has improved delivery to the liver and a better toxicological profile than previous lipids[27-28]. Arrowhead pharmaceuticals also initiated multiple programs (ARC-520 and ARC-AAT) using licensed technology from Mirus Bio/Roche—Dynamic Polyconjugates (DPC)—that improved the PK and activity of siRNAs[37]. At its core, DPCs consist of a polypeptide-based formulation containing a targeting ligand, a membrane-lytic peptide (melittin-like peptide) and a shielding molecule, such as PEG[37]. A wide variety of other delivery systems have been introduced in the clinic, including EnCore LNPs, LODER polymer, vitamin A-coupled LNPs, and biocollagen systems. By 2013, formulation-based approaches had surpassed unformulated siRNAs in the number of drug candidates and clinical trials (Figure 2c, 2d).

Nanoparticle formulation allowed the first RNAi drug cocktails targeting multiple genes to enter the clinical setting: ALN-VSP02 (against KSP and VEGF) and later STP705 (against TGF-beta and COX-2), both of which were formulated in LNPs. At this time, the first synthetic miRNA mimic (MRX34, mir-34 mimic) also reached the clinic using LNP-technology to treat primary liver cancer, followed by tailored VEDVsPayload in 2013 and TargomiRs (mir-16 mimic) in 2014, both for lung cancer. Therapeutic microRNA mimics have the potential to modulate the expression of a broad, yet specific, network of transcripts, an approach that might be particularly useful in diseases such as cancer[38]. More recently, exosomes isolated from mesenchymal cells have been used as a delivery vehicle for siRNAs against KRAS G12D for pancreatic cancer (NCT03608631), opening another delivery strategy for gene silencing drugs.

Formulation approaches became more successful over time as new materials and concepts were evaluated. However, vector-mediated toxicities still halted progression of most of these delivery platforms. For example, ARC-520 and ARC-AAT programs using the DPC formulation platform presented exciting safety and PK data[39-40], but were put on hold by the FDA after extended toxicology showed death in non-human primates at high doses. Indeed, even the most advanced SNALP systems are not innocuous, with a percentage of patients developing infusion-related mild-to-moderate inflammatory reactions and peripheral edema. To minimize the risk, patients are regularly pre-treated with dexamethasone (corticosteroid), acetaminophen/paracetamol (non-steroidal anti-inflammatory), ranitidine or famotidine (H_2 blocker) and diphenhydramine (H_1 blocker)[39,41]. Despite this requirement, 3rd generation SNALPs have not being associated with any major adverse events, and are considered safe.

Lingering safety concerns with formulation-based approaches and significant advances in chemical stabilization of therapeutic oligonucleotides allowed for the emergence of another valid strategy in the clinic: conjugate-mediated delivery[20].

Emergence of conjugation as the dominant delivery paradigm in the clinic

RXi pharmaceuticals pioneered the first clinical trial that investigated the therapeutic utility of a conjugated siRNA without any formulation using their hydrophobic self-delivering asymmetric siRNA scaffold. This consisted of a self-delivering hydrophobically-conjugated asymmetric siRNAs against connective tissue growth factor (CTGF) injected intradermally for prevention of hypertrophic scarring[42]. However, the most prominent platform currently in the clinic uses a trivalent GalNAc ligand for targeted delivery to hepatocytes through ASGPR[31]. The high recycling turnover of ASGPR yields high levels of uptake in the liver after a simple subcutaneous injection[30]. Subcutaneous delivery enables convenient self-administration by the patient and is less costly for the healthcare system. Thus, this approach is preferable to the intravenous injections used for formulation-based approa-

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Table 1. Clinical trials conducted using synthetic RNAi drug candidates

T. Area	Drug	Target Gene	Technology	Primary Conditions	Latest Phase	CT Identifier	Sponsor	Year
Ocular	Bevasiranib/Cand5	VEGF	Minimally/Partially modified siRNA	Age Related Macular Degeneration	Phase 3	NCT00557791	OPKO Health, Inc.	2004
	AGN211745/sirna-027	VEGFR1	Minimally/Partially modified siRNA	Age Related Macular Degeneration	Phase 2	NCT00395057	Allergan|Sirna Therapeutics Inc.	2004
	Bamosiran/SYL040012	ADRB2	Minimally/Partially modified siRNA	Ocular Hypertension|Open Angle Glaucoma	Phase 2	NCT02250612	Sylentis, S.A.	2009
	Tivanisiran/SYL1001	TRPV1	Minimally/Partially modified siRNA	Ocular Pain|Dry Eye Syndrome	Phase 3	NCT03108664	Sylentis, S.A.	2011
	PF-04523655/REDD14NP	RTP801	Minimally/Partially modified siRNA	Age-Related Macular Degeneration	Phase 2	NCT01445899	Quark Pharmaceuticals|Pfizer	2007
	QPI-1007	CASP2	Minimally/Partially modified siRNA	Ischemic Optic Neuropathy	Phase 2|Phase 3	NCT02341560	Quark Pharmaceuticals	2010
	RXI-109-1501	CTGF	Hydrophobic siRNA (sd-rxRNA)	Retinal Scarring in Age-related macular degeneration (AMD)	Phase 1|Phase 2	NCT02599064	RXi Pharmaceuticals, Corp.	2015
Oncology	CatS siRNA	CatS	Minimally/Partially modified siRNA	Liver cancer	Phase 1	ChiCTR-RCC-08000279	The First Hospital, Guangxi Medical University	2007
	CALAA-01	RRM2	Cyclodextrin NP	Cancer|Solid Tumor	Phase 1	NCT00689065	Calando Pharmaceuticals	2008
	ALN-VSP02	KSP and VEGF	SNALP MC3 Lipid	Solid Tumors	Phase 1	NCT01158079	Alnylam Pharmaceuticals	2009
	TKM-080301/TKM-PLK1	PLK1	SNALP MC3 Lipid	Cancer With Hepatic Metastases	Phase 1|Phase 2	NCT02191878	National Cancer Institute	2010
	DCR-MYC	MYC	LNP EnCore	Advanced Solid Tumors	Phase 1|Phase 2	NCT02314052	Dicerna Pharmaceuticals, Inc.	2014
	MRX34	(mir-34 mimic)	LNP (Liposomes)	Primary Liver Cancer|Melanoma	Phase 1|Phase 2	NCT02862145	Mirna Therapeutics, Inc	2013
	VEDVsPayload	EGFR (or mir15/16 mimic)	EnGeneIC Delivery Vehicles	Advanced Solid Tumours Expressing EGFR	Phase 1|Phase 2	ACTRN12613001249741	EnGeneIC, LTD.	2013
	Atu027	PKN3	LNP (Atuplex)	Advanced Solid Tumors|Pancreatic cancer	Phase 1|Phase 2	NCT01808638	Silence Therapeutics GmbH	2009
	Nek2 siRNA	NEK2	Biocollagen delivery system	Non curative pancreas cancer	Phase 1|Phase 2	JPRN-UMIN000016330	Nagoya University Hospital	2013
	siG12D LODER	KRASG12D	LODER polymer	Pancreatic Ductal Adenocarcinoma|Pancreatic Cancer	Phase 2	NCT01676259	Silenseed Ltd	2011
	iExosomes-KrasG12D siRNA	KRASG12D	iExosomes	Pancreatic Ductal Adenocarcinoma|Pancreatic Cancer	Phase 1	NCT03608631	M.D. Anderson Cancer Center	2019
	TDM-812 (RPN2siRNA/A6K complex)	RPN2	A6K Surfactant-like peptide	Treatment-resistant breast cancer	Phase 1	JPRN-UMIN000016790	National Cancer Center (Japan)	2015
	siRNA-EphA2-DOPC	EphA2	LNP (DOPC Neutral Liposomes)	Advanced Cancers	Phase 1	NCT01591356	M.D. Anderson Cancer Center	2015
	ATN-RNA	Tenascin-C	No / Not specified	Glioblastoma multiforme	Phase 1|Phase 2	Not reported	Not reported	2006
	BCR-ABL siRNA	BCR-ABL	Anionic Liposomes	Chronic Myeloid Leukemia	Phase 1	Not reported	University of Duisburg-Essen	2006
	TargomiRs/MesomiRs	(mir-16 mimic)	EnGeneIC Delivery Vehicles	Malignant Pleural Mesothelioma|Non-Small Cell Lung Cancer	Phase 1	NCT02369198	University of Sydney	2014
	NU-0129	BCL2L12	Gold Nanoparticle/Spherical Nucleic Acid	Gliosarcoma|Recurrent Glioblastoma	Early Phase 1	NCT03020017	Northwestern University|National Cancer Institute (NCI)	2017
Hepat. Gen.	ALN-TTR01	TTR	SNALP	TTR-mediated Amyloidosis	Phase 1	NCT01148953	Alnylam Pharmaceuticals	2010
	Patisiran/ALN-TTR02	TTR	SNALP MC3 Lipid	TTR-mediated Amyloidosis	Approved	NCT03862807	Alnylam Pharmaceuticals	2012
	Revusiran/ALN-TTRSC	TTR	GalNac-siRNA (STC) conjugate	TTR-mediated Amyloidosis	Phase 3	NCT02319005	Alnylam Pharmaceuticals	2013
	Vutrisiran/ALN-TTRSC02	TTR	GalNac-siRNA (ESC) conjugate	TTR-mediated Amyloidosis	Phase 3	NCT03759379	Alnylam Pharmaceuticals	2019
	Givosiran/ALN-AS1	ALAS-1	GalNac-siRNA (ESC) conjugate	Acute Intermittent Porphyria	Approved	NCT03338816	Alnylam Pharmaceuticals	2015
	ALN-PCS02	PCSK9	SNALP MC3 Lipid	Hypercholesterolemia	Phase 1	NCT01437059	Alnylam Pharmaceuticals	2011
	Inclisiran/ALN-PCSSC	PCSK9	GalNac-siRNA (ESC) conjugate	Hypercholesterolemia	Phase 3	NCT03814187	Alnylam Pharmaceuticals|The Medicines Company	2014
	PRO-040201/TKM-ApoB	ApoB	SNALP MC3 Lipid	Hypercholesterolemia	Phase 1	NCT00927459	Tekmira Pharmaceuticals Corporation	2009
	ARO-ANG3	Angiopoietin-like protein 3	Targeted RNAi Molecule (TRiM)/GalNAc-siRNA	Hypercholesterolemia|Hypertriglyceridemia	Phase 1	NCT03747224	Arrowhead Pharmaceuticals	2019
	ARO-APOC3	ApoC-III	Targeted RNAi Molecule (TRiM)/GalNAc-siRNA	Hypertriglyceridemia	Phase 1	NCT03783377	Arrowhead Pharmaceuticals	2019
	Cemdisiran/ALN-CC5	CC5	GalNac-siRNA (ESC) conjugate	Complement-mediated diseases, Proteinuria	Phase 2	NCT03841448	Alnylam Pharmaceuticals	2015
	Fitusiran/ALN-AT3SC	Antithrombin	GalNac-siRNA (ESC) conjugate	Hemophilia A|Hemophilia B	Phase 3	NCT03417102	Alnylam Pharmaceuticals	2014
	ALN-AAT	AAT	GalNac-siRNA (ESC) conjugate	Alpha-1 Antitrypsin Deficiency	Phase 1|Phase 2	NCT02503683	Alnylam Pharmaceuticals	2015
	ALN-AAT02	AAT	GalNac-siRNA (ESC+) conjugate	Alpha-1 Antitrypsin Deficiency	Phase 1|Phase 2	NCT03767829	Alnylam Pharmaceuticals	2016
	ARC-AAT	AAT	Dynamic Polyconjugates	Alpha-1 Antitrypsin Deficiency	Phase 2	NCT02900183	Arrowhead Pharmaceuticals	2015
	ARO-AAT	AAT	Targeted RNAi Molecule (TRiM)/GalNAc-siRNA	Alpha-1 Antitrypsin Deficiency	Phase 2|Phase 3	NCT03945292	Arrowhead Pharmaceuticals	2018
	AMG 890/ARC-LPA	ApoA	GalNAc-siRNA	Cardiovascular disease	Phase 1	NCT03626662	Amgen	2018
	ALN-AGT01	Angiotensinogen	GalNac-siRNA (ESC+) conjugate	Hypertension	Phase 1	NCT03934307	Alnylam Pharmaceuticals	2019
	Lumasiran/ALN-GO1	Hydroxyacid oxidase 1	GalNac-siRNA (ESC) conjugate	Primary Hyperoxaluria	Phase 3	NCT03905694	Alnylam Pharmaceuticals	2016
	DCR-PH1	Hydroxyacid Oxidase 1	LNP EnCore	Primary Hyperoxaluria Type 1	Phase 1	NCT02795325	Dicerna Pharmaceuticals, Inc.	2016
	DCR-PHXC	Lactate dehydrogenase A	GalXC	Primary Hyperoxaluria Type 1	Phase 2	NCT03847909	Dicerna Pharmaceuticals, Inc.	2017
	ND-L02-s0201	SERPINH1, heat shock protein 47	Vitamin A-Coupled LNP	Hepatic Fibrosis (METAVIR F3-4)	Phase 1	NCT02227459	Nitto Denko Corporation	2013
Hepat. Inf.	ALN-HBV/VIR-2218	HBV	GalNac-siRNA (ESC+) conjugate	Chronic Hepatitis B	Phase 1|Phase 2	NCT03672188	Alnylam Pharmaceuticals	2016
	ARC-520	HBV	Dynamic Polyconjugates	Chronic Hepatitis B	Phase 2	NCT02738008	Arrowhead Pharmaceuticals	2013
	ARC-521	HBV	Dynamic Polyconjugates	Chronic Hepatitis B	Phase 1	NCT02797522	Arrowhead Pharmaceuticals	2016
	ARO-520	HBV	Targeted RNAi Molecule (TRiM)/GalNAc-siRNA	Chronic Hepatitis B	Phase 1|Phase 2	NCT03365947	Arrowhead Pharmaceuticals	2018
	DCR-HBVS	HBV	GalXC	Chronic Hepatitis B	Phase 1	NCT03772249	Dicerna Pharmaceuticals, Inc.	2018
	ARB-001467	HBV	SNALP MC3 Lipid	Chronic Hepatitis B	Phase 2	NCT02631096	Arbutus Biopharma Corporation	2015
Pulmonary	ExcellairTM	Syk Kinase	Minimally/Partially modified siRNA	Asthma	Phase 2	Not reported	ZaBeCor	2009
Pulm. Inf.	ALN-RSV01	RSV nucleocapsid	Minimally/Partially modified siRNA	Respiratory Syncytial Virus Infections	Phase 2	NCT01065935	Alnylam Pharmaceuticals	2007
Other Inf.	TKM-100201	VP24, VP35, Zaire Ebola L-polymerase	SNALP MC3 Lipid	Ebola Virus Infection	Phase 1	NCT01518881	Tekmira Pharmaceuticals Corporation	2012
	TKM-100802	VP24, VP35, Zaire Ebola L-polymerase	SNALP MC3 Lipid	Ebola Virus Infection	Phase 1	NCT02041715	Tekmira Pharmaceuticals Corporation	2014
	SXL01	AR-V7	No / Not specified	Prostate Cancer	Phase 1	NCT02866916	SeleXel |Institut Claudius Regaud	2017
Renal	Teprasiran/QPI-1002/I5NP	p53	Part. mod. siRNA/Stabilized siRNA	Acute Kidney Injury/Delayed Graft Function	Phase 3	NCT03510897	Quark Pharmaceuticals	2007
Skin	TD101	KRT6a (N171K mutation)	Unmodified siRNA	Pachyonychia Congenita	Phase 1	NCT00716014	TransDerm/Pachyonychia Congenita Project	2008
	RXI-109	CTGF	Hydrophobic siRNA (sd-rxRNA)	Hypertrophic Scar	Phase 2	NCT02079168	RXi Pharmaceuticals, Corp.	2012
	RXI-SCP-1502	CTGF	Hydrophobic siRNA (sd-rxRNA) + DPCP, Samcyprone	Human papillomavirus Warths	Phase 2	NCT02640820	RXi Pharmaceuticals, Corp.	2015
	OLX10010-01	CTGF	Self-delivering Asymmetric siRNA	Hypertrophic Scar	Phase 1	NCT03569267	Olix Pharmaceuticals, Inc.	2018
	BMT101	CTGF	Self-delivering Asymmetric siRNA	Hypertrophic Scar	Phase 1	NCT03133130	Olix Pharmaceuticals, Inc.(Biomolec. Therap.)|Hugel	2017
	STP705	TGF-beta and COX-2	Histidine Lysine Polypeptide Nanoparticle	Hypertrophic Scar	Phase 1|Phase 2	NCT02956317	Sirnaomics	2017

Notes: AAT, Alpha-1 antitrypsin. ADRB2, Beta 2 Adrenoreceptor. ALAS-1, 5'-Aminolevulinate Synthase 1. Apo, Apolipoprotein. AR-V7, Androgen-receptor splice variant 7. BCR-ABL, BCR-ABL proto-oncogene. CASP2, Caspase-2. Cbl-b, E3 ubiquitin ligase casitas B-lineage lymphoma-b. CC5, Complement Component 5. COX-2, Cyclooxygenase 2. CTGF, connective tissue growth factor. DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine. EGFR, epidermal growth factor receptor. EphA2, Ephrin Type-A Receptor 2. EUS, Endoscopic Ultrasound. HBV, Hepatitis B virus. KRASG12D, KRAS Proto-Oncogene. KRT6a, Keratin 6a. KSP, Kinesin spindle protein. LODER, Local Drug EluteR. MYC, MYC Proto-Oncogene. NEK2, Never in Mitosis Related Kinase 2. PCSK9, Proprotein Convertase Subtilisin/Kexin Type 9. PKN3, Protein Kinase N3. PLK1, polo-kinase 1. RPN2, Ribophorin II. RRM2, M2 Subunit of Ribonucleotide reductase. RSV, Respiratory syncytial virus. Syk, Spleen tyrosine kinase. TGF-beta, transforming growth factor-beta. TRPV1, Transient Receptor Potential Vanilloid-1. TTR, Transthyretin. VEGF, Vascular Endothelial Growth Factor. VEGFR, Vascular Endothelial Growth Factor receptor. VP24, viral protein 24. VP35, viral protein 35.

ches. Alnylam Pharmaceuticals were the first RNAi company to introduce such a concept, resulting in 7 drug candidates being evaluated for different liver indications (Table 1). Many other industrial competitors, including Dicerna Pharmaceuticals and Silence Therapeutics, followed with their own proprietary GalNAc conjugate variants.

The GalNAc platform achieved deeper and uniform penetration into the liver, and a better toxicity profile than LNP formulations with no prior or co-administration of immunesupression required. This brought a new wave of enthusiasm to the field. By 2015, the clinical landscape reached an inflexion point from where formulation-based approaches started to lose popularity, and conjugate-mediated delivery (within the category of unformulated siRNAs) became the dominating paradigm (Figure 2c, 2d). Moreover, the popularity of these new GalNAc platforms across many companies significantly skewed the focus from local delivery approaches to systemic delivery strategies, with the liver as main target (Figure 2e, 2f).

RNAi was once again blossoming with a new and revitalized approach that enabled modulation of virtually any gene in the liver. However, during the Revusiran Phase 3 trial (ENDEAVOUR) in late 2016, an imbalance of mortality between the treatment arm (*n*=18 out of 140) versus placebo (*n*=2 out of 66) was reported, and re-ignited concerns about the future of RNAi[43]. Revusiran consisted of a GalNAc-conjugated siRNA targeting TTR, essentially an alternative to Patisiran[30]. In contrast to minimally-modified Patisiran, Revusiran used first generation chemistry (Standard Template Chemistry, STC) of fully modified siRNAs, with a high percentage of 2′-Fluoro (~50%) modifications and a limited number of PS substitutions. Although investigations failed to clearly identify the cause of the associated toxicities, Alnylam decided to discontinue further development of this candidate.

Revusiran required substantially higher doses (annualized dose ~28 g) and more frequent dosing, than other GalNAc conjugate programs that used enhanced stabilization chemistries (ESC) (e.g. Inclisiran yearly exposure of ~0.6-1.2 g, dosing every 3-6 months)[44-45]. ESCs and advanced ESCs (ESC+) contain a lower fraction of 2′-fluoro modifications and higher amount of 2′-O-Methyl and terminal PS modifications. The higher stability and potency of these compounds enable up to 6-12 months of gene silencing in the liver after a single administration, and thus have been the focus of most drug development programs at Alnylam.

The approval of the first RNAi drug

Despite conjugate-mediated approaches dominating the clinic landscape, the first approved RNAi drug was Patisiran, a LNP-formulated minimally-modified siRNA. Patisiran was approved in August 2018 for the treatment of the polyneuropathy of hereditary TTR-mediated amyloidosis[41]. A wide variety of mutations associated with the TTR gene (e.g. V30M and V122I0) can destabilize the tretrameric protein, causing misfolding and aggregation of the monomers into amyloid fibrils[46]. TTR is produced in the liver, but is released into the bloodstream and amyloid fibrils may deposit in several organ-systems, including the heart and nervous system[46]. Consequently, patients develop heart-related pathology, including hypotension and arrhytmia, and sensorimotor and autonomic disturbances that deteriorate movement and other daily activities[46].

Patisiran silences the expression of both wild-type and mutant forms of TTR in the liver by targeting conserved regions at the 3′-UTR of the TTR mRNA. Multi-dose Phase 2 trials showed dose-dependent reductions of TTR levels in serum after intravenous administration of Patisiran[47]. Two consecutive doses of Patisiran (0.3 mg/kg, every 3 weeks) reduced TTR levels in serum by 80% (NCT01617967)[47]. The subsequent Phase 3 APOLLO trial showed a significant improvement in quality of life, nutrition status, walking, and other daily activities, meeting both primary and secondary endpoints of the trial[41]. Patisiran was also found to be well-tolerated during the time course of the trials, with only mild-to-moderate infusion-related reactions being reported[41,47]. Together, these trials demonstrated that gene silencing is a valid approach to halt or reverse TTR-mediated pathology.

Other drug types have been approved for hTTR amyloidosis, including the small molecule drug, Tafamidis, which stabilizes TTR[46]. However, these do not directly target the root cause of the disease. Oligonucleotide-based products for hTTR amyloidosis have also entered the market (Inotersen, TEGSEDI™). Inotersen consists of an ASO that targets the TTR mRNA for degradation through a RNase H-mediated mechanism[48]. Clinical trials showed robust silencing and clinical improvements[48], but substantially higher doses were required for therapeutic effect (284 mg/weekly) compared to Patisiran (~21-30 mg/3 weeks). Thus, the potency and long-lasting efficacy of synthetic siRNAs make RNAi-based therapeutics an extremely attractive approach for gene silencing.

Approval of the first conjugated RNAi drug

Late in November 2019, FDA approved the first GalNAc-conjugated siRNA, the second synthetic RNAi drug. Givosiran (GIVLAARI™) received approval for acute intermittent porphyria, a disorder that causes acute neurovisceral attacks and takes root in an enzymatic deficiency in heme production[49-50]. Accumulation of delta-aminolevulinic acid (ALA) and porphobilinogen leads to acute neurotoxicity, and by targeting delta-ALA synthase 1 (ALAS1), Givosiran silences the expression of these toxic products and reduces disease burden[49-50]. The Phase 3 ENVISION trial (NCT03338816) showed that a subcutaneous monthly administration of 2.5 mg/kg of Givosiran enabled reduction of urinary levels of ALA, but also significantly reduced the rates of porphyria attacks requiring hospitalization over a period of 6 months (70% fewer attacks than the placebo group)[50].

The approval of this first GalNAc-conjugated siRNA established a major milestone for targeted delivery approaches in the field. The 10x lower doses to achieve therapeutic efficacy, the long-lasting silencing effects and the ease of subcutaneous administrations made the GalNAc platform a remarkable advance for the treatment of liver diseases. In fact, the potential of this platform and the recent approval of Givosiran prompted the 9.7 billion dollar acquisition of The Medi-

cines Company by Novartis in an effort to be able to commercialize Inclisiran upon FDA approval. Inclisiran is a GalNAc--siRNA targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) as a strategy to reduce low-density lipoprotein cholesterol (LDL-C) levels in the blood stream[44-45]. Inclisiran enables at least 3-6 months of silencing of the PCSK9 target and a significant reduction in LDL-C[44-45]. Although Inclisiran was initially being developed for patients with familial hypercholesterolemia, the long duration of effect (up to 6 months) encouraged investigators to broaden the scope of indications to include Atherosclerotic cardiovascular disease (ASCVD). It is believed that, if approved, Inclisiran may become the first "RNAi blockbuster" drug that will enable treatment of millions of patients around the globe.

The future of RNAi medicines

In the near future, several other RNAi drug candidates are expected to receive FDA approval for liver diseases. Most of these consist of GalNAc-conjugated fully modified siRNA scaffolds, such as Inclisiran, and Lumasiran, all of which are currently in Phase 3 trials. Importantly, the clinical success of the GalNAc platform has inspired the field to explore other conjugated modalities for extra-hepatic delivery. Conjugation of chemically diverse lipids, carbohydrates, aptamers, and antibodies has been shown to drive tissue distribution beyond the liver after systemic administration[29]. Expanding the chemical diversity of siRNAs has also brought fruitful results after local administration. In the central nervous system, fine-tuning hydrophobicity and construct size has been shown to be pivotal for distribution and retention[20]. As seen for liver diseases, once a delivery ligand with suitable uptake in a target cell/tissue has been identified, changing the target sequence will allow for modulation of any given gene target in that tissue. This opens the possibility of developing disease-modifying treatments for a wide-range of debilitating and progressive diseases, provided that the genetic target (or targets) has been identified.

Most applications in the clinic are currently limited to rare disorders with a single causative gene. In the future, it is likely that treatment of more common and complex diseases will be considered. The long-lasting effects (6-12 months) of RNAi-based drugs makes them an attractive approach for this purpose, and places RNAi medicines on par with other therapeutics, such as vaccines, that only require yearly (or multi-year) dosing to maintain clinical efficacy.

Conclusion

Many expect RNAi to become "the monoclonal antibody of the 2020s". Unlike small molecules, which are generally restricted to a selected range of protein targets, RNAi directly inhibits the protein precursors (mRNA), allowing modulation of any disease target. Although the first drug approved by regulators consisted of a LNP-formulated siRNA, conjugate-mediated delivery has become the dominant strategy in the clinic for liver diseases. This was made possible through critical developments in RNA chemistry, that enhanced stability and reduced immune responses, accompanied by creative new approaches that improved delivery. In the future, discovery of new delivery platforms that enable meaningful distribution, accumulation, and durable gene silencing in tissues beyond the liver has the potential to accelerate the impact of this technology exponentially. Patisiran, the first success in the clinic, opened a new chapter for this technology that will enable positive change in the lives of many patients, and in human medicine, by treating currently incurable disorders.

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References

1. Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell. 1990;2(4):279-89.
2. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391(6669):806-11.
3. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. 2001;20(23):6877-88.
4. Wood H. FDA approves patisiran to treat hereditary transthyretin amyloidosis. Nat Rev Neurol. 2018;14(10):570.
5. Corey DR. RNA learns from antisense. Nat Chem Biol. 2007;3:8.
6. Haussecker D. The business of RNAi therapeutics in 2012. Mol Ther Nucleic Acids. 2012;1(2):e8.
7. Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. Nat Biotechnol. 2017;35(3):238-48.
8. Plasterk RH. RNA silencing: the genome's immune system. Science. 2002;296(5571):1263-5.
9. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature. 2004;431(7006):343-9.
10. Huang W. MicroRNAs: biomarkers, diagnostics, and therapeutics. In: Huang J, Borchert GM, Dou D, Huan J, Lan W, Tan M, et al., editors. Bioinformatics in MicroRNA research. New York: Springer; 2017. p. 57-67. ISBN 9781493970469
11. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet. 2003;4(5):346-58.
12. Couto LB, High KA. Viral vector-mediated RNA interference. Curr Opin Pharmacol. 2010;10(5):534-42.

13. Marshall E. Gene therapy death prompts review of adenovirus vector. Science. 1999;286(5448):2244-5.
14. Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR. Gene therapy clinical trials worldwide to 2017: an update. J Gene Med. 2018;20(5):e3015.
15. Borel F, Kay MA, Mueller C. Recombinant AAV as a platform for translating the therapeutic potential of RNA interference. Mol Ther. 2014;22(4):692-701.
16. Boudreau RL, Spengler RM, Hylock RH, Kusenda BJ, Davis HA, Eichmann DA, et al. siSPOTR: a tool for designing highly specific and potent siRNAs for human and mouse. Nucleic Acids Res. 2012;41(1):e9.
17. Naito Y, Ui-Tei K. siRNA design software for a target gene-specific RNA interference. Front Genet. 2012;3(102).
18. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol. 2004;22(3):326-30.
19. Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D, et al. A protocol for designing siRNAs with high functionality and specificity. Nat Protoc. 2007;2(9):2068-78.
20. Godinho BM, Coles AH, Khvorova A. Conjugate-mediated delivery of RNAi-based therapeutics: enhancing pharmacokinetics-pharmacodynamics relationships of medicinal oligonucleotides. In: Agrawal S, Gait MJ, editors. Advances in nucleic acid therapeutics. Royal Society of Chemistry; 2019. p. 206-32. ISBN 9781788015714
21. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature. 2008;452(7187):591-7.
22. Robbins M, Judge A, Liang L, McClintock K, Yaworski E, MacLachlan I. 2'-O-methyl-modified RNAs Act as TLR7 Antagonists. Mol Ther. 2007;15(9):1663-9.
23. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BRG. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. 2003;5(9):834-9.
24. Schlee M, Hornung V, Hartmann G. siRNA and isRNA: two edges of one sword. Mol Ther. 2006;14(4):463-70.
25. Watts JK, Deleavey GF, Damha MJ. Chemically modified siRNA: tools and applications. Drug Discov Today. 2008;13(19-20):842-55.
26. Haraszti RA, Roux L, Coles AH, Turanov AA, Alterman JF, Echeverria D, et al. 5´-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs in vivo. Nucleic Acids Res. 2017;45(13):7581-92.
27. Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nat Rev Genet. 2014;15(8):541-55.
28. Guzman-Villanueva D, El-Sherbiny IM, Herrera-Ruiz D, Vlassov AV, Smyth HD. Formulation approaches to short interfering RNA and MicroRNA: challenges and implications. J Pharm Sci. 2012;101(11):4046-66.
29. Osborn MF, Coles AH, Biscans A, Haraszti RA, Roux L, Davis S, et al. Hydrophobicity drives the systemic distribution of lipid-conjugated siRNAs via lipid transport pathways. Nucleic Acids Res. 2018;47(3):1070-81.
30. Zimmermann TS, Karsten V, Chan A, Chiesa J, Boyce M, Bettencourt BR, et al. Clinical proof of concept for a novel hepatocyte-targeting GalNAc-siRNA conjugate. Mol Ther. 2017;25(1):71-8.
31. Springer AD, Dowdy SF. GalNAc-siRNA conjugates: leading the way for delivery of RNAi therapeutics. Nucleic Acid Ther. 2018;28(3):109-18.
32. Garba AO, Mousa SA. Bevasiranib for the treatment of wet, age-related macular degeneration. Ophthalmol Eye Dis. 2010;2:75-83.
33. Kaiser PK, Symons RA, Shah SM, Quinlan EJ, Tabandeh H, Do DV, et al. RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. Am J Ophthalmol. 2010;150(1):33-9. e2.
34. Peddi V, Ratner L, Cooper M, Gaber O, Feng S, Tso P, et al. Treatment with QPI-1002, a short interfering (SI) RNA for the prophylaxis of delayed graft function: abstract# 2967. Transplantation. 2014;98:153.
35. Koldehoff M, Steckel NK, Beelen DW, Elmaagacli AH. Therapeutic application of small interfering RNA directed against bcr-abl transcripts to a patient with imatinib--resistant chronic myeloid leukaemia. Clin Exp Med. 2007;7(2):47-55.
36. Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, Alabi CA, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010;464(7291):1067-70.
37. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, et al. Dynamic polyConjugates for targeted in vivo delivery of siRNA to hepatocytes. Proc Nat Acad Sci. 2007;104(32):12982-7.
38. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov. 2017;16(3):203-22.
39. Schluep T, Lickliter J, Hamilton J, Lewis DL, Lai CL, Lau JY, et al. Safety, tolerability, and pharmacokinetics of ARC-520 injection, an RNA interference-based therapeutic for the treatment of chronic hepatitis B virus infection, in healthy volunteers. Clin Pharmacol Drug Dev. 2017;6(4):350-62.
40. Turner AM, Stolk J, Bals R, Lickliter JD, Hamilton J, Christianson DR, et al. Hepatic-targeted RNA interference provides robust and persistent knockdown of alpha-1 antitrypsin levels in ZZ patients. J Hepatol. 2018;69(2):378-84.
41. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang C-C, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. N Engl J Med. 2018;379(1):11-21.
42. Pavco P, Libertine L, Young V, Woolery-Lloyd H, Cauwenbergh G. RXI-109 treatment to reduce the formation of keloids following keloidectomy. J Am Acad Dermatol. 2015;72(5 Suppl 1):AB273.
43. Mullard A. RNAi hits another rut. Nat Rev Drug Discov. 2016;15(11):738.
44. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, et al. Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol. N Engl J Med. 2017;376(15):1430-40.

45. Huang Y. Preclinical and clinical advances of GalNAc--decorated nucleic acid therapeutics. Mol Ther Nucleic Acids. 2017;6:116-32.
46. Ando Y, Coelho T, Berk JL, Cruz MW, Ericzon B-G, Ikeda S-i, et al. Guideline of transthyretin-related hereditary amyloidosis for clinicians. Orphanet J Rare Dis. 2013;8:31.
47. Suhr OB, Coelho T, Buades J, Pouget J, Conceicao I, Berk J, et al. Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: a phase II multi-dose study. Orphanet J Rare Dis. 2015;10:109.
48. Benson MD, Waddington-Cruz M, Berk JL, Polydefkis M, Dyck PJ, Wang AK, et al. Inotersen treatment for patients with hereditary transthyretin amyloidosis. N Engl J Med. 2018;379(1):22-31.
49. Sardh E, Harper P, Balwani M, Stein P, Rees D, Bissell DM, et al. Phase 1 Trial of an RNA interference therapy for acute intermittent porphyria. N Engl J Med. 2019;380(6):549-58.
50. Brandão PR, Titze-de-Almeida SS, Titze-de-Almeida R. Leading RNA interference therapeutics part 2: silencing delta-aminolevulinic acid synthase 1, with a focus on Givosiran. Mol Diagn Ther. 2019 December 2. [Online ahead of print].

Conflict of interests

This is a contributed review article submitted upon invitation of the Editorial Board.