Journal of Controlled Release 321 (2020) 616–628

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review article

Small interfering RNA from the lab discovery to patients' recovery

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ARTICLE INFO

Keywords:
siRNA
Delivery
Nanotechnology
Formulation
Galenic
Clinical studies

ABSTRACT

In 1998, the RNA interference discovery by Fire and Mello revolutionized the scientific and therapeutic world. They showed that small double-stranded RNAs, the siRNAs, were capable of selectively silencing the expression of a targeted gene by degrading its mRNA. Very quickly, it appeared that the use of this natural mechanism was an excellent way to develop new therapeutics, due to its specificity at low doses. However, one major hurdle lies in the delivery into the targeted cells, given that the different extracellular and intracellular barriers of the organism coupled with the physico-chemical characteristics of siRNA do not allow an efficient and safe administration. The development of nanotechnologies has made it possible to counteract these hurdles by vectorizing the siRNA in a vector composed of cationic lipids or polymers, or to chemically modify it by conjugation to a molecule. This has enabled the first clinical developments of siRNAs to begin very quickly after their discovery, for the treatment of various acquired or hereditary pathologies. In 2018, the first siRNA-containing drug was approved by the FDA and the EMA for the treatment of an inherited metabolic disease, the hereditary transthyretin amyloidosis. In this review, we discuss the different barriers to the siRNA after systemic administration and how vectorization or chemical modifications lead to avoid it. We describe some interesting clinical developments and finally, we present the future perspectives.

1. Introduction

Since the RNA interference (RNAi) discovery, a new specific therapeutic approach for severe diseases has emerged and brings new hope for patients [1,2]. In 2001, Elbashir and Tushl have already mentioned the possible use of small interfering RNA (siRNA) as “a new alternative to antisense or ribozyme therapeutics” [3]. The design and synthesis facilities lead to several research and development of siRNA drug [4].

Indeed, nine years after Elbashir and Tushl discovery, the first RNAi evidence in human was done by Davis et al. [5]. The authors reported that the systemic administration of targeted nanoparticles of siRNA against M2 subunit of ribonucleotide reductase (RRM2) silence efficiently the targeted gene [5]. In 2018, the first-ever siRNA has been approved by the FDA (Food and Drug Administration) for the treatment of a severe neuropathy, the hereditary transthyretin-mediated amyloidosis [6,7]. Very recently, a second siRNA has been marketed to cure an inherited rare hepatic disease after significant results in phase III clinical trial [8].

Despite the fact that siRNA are specific and efficient at low doses, their highly hydrophilicity and their short plasmatic half-life constitute the main hurdles for their systemic administration. Therapeutic applications of siRNA require development of nanosized delivery carriers, named as nanovector or nanoparticle (NPs), to protect oligonucleotides and improve pharmacokinetic parameters. In this review, we describe the siRNA hurdles that must be overcome to exert its effects. Then, different vectorization approaches and their limits to counteract the siRNA barriers will be presented. Finally, we depict some interesting clinical studies and the future challenges of the RNAi therapy.

2. Hurdles for systemic siRNA-based therapeutics

2.1. Obstacles due to intrinsic siRNA properties

The siRNA is a double-stranded RNA composed of 19–21 base pairs with 3′-cohesive ends [3,9]. Antisense strand, also called guide strand, is fully complementary to a mRNA sequence of a targeted gene and induces its specific degradation. The synthetic siRNA bypasses through Dicer cleavage and is taken directly by the RNA-Induced Silencing Complex (RISC). The RISC nuclease, Ago2, unwinds siRNA strands and degrades the sense strand. The guide strand anneals with its complementary mRNA leading to its degradation and the silencing of the targeted gene [1,10]. The activated RISC complex (with antisense strand included) can move on, to degrade additional targeted mRNA, allowing a transient gene silencing for 3–7 days in rapidly dividing cells

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https://doi.org/10.1016/j.jconrel.2020.02.032
Received 7 January 2020; Received in revised form 17 February 2020; Accepted 18 February 2020
Available online 19 February 2020
0168-3659/

and, for several weeks, in slowly dividing cells [11,12].

The siRNA administration aims to deliver it from the injection point to the cytosol of targeted cells in enough quantities, to be able to inhibit the targeted gene expression. Unfortunately, some intrinsic characteristics do not help to reach this aim. Indeed once in the blood, naked siRNA are degraded by nucleases into shortened oligonucleotides then, eliminated by glomerular filtration, leading to a very short blood half-life (less than 15mins) [13–15]. Moreover, the siRNA is of high molecular weight (13 kDa), hydrophilic and polyanionic thus preventing a good cellular uptake. It can also stimulate the immune system by sequence-dependent manner TLR-activation [1]. Several immune-stimulatory sequence patterns have been described, in general a U-rich sequence correlates with TLR-7 and 8 activation, which transmembrane receptors present in endosome of dendritic and monocytic cells [1].

To overcome these disadvantages, siRNA can be protected by a vector that should be non-toxic, biocompatible, biodegradable and non-immunostimulatory. However, each nanovector has its own toxicity, tissue biodistribution and internalization according to its physico-chemical characteristics [13].

We will focus on two siRNA vectorization strategies: the physical one (encapsulation in lipidic and polymeric nanovector) and the chemical one (modification on the siRNA).

2.2. Obstacles due to administration mode

The aim of siRNA encapsulation or modification is to deliver this biologic molecule in the targeted tissue and cells. However, several extracellular and intracellular barriers obstruct this route depending on the administration mode.

Indeed, local injection corresponds to the direct administration of the drug inside the tissue. It limits systemic toxicity of siRNA and nanovector but only few tissues are candidates to localized therapy, including eye, skin, lung (by direct instillation) and local tumors. For deep tissues, systemic administration in the bloodstream *via* intravenous and subcutaneous injections is mandatory. Intravenous injections allow distribution through the whole bloodstream and avoid rapid hepatic clearance by the first-pass effect. The major disadvantage is the occurrence of frequent hypersensitivity reactions, that can be prevented by prophylactic antihistaminic treatment. Subcutaneous injections into adipose tissue are faster and easier to administrate, even by patients themselves. Molecules administered subcutaneously have a slower release into blood and can access circulation *via* capillaries or lymphatic drainage from interstitial space [10,16].

After systemic administration, active molecules disappear from the bloodstream by several ways. The most common one is through kidney clearance. Glomerular filtration allows water and small molecules (size < 8 nm) to pass into urine. This process of excretion concerns only naked siRNA because of its small size (less than 10 nm). On the other hand, siRNA vectorized into nanocarrier avoids renal clearance because of changes in its intrinsic properties. This would allow its retention in the blood circulation. The siRNA-nanovector can be uptaken by tissues of the mononuclear phagocyte system (MPS) after recognition by the monocytes and macrophages. These cells can phagocyte and degrade foreign molecules after their opsonization [17].

siRNA-loaded nanocarriers have to cross the bloodstream to reach the target tissue and achieve their inhibitory effect. This step is called extravasation. Knowledge and exploitation of the vascular characteristics of the target tissue are fundamental in the design and development of the vector that will carry the siRNA. Not all organs have the same accessibility, particularly depending on the endothelium surrounding them. For example, it is easier to reach the liver or some solid tumors, because there are surrounded by disjointed endothelial cells [18]. This endothelial specificity leads to passive accumulation of NPs > 100 nm in size. For tumor, this phenomenon, called “Enhanced Permeability and Retention (EPR) effect” is due to uncontrolled angiogenesis and strong inflammatory reaction [19]. For other tissues/organs, passive exit from the bloodstream is very limited due to continuous capillaries with constant endothelial cells and basal lamina. Only water and small molecules can easily diffuse [17]. In the nervous system, both blood-brain barrier (BBB) and the blood-nerve barrier (BNB) constitute an obstacle for drug delivery. In the brain, it is due to the continuous endothelium with tight junctions. However, transporters in the cerebral endothelial cells for hormones, amino acids and others, could facilitate the internalization of drugs [20]. In the peripheral nervous system, the perineurium and its projections into the endoneurium are responsible of the blood nerve barrier [21].

Once siRNA nanocarriers reach the targeted organs, a second limiting step appears, concerned with the uptake by the targeted cells within the tissues and endosomal escape to provide a silencing effect. Nanoparticles can enter the cells by different endocytosis mechanisms depending on their chemical structure, surface modification, surface charge and size. Macropinocytosis is a non-specific internalization mechanism, characterized by vesicles size from 100 nm to 5 μm. The mechanism is similar to phagocytosis, except that it can occur in all cells [22]. Clathrin- and caveolae-mediated endocytosis are both receptor-mediated endocytosis and are defined by internalization vesicles size of respectively, 120 and 80 nm [23]. The clathrin-endocytic pathway is used by several biological molecules, as lipoproteins and transferrin. Once the ligand attaches to its receptor, it triggers uptake inside the cells and endosome formation. The maturation of this internalization vesicle is correlated with a decrease of pH, the activation of enzymes involved in siRNA degradation and finally fusion with lysosome [24,25]. The ability of NPs to deliver their cargo in the cytoplasm is strongly determined by the mechanism through which they are taken up and how the siRNA goes from the endosome to the cytosol (named as endosomal escape) [26].

Finally, to elicit a biological response (Fig. 1), siRNA chemically modified or encapsulated in a cationic vector, have to circulate into the bloodstream and leave it, be internalized by the targeted cells, and escape from endosome before fusion with lysosome to bind to RISC complex.

3. siRNA vectorization for systemic delivery

3.1. Physical vectorization

Polyanionic siRNA is complexed with a nanomaterial as cationic lipid or cationic polymer *via* electrostatic interactions. Different generations of nanocarrier have been developed. The first generation is composed of simple nanovector that rapidly reach the MPS tissues mainly in the liver and spleen. The fast distribution into theses tissues can be beneficial for the targeting of MPS organs. Otherwise, it can cause toxicity and treatment inefficiency [27]. To address this issue, these nanocarriers can be coated with highly flexible, hydrophilic and neutral polyethylene glycol (PEG) chains. This second generation vector, is also called “stealth nanovector”, because this coating permits to avoid opsonization by MPS cells, reducing non-specific interaction and therefore, improve their circulation time. The challenge here is to control the concentration of PEG surrounding the nanoparticle. A high concentration of polymer chains can reduce cellular uptake and siRNA delivery. Additionally, it causes a size increase of the siRNA nanocarrier, preventing uptake in some organs surrounded by continuous endothelium but allowing accumulation in sites where the endothelial barrier is fenestrated as liver, spleen and tumors with high EPR [15]. The distribution profile of siRNA nanocarriers could be improved by chemically modifying PEG chains through the addition of targeting ligands for specific interactions with receptor expressed at the targeted cells surface (third nanocarrier generation) [27,28].

3.1.1. Lipid nanoparticles (LNP)

Lipid NPs are one of the leading non-viral siRNA delivery nanosystems. There are composed of different lipid types, able to cross the

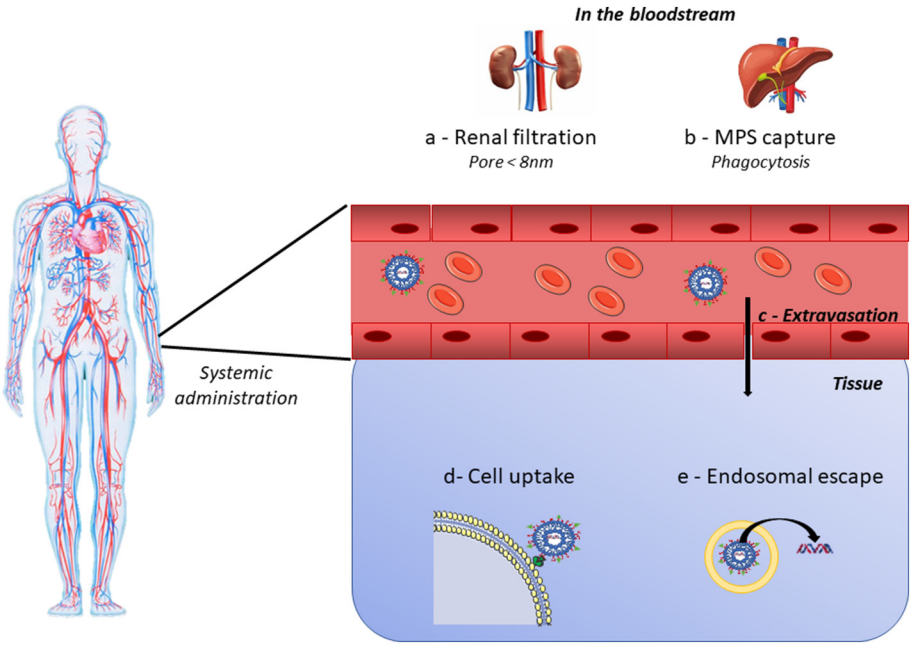

Fig. 1. Barriers to the delivery of siRNAs after systemic administration. After systemic injection, encapsulated siRNA (by physical or chemical interactions) has to avoid glomerular filtration (a) and phagocytosis by MPS cells (b). Once in the targeted tissue (c), it has to be internalized into the cell (d) and escape from endosome before degradation (e).

lipid bilayer of the cell membrane. We describe here the two main groups: the lipoplex or cationic liposome and the stable nucleic acid lipid particle (SNALP).

3.1.1.1. Liposome. Liposome are unilamellar or multilamellar vehicles composed of lipid bilayer, entrapping hydrophilic and lipophilic drugs [29,30]. Some liposomal formulations of different active molecules have been FDA-approved and show to be effective in maintaining high plasma concentrations of low bioavailable drugs with hydrophilic feature or to reduce toxicity, like the antineoplastic doxorubicin molecule (Doxil®, PEGylated liposome) [31].

Cationic lipids interact *via* electrostatic interactions between their positive charges carried by the polar head and polyanionic siRNA, leading to "lipoplex" formation. Positive surface charges are advantageous for siRNA encapsulation, cell uptake and endosomal escape by binding to negatively charged cell membrane. Two basic categories of cationic lipids exist: the monovalent as 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) and multivalent as 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA), a lipid present in the commercial lipoplex Lipofectamine (ratio DOSPA/DOPE 3/1). Lipid structure also has significant influence on lipoplex efficiency and toxicity which could be prevented by lipid library construction approaches, developed to select efficient cationic lipid characterized by enhanced gene silencing and reduced toxicity [32]. Other lipid types are also incorporated into lipoplexes. The "helper lipids" such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) or cholesterol, facilitate siRNA complexation, increase liposome stability and decrease their toxicity. The fusogenic characteristic of DOPE explains its common use in lipoplex formulation as its "fusogenicity" contributes to cytoplasmic delivery of nucleic acids by breakdown of endosomal membrane [32–34].

PEGylated lipid can also be incorporated. The benefit of this incorporation has been demonstrated several times. Recently, Lee and Ahn [35] observed a significant difference in tumor accumulation of a PEGylated and non-PEGylated formulation. This cationic liposome is composed of 3ß-[N-(N′,N′,N′-diméthylaminoéthane)-carbamoyl]cholesterol (DC-chol) as cationic lipid and DOPE as helper. It encapsulates a siRNA against kinesin spindle protein (KSP), involved in cell cycle (formation of bipolar mitotic spindle during centrosome separation). Its inhibition induces mitotic arrest during cell cycle followed by apoptosis. The results were interpreted in three points. Firstly, intravenous injection of PEGylated KSP siRNA-lipoplexes did not activate the immune system in immunocompetent mice, confirming the non-immunostimulatory effects of this formulation. Secondly, biodistribution differences were observed between PEGylated and non-PEGylated form: the PEGylation of the lipoplex increased its circulation time, by reducing renal excretion and scavenging in liver and led to higher tumor accumulation. Thirdly, systemic administration of PEGylated KSP siRNA-lipoplexes caused gene silencing, correlated with substantial tumor growth inhibition [35].

PEGylation improves significantly pharmacokinetic parameters but does not prevent siRNA nanocarrier accumulation in MPS organs (mainly liver). Therefore, adding a ligand at the nanoparticle surface allows to target other tissues such as placenta and glioma [36,37].

Placenta connects the embryo to the uterine wall ensuring its nutrition and respiration. It can be the cause of dysfunction like preeclampsia, characterized by hypertension and proteinuria after 20 weeks of gestation. The lack of preclinical models leads Yu et al., to develop a relevant model based on siRNA anti-H19X vectorized in arginine-glycine-aspartic acid (RGD)-modified PEGylated liposome [36]. The efficient delivery of this formulation is possible because placenta possesses similar features to tumors including rich blood flow, rapid proliferation and overexpressed αvβ3 integrin receptor, which could be targeted by RGD peptide.

Glioma is one of the most aggressive and lethal cancer with a 5-years survival rate less than 10%. To have an antineoplastic effect, the drug must cross the BBB to reach the brain and the cancerous cells. In 2016, Wei *et al,* were able to formulate a third generation liposome with specific ligand of transferrin receptor and encapsulated a siRNA to silence epidermal growth factor receptor (EGFR) expression [37]. The transferrin receptor (TfR) is expressed on brain endothelial cells and cerebral glioma cells, so the conjugation of a peptide binding TfR leads to a dual targeting: BBB and glioma. Therefore, a T7 peptide was designed to bind TfR with high affinity and has been added to cationic liposome surface. This active targeting shows high capacity *in vitro* to cross the BBB and to be uptaked by glioma cells. *In vivo*, intravenous injections of this nanoformulation lead to higher accumulation in the brain tumor, associated with a longer survival without side effect [37].

The active targeting permits also to reach a specific cell type in a tissue. A good example is Vitamin A-coupled liposome at the nanovector surface to target specific cells in the liver, the hepatic stellate cells, responsible for liver fibrosis. The intravenous injection with a

siRNA against HSP47 (heat shock protein 47; collagen-specific chaperone), completely resolved hepatic fibrosis and prolonged survival in rat using three models of liver cirrhosis (with dimethylnitrosamine, CCl4 or bile duct ligation) [38].

3.1.1.2. Stable nucleic acid lipid particle (SNALP). The positive charge explains the success of lipoplex in delivering siRNA to cells, but it is also a cause of adverse events, such as non-specific interactions with negatively charged serum components [39]. Nowadays, new formulation are developed based on nanocarriers with distinct physico-chemical properties from lipoplex [30]. The stable nucleic acid lipids particles (SNALP) are composed of a lipid bilayer containing a lipids mixture of: i) *pH sensitive ionizable lipids* that are optimized in order to have a pKa polar head of around 7, allowing a neutral charge at physiological pH and, a cationic part at low pH allowing better siRNA condensation during nanocarrier formation and allowing a good siRNA release of the endosome. In blood circulation, the SNALP is uncharged, stable with an increased plasmatic half-life and a reduced toxicity, compared to lipoplexes [15]; ii) *Helper lipids* that contribute to the stability and delivery efficiency of the particle, enabling cellular uptake and endosomal release of siRNA [40] and, iii) *Shielding lipids,* PEGylated lipids used to stabilize particle and to provide a neutral hydrophilic surface. It increases circulation time into the blood and decreases uptake by MPS cells. Shielding lipid can be served as an anchor for a targeting ligand [30].

SNALP formation procedure is divided in three steps. Firstly, liposomes are prepared by injection of 40% ethanol solution containing lipid, into an acidic buffer (pH = 4). The resulting liposomes have a positive charge and a high membrane permeability, allowing siRNA to bind on the inner cationic membrane. The last step consists of removing the siRNA by dialysis against neutral buffer [32,40,41].

Different ionizable lipid exists. Alnylam Pharmaceuticals developed a novel type named “lipidoid”. This “lipid-like material” is composed of a polar core surrounded by short hydrophobic carbon tails. The link between the different chemical groups is easily biodegraded by esterases [42].

Another interesting ionizable lipid –named malate deshydrogenase- contains a nitroimidazole chemical function linking the hydrophobic tails (whose nitro group from is converted to amino group in hypoxic conditions) and the polar head containing tertiary amines function protonable at low pH [43]. Tumor environment is hypoxic (partial pressure of oxygen near 0 mmHg) and acidic (pH < 7). Thus, the combination of this lipid with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) and cholesterol leads to liposome formation named MLP and has the unique feature to be “doubling” positive in hypoxic tumor microenvironment [43]. Polo-like kinase 1 (PLK1) is a serine/threonine protein kinase, highly expressed in glioma tissues. PLK1-targeted siRNA has been encapsulated in MLP and injected intravenously *in vivo*. The safety of the formulation is demonstrated on heart, liver, lung and kidney with no-noticeable tissue damages. MLP siRNA PLK1 shows also an effective inhibition of tumor growth *in vitro* and *in vivo* and seems to be a promising siRNA delivery for cancer treatment [43].

Other monogenic pathologies can be cured by siRNA such as thrombotic and hemostatic disorders. The coagulation factors are synthetized by the liver. SNALP was prepared by dissolving ionizable amino lipid, cholesterol, DSPE and PEG2000 and then assembled with different siRNA duplexes depending on animal models. Indeed, two common models for thrombosis and hemostasis study were used: rat model with siRNA anti-kallikrein and rabbit model with siRNA anti-factor X [44]. In both species, the lipidic formulation was well tolerated after single intravenous injection and exerted selective mRNA expression inhibition of over 90% until 7 days in the liver [44].

3.1.2. Polymeric nanocarriers

For siRNA vectorization, few polymers have been used and even less have reached clinical stages. Contrary to LNP, polymeric nanocarriers contain only cationic polymers with no hydrophobic moiety and are fully soluble in water. Two polymer categories can be distinguished: synthetic (polyethyleneimine (PEI) or dendrimers) and natural (cyclodextrin, chitosan) [45,46].

3.1.2.1. Synthetic polymer. PEI is the most successful and widely studied polymer for oligonucleotide delivery due to its good cell membrane interaction, high cellular uptake and endosomal escape rate by the “proton sponge effect” [47]. The efficiency of different types of PEI depends on their chemical characteristics in terms of molecular weight and structure. Branched PEI in low molecular weight (25 kDa) is usually used *in vitro* for nucleic acid transfection. When PEI is bioconjugated to siRNA, delayed cytotoxicity was noticed after their cell internalization. This could be explained by the fact that after siRNA release, cationic charges of free PEI can disturb cellular process, leading to several changes such as reduced cell size, decrease of mitosis number and of cytoplasm vacuolization [39,41]. *In vivo*, PEI cannot be used because of its non-specific interaction with serum protein leading to aggregation and toxicity [39].

However, strategies to reduce toxicity and improve pharmacokinetic parameters, while retaining its potent transfection ability, are in development. They mainly concern surface modification. Administration of mixed micelles composed of methotrexate conjugated to PEI linked to a fatty acid, the linolenic acid, and siRNA against survivin (an apoptotic inhibitor) showed elevated tumor uptake and significant tumor growth inhibition. This co-delivery formulation may be a model for future anticancer bi-therapy in order to overcome some drug resistance and increases antineoplastic efficiency [48]. Moreover, another study proved that lipidation of PEI-based polyplex improved serum stability of siRNA, leading to an efficient delivery into orthotopic hepatocellular carcinoma xenograft tumors [49].

Apart from PEI, dendrimers, which are well-defined globular structures with low polydispersity index, are also frequently studied for nucleic acid delivery. The most commonly used, polyaminated dendrimers (PANAMs), can interact with siRNA to form dendriplexes, leading to efficient transfection *in vitro* and *in vivo* [50]. Despite their biocompatibility, they lack biodegradability and interact with blood components, resulting in toxicity when injected *in vivo*. Adding PEG chains to dendriplexes is one possibility to improve pharmacokinetic parameters and reduce toxicity [50]. This concept was successfully applied to target BACE1 (β-site APP cleavage enzyme 1) responsible for the accumulation of age-related amyloid-beta aβ peptide characterizing Alzheimer's disease [51]. It has been shown that inhibition of this enzyme reduced the amyloid-beta aβ peptide deposition and improved neurodegenerative and behavioral deficits in transgenic mice [52]. However, intracerebral administration of small or large molecules is very invasive. Therefore, a recent study was conducted to specifically deliver, by intravenous injection, a siRNA anti-BACE1 to neuronal cells [51]. The PEGylated polymer Poly(2-(*N*,*N*-dimethylamino)ethyl methacrylate) (PDMAEMA) is known for its low toxicity. This nanomaterial has been coupled with cingulin peptide, a brain targeting ligand, to cross the BBB and, Tet-1 to target neuronal cells by binding on GT1B gangliosides. The bi-functionnalized polyplex was injected intravenously and inhibited BACE1 expression inside the neurons. Moreover, the four injections of siRNA at 240 µg cumulative dose succeeded in restoring cognitive performance of transgenic mice, similarly to wild-type. These results are encouraging and demonstrate the capacity of polymer to cross the BBB and achieve silencing to treat neurodegenerative disorders [51].

Nowadays, a commercially available lipid polymers was also used for *in vivo* siRNA delivery in the peripheral nervous system to treat the Charcot-Marie-Tooth (CMT) disease [53]. CMT is the most common hereditary neurogenic disorders with a prevalence of 1:2500 worldwide. This pathology is no curable, only supportive care are available for the patients [54,55]. The cause is mutations in Schwann cells genes

leading to demyelination of peripheral nerves [56] [57]. Several genes are found to be involved in the CMT development. The most common subtype is CMT1A, caused by duplication of peripheral myelin protein 22 (PMP22) gene, responsible for formation and maintenance of compact myelin [54,55]. Moreover, a mutation within this gene (Leu16Pro) is reliable to CMT1E development, therefore, a specific siRNA against one mutant allele of PMP22 gene, encapsulated in a commercial lipid polymers showed notable results. This formulation restored peripheral nerves myelination by the Schwann cells and improved the motor activity in the treated mice compared to the control [53]. These studies are promising and highlight the potentiality of RNAi molecules to treat non-hepatic or tumoral pathologies.

3.1.2.2. Natural polymer. Natural cationic polymers as chitosan and cyclodextrin are considered safer for oligonucleotides delivery. The polysaccharide chitosan is derived from chitin, usually found in crustacean exoskeleton. Its biocompatibility and biodegradability explain the study of this polycationic polymer for siRNA delivery. Compared to PEI, *in vitro* transfection efficiency is lower but it can to be modified in order to optimize *in vivo* siRNA delivery. The most widely used approach is the PEGylation. The advantage of PEGylated chitosan is its high solubility, increased stability in the biological environment and reduced non-specific interaction [58]. Recently, survivin targeted siRNA loaded in PEGylated chitosan, showed higher antineoplastic activity *in vitro* and *in vivo* in a breast cancer model compared to naked siRNA [59].

Cyclodextrins are neutral cyclic oligosaccharides. Accordingly, the team of Pr. Davis did chemical modifications to make them cationic [60–62]. Then, the nanoparticle surface has been modified by PEGylation and conjugated to transferrin to target specifically tumor cells [63,64]. A siRNA targeting the fusion oncogene, EWS-FLI1, present in 85% of Ewing's sarcomas, has been encapsulated in this functionalized polyplex. The formulation showed a strong anti-tumor activity *in vivo*, without any observations of toxicity or immune system stimulation [65].

3.1.3. Common limits of lipidic- and polymeric- siRNA nanocarriers

All of these formulations are the most commonly used to encapsulate siRNA in the past decades. However, they shared some disadvantages (Table 1) that can explain their non-approval by the health authorities until recently (Onpattro®, first siRNA drug encapsulated in a SNALP and approved in August 2018) [6].

Firstly, electrostatic interactions between the polyanionic siRNA and cationic carrier are considered weak and the risk of siRNA "burst release" just after systemic administration exists which can induce non-specific side effects and no siRNA efficiency [28].

Secondly, PEGylation is a strategy to escape MPS system and to extend plasmatic half-life of siRNA nanocarrier. Additionally to the size increase induced by PEG chains, Ishida *et al,* observed an "accelerated blood clearance" after repeated injections of PEGylated liposome [66]. Indeed, due to anti-PEG antibody synthesis after two injections, PEGylated liposomes were rapidly cleared from the blood circulation [67]. This resulted in the loss of long-circulating characteristic of the 2nd generation vector and faster excretion. Some hypersensitivity reactions related to complement activation have also been observed after repeated injection of a PEG-liposomal doxorubicin [66–69].

Thirdly, high numbers of cationic nanomaterials (lipidic or polymeric) have to be used to entirely encapsulate the siRNA and this is considered to be the main cause of toxicity. For example, PEI exhibits a dose-dependent toxicity, which may explain why it has not been used yet in clinical trials. Its toxicity depends on its physico-chemical characteristics. Indeed, high-molecular weight PEI is more toxic and even more if branched because of a high cell internalization [41]. Cationic lipids are divided in three parts: polar head-group, a linker and hydrophobic tails (Fig. 2). All can affect the cytotoxicity in a dose-dependent manner but toxic effects are mainly determined by the head-group chemical structure. Positive charges are responsible for non-specific interaction with anionic serum proteins, leading to unfavorable aggregation, clearance through the MPS system - mainly the liver - and of lower efficiency compared to *in vitro* results**.** Cationic nature of SNALP depends on the biological environment. However, the toxicity related to cationic charges (at acidic pH) is not negligible and remains a significant hurdle [39,45].

Most of the cationic NPs contain several excipients as polymer or lipid, PEG chains, ligand. Each additional component is another toxic risk and adds a difficulty to characterize precisely the nanovector [16,41]. Obviously, systemic and non-specific toxicity is reduced when nanoparticle surface is modified by the conjugation of a ligand targeting a specific receptor. Nevertheless, it is important to keep in mind that this ligand targets a receptor overexpressed in specific cells and therefore can also be uptaken by non-targeted cells expressing the same receptor but at another level [27].

Nanocarrier toxicity is not limited to the cytoplasm or biological fluids. It can also occur in the nucleus, where the modulation of gene expression alteration by chemical compounds is called as toxicogenomic. Akhtar and his colleagues demonstrated that polymeric and lipidic nanocarriers are able to alter cell gene expression, with consequences on cell phenotype and siRNA efficiency [70].

3.2. Chemical modification: siRNA conjugates

Even if the first therapeutic approved siRNA are encapsulated in a SNALP, their development is declining, because of the disadvantages cited above (Table 1). In order to avoid them, chemists and galenists searched for a novel way to deliver siRNA [71]. They modified the siRNA by linking it covalently to different molecules as lipid, aptamer,

Table 1
Advantages and disadvantages of siRNA physical and chemical encapsulation.

		Advantages	Disadvantages
Physical encapsulation	Common to all generations	Good cell internalization Possibility of cell targeting Studied for a long time	Burst release Toxic effects of cationic groups Gene expression alteration
	First generation		Fast uptake by MPS cells
	Second generation « Stealth nanovector »	Improvement of pharmacokinetic parameters	Accelerated blood clearance due to PEG antibody synthesis
	Third generation Active targeting	Biodistribution improvement	Impossibility to target only a specific cell type
Chemical encapsulation		Equi-molar amounts of delivery material and siRNA Conjugation to different types of molecules No need for complexation with cationic lipids or polymers	Poor delivery efficacy compared to particle-based systems siRNA more exposed and vulnerable to enzyme nucleases blood Poorer knockdown efficiency *in vitro* Cost of the scale up Impossibility to target only a specific cell type

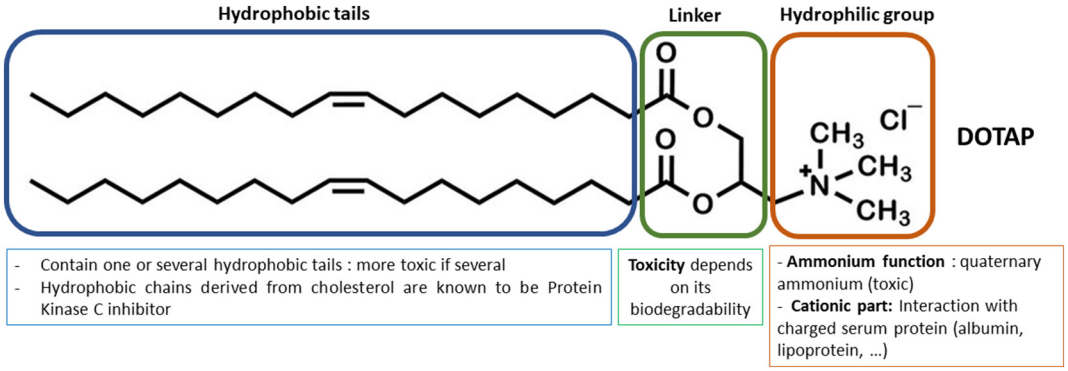

Fig. 2. Schematic representation of cationic lipid and its associated toxicity. DOTAP is 1,2-dioleoyloxy-3-trimethylammoniumpropane, a common cationic lipid used for siRNA delivery.

carbohydrate, and others [72,73]. As for physical encapsulation, siRNA bioconjugation changes its intrinsic characteristics and decreases its hydrophilicity in favor to the ligand lipophilicity, which changes some pharmacokinetics parameters. It improves its plasmatic half-life and its biodistribution depending on the linked molecule. Indeed, it is possible to link ligands targeting specific cells as hepatocytes, cancer cells. The link occurs usually on the sense strand to enhance RISC attachment and the keeping of the guide strand (the siRNA active part). Thus, the bioconjugate can be injected by themselves and so no additive cationic molecules will be administered. Compared to cationic nanocarriers, these formulations decrease the siRNA toxicity *in vivo* but render it more vulnerable and exposed to serum nucleases [72].

3.2.1. Conjugation to cholesterol

Different lipid types have been linked to siRNA to improve plasmatic half-life and intracellular uptake. Cholesterol is one of the major component of cell membrane and precursor of steroid hormones such as testosterone or cortisol. As mentioned above, it is usually used as a helper lipid in lipoplex to facilitate siRNA condensation, increase liposome stability and decrease cationic toxicity. In blood, lipoprotein (mainly low density lipoprotein LDL and high density lipoprotein HDL) carry the cholesterol to the different organs. Cholesterol-siRNA bioconjugates bind to these lipoproteins and are delivered to tissues expressing lipoprotein receptor, mainly the liver and cancer cells [74]. The first reported systemic administration of cholesterol-siRNA conjugate was at a higher dose (50 mg/kg) compared to liposomal nanosystems (less than 10 mg/kg) [75]. Efforts have been exerted to improve siRNA potency and delivery efficiency. Chernikov et al. [76] developed an anti-MDR1 siRNA-cholesterol NPs, that showed efficient silencing for 8 days before recovery of initial expression level, in cancer xenograft overexpressing the MDR1 gene. After intravenous injection, siRNA-cholesterol bioconjugates spread throughout all mice bloodstream and accumulated mainly in the liver and kidney (but to a lesser extent than with non-conjugated siRNA) in healthy mice. In mice bearing xenograft tumor, the lower renal clearance induced a higher drug accumulation within the tumor thanks to the EPR effect. However, when the siRNA-cholesterol bioconjugates were injected subcutaneously, the biodistribution of the formulation showed that it remained at the injection point and was not distributed in the mice organs [76].

The biotech company Arrowhead Pharmaceuticals, developed formulations with siRNA conjugated to cholesterol. The first one, named DynamicPolyConjugates (DPC), was composed of cholesterol-siRNA against Apolipoprotein B or factor VII genes. The cholesterol-siRNA bioconjugates were *co*-injected with a hepatocyte-targeted ligand (*N*-acetyl-galactosamine) linked to endosomolytic polymer (poly(butyl-aminovinyl ether or PBAVE) [77]. This formulation allowed an efficient endosomal escape *via* polymer protonation, resulting in a significant decrease of siRNA dose injected in mice (500-fold less than that usually used) and a high gene silencing effect [77].

The second DPC formulation is constituted by cholesterol-conjugated siRNA but co-injected with the hepatocyte-targeted ligand *N*-acetyl galactosamine linked to melitin-like peptide (NAG-MLP). Melitin is a peptide component of bee venom, frequently used as endosomolytic agent and fully biodegradable. Two hepatitis B virus (HBV) targeted siRNA have been designed, conjugated to cholesterol and co-injected with 6 mg/kg of NAG-MLP to HBV transgenic mice. It leads to silencing more than 85% of targeted HBV mRNA, decreased significantly viral protein production and inhibited viral replication. This co-injection is well tolerated, all serum parameters are normal and no cytokine/chemokine secretions are detected [78,79].

3.2.2. Conjugation to galactose derivative

Nowadays, most of siRNA drug-candidates in clinical development are oligonucleotides modified by conjugation to *N*-acetylgalactosamine (GalNAc). This carbohydrate is a galactose derivative, which has an enhanced binding affinity on the asiaglycoprotein-receptor. This receptor is a C-type lectin receptor allowing the clearance of circulating glycoprotein *via* clathrin-mediated endocytosis followed by receptor recycling. It is highly expressed in the hepatocyte membrane, some subunits have been found in human thyroid, large intestine, renal epithelium, testis and blood monocytes [80].

The most successful formulation has been developed by Alnylam Pharmaceuticals in various range of hepatic disorders [80–84].

Small interfering RNA have been covalently conjugated to a triantennary GalNAc sugar and successfully delivered to the liver [82]. In contrast to all previous presented siRNA nanocarriers, these molecules are injected subcutaneously. The link between chemically modified siRNA to GalNAc results in bioconjugates with high systemic stability and improved pharmacokinetics. Moreover, a higher liver accumulation was detected when it is injected subcutaneously compared to intravenous route that paralleled with robust and durable targeted gene silencing [82]. For example, siRNA targeted rodent transthyretin (TTR) has been successfully linked to triantennary GalNAc and demonstrated a relevant gene silencing after a single dose administration from 1 to 5 mg/kg. The chronic injections of an effective dose of 1 mg/kg over 280 days resulted in sustained pharmacological effect without toxicity, indicating the possibility to treat chronic disease [81,82].

The same formulation was also applied to treat primary hyperoxaluria, an autosomic recessive inborn error of metabolism, caused by mutation on alanine glyoxylate aminotransferase (AGT) gene leading to deficiency of this hepatic enzyme (Fig. 3**)**. It induces high oxalate production, which will form calcium oxalate crystals in kidney and urinary tract, blocks urine elimination and leads to a progressive kidney disease that could be extended to other organs. No cure is available except liver transplantation after years of dialysis and vitamin B6 supplementation [85]. Herein, the authors inhibit by siRNA conjugated to GalNAc (named ALN-GO1) the expression of hepatic enzyme upstream of AGT, the glycolate oxidase (GO). Four weekly subcutaneous

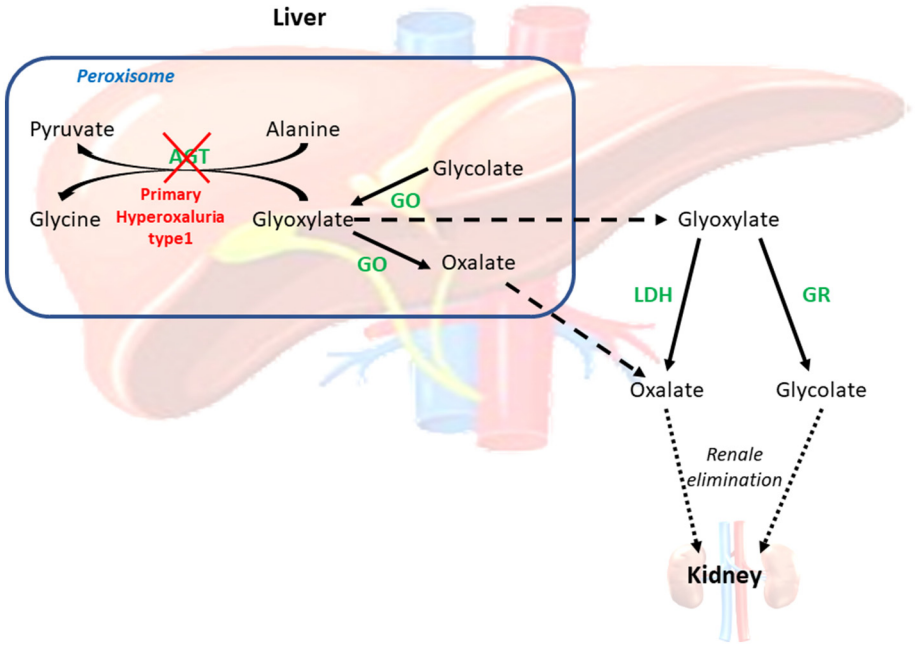

Fig. 3. Endogenous oxalate pathway in hepatocytes and primary hyperoxaluria type 1. AGT, alanine glyoxylate aminotransferase; GO, glycolate oxidase; LDH, lactate deshydrogenase; GR glyoxylate reductase.

injections of ALN-GO1 (from 0,3 to 3 mg/k for 14 days) in rodent with primary hyperoxaluria, silenced more than 95% of GO mRNA and inhibited urinary oxalate accumulation. In healthy non-human primates, subcutaneous injection of ALN-GO1 (single injection at 4 mg/kg) completely abolished GO mRNA up to 15 days [83,85].

General toxicology studies have also been performed for all siRNA-GalNAc bioconjugates, in two species: one rodent and one non-human primate. Rats are known to be more sensitive to detect hepatic toxicity. At high doses, asiaglycoprotein receptor is saturated and the siRNA-GalNAc excess is eliminated by the kidney. Histologically, hepatocellular vacuolation has been observed after one injection per week during 3 weeks in rats at doses ≥ 30 mg/kg (30- to 300-fold greater than efficient pharmacological doses) but without significant clinical changes. Interestingly, this observation has not been detected in non-human primate even at the highest repeated dose tested [80].

3.2.3. Conjugation to aptamer

Aptamer is a short single-strand oligonucleotide with a unique three-dimensional structure, that allows binding and uptake by specific cells when the targeted receptor is expressed. It has low production cost, minimal nonspecific toxicity and immunogenicity [86]. It has already been shown that aptamer conjugates have intracellular uptake *via* receptor-mediated endocytosis and efficient gene silencing *in vitro* and *in vivo* [72,86]. Aptamer-conjugated siRNA were first described in 2006 [87]. Passenger strand of siRNA is covalently linked to A10 aptamer targeting prostate-specific membrane antigen (PSMA) expressed in LNCaP prostate cancer cells. Cell internalization of this formulation and efficient anti-cancer activity correlated with gene silencing were observed [87,88]. More recently in another study, bivalent PSMA aptamer allows the delivery of two different siRNAs silencing EGFR whose expression is associated with bone metastasis formation, and survivin which is an inhibitor of apoptosis protein family. This bi-combinatorial formulation leads to effective delivery to xenograft mice model, where it significantly suppressed tumor growth and angiogenesis [89]. Despite promising results in different diseases, no aptamers-mediated siRNA deliveries are under clinical trials. It can be explained by the lack of in-depth pharmacokinetic/pharmacodynamic and toxicities studies [86,90].

3.2.4. Common limits of siRNA bioconjugates

These bioconjugate formulations have been developed to overcome some undesirable effects of cationic lipid and polymer, such as the burst release and toxicity risk associated with cationic charges in order to have easier formulation (with only one component). Nevertheless, as physically encapsulated siRNA, conjugates may also have some disadvantages that can limit their clinical translation (Table 1) [91]. Indeed, compared to the "physical" encapsulation, siRNA conjugates have poorer silencing effects *in vitro* that can impede translation to develop preclinical and clinical studies [72]. Another limit, is the cost of the "scale-up" to have enough quantities of siRNA bioconjugates for clinical development. The "scale-up" nanoformulation has to respond to good manufacture's practice and to the same quality control than the non-"scale-up" siRNA conjugate, meaning same size distribution, morphology and efficiency. The translation to clinics will depend on this step and on cost of the final product [91,92]. We should also keep in mind that the conjugated siRNA may be more exposed to serum nucleases than encapsulated siRNA and therefore have a lowest bioavailability due to unfavorable pharmacokinetic, leading to higher dose injected [72].

4. Therapeutic applications – overview of some clinical trials

Some of the formulations passed successfully all preclinical development steps, proving that they were found safe enough and efficient enough to be administered to patients whether they have been successfully translated to clinical phases or not. According to FDA website, the aim of clinical trials is to "test the potential treatments in human volunteers" in terms of safety, tolerance and efficiency.[1]

We focused on different interesting drug candidates, which were or are currently in phase I, II or III clinical trials (Table 2).

[1] https://www.fda.gov/drugs/development-approval-process-drugs/conducting-clinical-trials, accessed 26 December 2019

Table 2
Selected siRNA-based drug candidates in clinical trials.

	Name	Delivery method: administration mode	Indication: target	Clinical phase	NCT number	Status	Study start	Study completion	Sponsor company	Comments
Physical vectorization	Atu027	Liposome: intravenous injection	Advanced solid tumor: PKN3	phase I	NCT00938574	Completed	June 2009	September 2012	Silence Therapeutics	
				Phase I/II	NCT01808638	Completed	March 2013	January 2016		
	Patisiran	SNALP: intravenous injection	Transthyretine amyloidosis: transthyretine	phase I	NCT01559077	Completed	March 2012	November 2012	Alnylam Pharmaceuticals	First-siRNA nanocarrier approved by health authorities in United States
				phase II	NCT01617967	Completed	May 2012	January 2014		
				phase III	APOLLO NCT01960348	Completed	November 2013	August 2017		
	CALAA-01	Cyclodextin polyplexe: intravenous injection	Solid tumors: RRM2	phase I	NCT00689065	Terminated	May 2008	September 2012	Calando Pharmaceuticals	First proof of concept of siRNA efficiency in human, not continued because of toxicity
Chemical vectorization	ARC-520	Cholesterol-conjugated siRNA: subcutaneous injection	Chronic Hepatitis B: hepatitis B virus transcripts	phase I	NCT01872065	Completed	July 2013	November 2014	Arrowhead Pharmaceuticals	Ended clinical trial due to toxicity on the formulation
				phase II	NCT02349126	Withdrawn	February 2015	March 2016		
	ARO-HBV	*N*-acetylgalactosamine-conjugated siRNA: subcutaneous injection	Chronic Hepatitis B: hepatitis B virus transcripts	Phase I/II	NCT03365947	Recruiting	March 2018	September 2020	Arrowhead Pharmaceuticals	
	ARO-AAT	*N*-acetylgalactosamine-conjugated siRNA: subcutaneous injection	Alpha-1 antitrypsine (AAT) deficiency: AAT	phase I	NCT03362242	Active, not recruiting	March 2018	September 2019	Arrowhead Pharmaceuticals	
				phase II	NCT03946449	Recruiting	November 2019	November 2021		
				phase II/III	NCT03945292	Recruiting	August 2019	May 2023		
	Revusiran	*N*-acetylgalactosamine-conjugated siRNA: subcutaneous injection	Transthyretine amyloidosis: transthyretine	phase I	NCT01814839	Completed	March 2013	May 2015	Alnylam Pharmaceuticals	Discontinued clinical trial because of higher mortality rate in treated group compared to placebo
				phase II	NCT01981837	Completed	December 2013	January 2015		
				phase III	NCT02319005	Completed	December 2014	March 30, 2017		
	ALN-GO1 (Lumasiran)	*N*-acetylgalactosamine-conjugated siRNA: subcutaneous injection	Primary hyperoxaluria type 1: glycolate oxidase	phase I/II	NCT02706886	Completed	March 2016	January 2019	Alnylam Pharmaceuticals	Change in backbone chemistry (by comparison with Revusiran) to have more potency and safety of siRNA bioconjugates
				phase II	NCT03350451	Enrolling by invitation	November 2017	September 2021		
				phase III	NCT03681184	Active, not recruiting	November 2018	May 2024		

Abbreviations: siRNA target PKN3 protein kinase 3, RRM2 ribonucleotide reductase subunit M2, AAT alpha-1 antitrypsine.

4.1. Lipid nanoparticles

4.1.1. Lipoplex

Atu027 is a liposomal siRNA formulation, composed of an anti-protein kinase 3 (PKN3; downstream effector of phosphatidylinositol 3 kinase) siRNA encapsulated with a new cationic lipid AtuFECT01 in combination with helper and PEG-modified lipids. It proved its efficiency in different cancer models (orthopic mouse models for prostate and pancreatic tumors) with significant tumor growth inhibition and changes in tumor lymphatic vasculature [93] without elevated systemic levels of immune cytokines (IFN-alpha or IL12) [94]. These results led to the clinical development of this novel lipoplex in oncology. The first-in-human phase I study on Atu027 started in 2009 on thirty-four patients with the aim to evaluate the safety, the tolerability, as well as the pharmacokinetic and therapeutic effects on primary tumors and metastatic lesions. Atu027 was considered to be well tolerated with only low-grade toxicities (1 and 2) and safe for patients with advanced solid tumors. Tumor growth was even stable in 41% patients [95]. A new phase I/II (NCT01808638) started in 2013. During this stage, Atu027 has been co-administered (one or two times per week) with the chemotherapeutic drug, gemcitabine. Twice weekly injection of Atu027 at a dose of 0.253 mg/kg with gemcitabine significantly increased patient survival compared to the single injection per week. In addition to this antineoplastic efficacy, patients' quality of life improved [96]. However, no follow-up was available since 2016.

4.1.2. SNALP

Transthyretin amyloidosis is a rare progressive disease caused by the abnormal deposition of transthyretin amyloid in various organs, including peripheral nerves. Patients suffer mainly from progressive neuropathy and cardiomyopathy with median survival less than 15 years after diagnosis. TTR is a circulating protein synthetized by the liver, its role is to carry mainly retinol (vitamin A) and in a lower proportion, thyroid hormone (thyroxine). The gene encoding for TTR can be mutated, resulting in protein misfolding and aggregation to form amyloid fibrils that will deposit in tissues. No curative treatment existed before the Onpattro® approval. Onpattro®, commercialized by Alnylam Pharmaceuticals, is composed of patisiran, siRNA targeted mutant and wild-type TTR in the 3′-untranslated region of the mRNA and vectorized in a SNALP. The lipid components of this formulation include the ionizable amino lipid DLin-MC3-DMA, PEGylated lipid 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG2000-C-DMG), a polar lipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol. In the bloodstream, PEGylated lipids are removed and replaced by serum protein such as apolipoprotein E, inducing hepatocyte internalization [16,97]. The receptor-mediated endocytosis leads to endosome acidification, inducing re-ionization of the main ionizable lipid and liberation of the siRNA in the cytoplasm. In non-human primate, which has the same TTR sequence as human, injection of patisiran induced efficient knockdown after single dose of 0.1–0.3 mg/kg that prevents protein deposition [98]. Besides, TTR silencing was more effective than tafamidis, the gold treatment used to inhibit products of the $Val^{30}Met$ mutation of TTR gene [98,99]. Taking together these results, the patisiran passed successfully phase II [99,100] then phase III [6,7]. During these studies, it showed its non-toxicity and its efficiency in decreasing circulating TTR and improving patients' quality of life [7,100]. Six years after clinical studies beginning, drug-candidate was approved by the European Medicine Agency (EMA) and FDA bringing new hope for patients with hereditary transthyretin amyloidosis [6].

Another formulation has been also developed by Anylam Pharmaceuticals but instead of being complexed in a LNP, the TTR-targeted siRNA is conjugated to triantennary GalNAc. This nanovector is amenable to less permissive administration mode (subcutaneous) and has been named revusiran. Preclinical studies demonstrated efficiency and non-toxicity in rodent and non-human primate species [98,99]. Phase I clinical trial (NCT01814839) did not display severe adverse effects, except those caused by the injection (mostly mild to moderate injection site reactions and one severe with erythema > 10 cm) [101]. Phase II (NCT01981837) confirmed the previous results but phase III (ENDEAVOUR; NCT02319005) was dismissed before the end due to report of peripheral neuropathy. Moreover, an increase in lactate concentration and mortality imbalance were observed between treated patients and placebo group [102,103]. Post-trial investigations did not link the higher mortality in the treatment arm at the revusiran but did not totally exclude that this molecule have a toxicity[2] [16]. Formulation has been improved by chemical modifications on siRNA sequence and is currently in phase III clinical trial (Vutrisiran; NCT03759379).

4.2. Polyplex

Cyclodextrin is a nontoxic and non-immunostimulantcyclic oligomer of glucose. Formulation with linear cationic cyclodextrin-based polymer, PEG chains and transferrin receptor ligand has been developed for siRNA delivery [104]. Encapsulation in this polyplex of siRNA targeted ribonucleotide reductase subunit M2 (RRM2) leads to reduced tumor growth and gene expression silencing [104]. Preclinical toxicity studies in non-human primate displayed, at the highest dose (27 mg/kg), mild increase in transaminases and elevated blood urea nitrogen and creatinine, correlated with hepatic- and renal-toxicity respectively. Mild immune stimulation has also been detected at this dose (increased IL-6 levels in all animals). However, no clinical signs of toxicity have been observed; therefore it is used in clinical studies at lowest doses (efficient dose in non-human primates, 0.6–1.2 mg/kg) to avoid biological toxicity [105]. The phase I study conducted by Dr. Heidel at Calando Pharmaceuticals started in May of 2008 (NCT00689065), was the first to show that multi-siRNA injections could be safely and successfully accomplished in a non-rodent species and after then in human [5,105]. Indeed, the targeted nanovector denoted as CALAA-01, accumulated preferentially in human metastatic melanoma tumors in a dose-dependent manner due to its targeted ligand and inhibited RRM2 expression [106]. Unfortunately, phase IB was ended prematurely because of toxicity due to the long-term drug instability [106,107].

4.3. siRNA conjugates

These formulations aim to reduce toxicity due to cationic charges and are actually used to treat several hepatic disorders.

The eradication of hepatitis B virus was found to be unachievable in the case of chronic infection because the virus integrates the genome [108]. ARC-520 is developed by Arrowhead Pharmaceuticals and is composed of two HBV targeted siRNA conjugated to cholesterol and co-injected with 6 mg/kg of endosomolytic agent (NAG-MLP) to provide effective endosomal escape [78]. This promising formulation went into phase I clinical trial and was injected to healthy volunteers to assess its safety, tolerability, pharmacokinetics and pharmacodynamics. No serious side effects were found [109]. The phase IIb clinical trial started in 2015 and showed a significant efficiency of ARC-520 with a reduction of Hepatitis B antigen up to 57 days [110]. However, the clinical phases were stopped prematurely, due to toxicity concerns, after non-human primate death in preclinical studies[3] [16,111].

The company, Arrowhead Pharmaceuticals, developed a new bioconjugation method, named “Targeted RNAi Molecules (TRiM) technology” that can be injected subcutaneously. This time, the siRNA is directly linked to *N*-acetylgalactosamine but the specific structure is not yet published. ARO-HBV is one of the first molecule derived from “TRiM technology” and as for ARC-520, is composed of two siRNA

[2] https://www.genengnews.com/news/after-18-deaths-in-phase-iii-alnylam-halts-revusiran-development/, accessed 30 December 2019

[3] https://xconomy.com/wisconsin/2016/11/09/arrowhead-stock-falls-after-fda-places-hold-on-clinical-trial/, accessed 30 December 2019

silencing HBV transcript and its integrated form, leading to reduce virus resistance to treatments and cure from the infection. Phase I/II of this drug candidate started in 2018 and study completion normally occurs in January 2020 (NCT03365947) [112]. It should be noted that another formulation is under development for deficiency in alpha-1 antitrypsin (OMIN code 613490) which is an inherited and well-defined disease that can trigger pulmonary and liver disorders [113,114].

After revusiran failure, Alnylam Pharmaceuticals continued the research and focused on changing the backbone chemistry (from standard template chemistry to enhanced stabilization chemistry platform by adding phosphorothioates linkages at the 5′-end of both strands) [82]. This leads to a more stable and safe GalNAc-conjugate siRNA [111]. Five drug-candidates are accounted currently in clinical studies: i) vutrisiran (phase III; NCT03759379) for the treatment of hereditary transthyretin amyloidosis, ii) lumasiran (phase III; NCT03681184) for the treatment of primary hyperoxaluria type 1 (PH1), iii) fitusiran (phase III; NCT03754790) for the treatment of hemophilia, iv) inclisiran (phase III; NCT03814187) for the treatment of hypercholesterolemia and, v) cemdisiran (phase II; NCT03999840) for the treatment of atypical hemolytic uremic syndrome.

ALN-GO1, also called lumasiran, is a siRNA-bioconjugated to triantennary GalNAc to treat the PH1 pathology (Fig. 3), which is a rare autosomic recessive disease (OMIN code 259900) [115,116]. The impressive efficiency results in animal models (mice, rat and non-human primate) opened the door for clinical trials [83]. Phases I/II started in 2016. Urinary oxalate level decreased substantially after the first dose. The results showed that the treatment was well-tolerated among the patients with PH1. No drug-related severe adverse effects were observed. The majority of side effects were mild to moderate and unrelated to the drug candidate.[4] The same patients were enrolled into phase II open-label extension and then in phase III (ILLUMINATE-A) which is currently ongoing in adult and children (NCT03681184).[5]

On November 20, 2019, the first-ever GalNAc-conjugate siRNA (givosiran, commercial name Givlaari®) has been approved by the FDA (followed in January 2020 by the EMA) for the treatment of a rare inherited hepatic pathology, the acute hepatic porphyria [8].[6]

5. What is next?

Therapeutic RNAi history spans over different eras. In 1998, its discovery paved the way for a new therapeutic field, which started development in the beginning of the 2000's (2005 to 2008). It was a lavish period for RNAi research, notably thanks to the Nobel Prize in 2006. Unfortunately, uncertainties about the real therapeutic effect of siRNA in human (more due to immunostimulatory effects than silencing) accompanied by bad investments by pharmaceutical companies, led to a funding crisis in early 2010's. Development of new nanomaterial less toxic, more efficient and RNAi efficiency demonstration in silencing targeted genes (proof of concept by CALAA-01) allowed a recovery of the pharmaceutical industries in this field [117–119]. Twenty years after RNAi discovery by Fire and his colleagues, the first siRNA drug has been approved by the FDA and opens a new era for RNAi research and development. However, some challenges still need to be addressed related to: 1) the lifelong administration of encapsulated siRNA in term of toxicity and 2) the enlargement of the treatment for non-hepatic or tumor pathologies.

The first challenge concerns toxicity due to a lifelong administration that can be developed by the siRNA and essentially the vector. Natural lipids such as cholesterol and derivates [72] or exosomes [120] could represent a safer solution.

One of the interesting cholesterol derivate is the squalene (SQ) that adopts a picked shape in the hydrophobic pocket of the enzyme inducing its cyclisation in lanosterol (involved in the cholesterol synthesis metabolism). This unique conformation in aqueous medium oriented the interest of chemists and galenists to link it to small vulnerable active molecule in order to protect it from degradation and improve pharmacokinetics parameters [121–123]. This new technology has been successfully transposed to siRNA against fusion oncogenes: RET/PTC1 and RET/PTC3 found in papillary thyroid carcinoma and TMPRSS2-ERG observed in prostate cancer. The bioconjugates siRNA-SQ have been injected intravenously to mice. Each nanoformulation showed significant tumor growth inhibition correlated with fusion oncogene expression silencing [124–129].

Exosomes are extracellular vesicles excreted by different types of cells that mimic natural liposomes produced by human cells. They offer a more potent and safer alternative to synthetic NPs [120]. Preclinical studies proved their efficiencies in KRAS siRNA delivery to mouse models of pancreatic cancer with an increase of survival rate [130]. The major hurdle to overcome with this new nanocarrier is to be able to prepare good manufacturing practice exosomes during the scale-up. The same team of the MD Anderson Cancer Center develops a bioreactor capable of generating engineered exosome carrying KRAS siRNA and to produce them at large scale [131]. This nanoformulation is currently in phase I clinical trial against pancreatic cancer (NCT03608631).

The second challenge concerns the siRNA-treatment expansion to other non-hepatic and tumoral pathologies. Indeed, aside from these tissues, it is difficult to cure diseases occurring in other organs because of lower blood flow and non-fenestrated endothelium. Few preclinical studies were able to study the effect of siRNA nanocarriers when crossing the BBB or the BNB and none were so far translated to clinical studies [51] [53]. Reaching the central-nervous and the peripheral nervous systems is important, because there is no cure for several pathologies including brain cancer (glioma), ischemic stroke and neurodegenerative diseases.

Most of the strategies used to cross the BBB are receptor-mediated delivery [37,51], but another way to reach the brain is to inject in the cerebrospinal fluid by intrathecal injection. Drug injected by this administration mode goes directly to the brain without crossing the BBB. Alnylam Pharmaceuticals reported recently preclinical results in CNS-targeted RNAi drug candidates with achievement of gene silencing after one intrathecal injection of siRNA-conjugate in rodent and non-primate human animal models. The company did not precise the vector linked to the siRNA.[7] The injection route is usually well-tolerated but it is an invasive method, patients have to be hospitalized. Therefore, there is a need of nanocarrier development for the aim of using an easier route [132]. However, for the peripheral nervous system, drugs including siRNA, can be injected systemically [53,133].

6. Conclusion

Since its discovery in 1998, RNAi has been considered as a powerful therapeutic mechanism because of the high specificity and efficiency of siRNA, to silence a targeted gene. However, to achieve therapeutic success, this oligonucleotide molecule has to overcome some delivery barriers. To this end, it has to be modified by conjugation to a molecule or entrapped in a cationic vector. The success story of Onpattro®, the first approved siRNA-based drug encapsulated in cationic vector opens new avenues. However, the actual tendency is more oriented to chemical conjugation of the siRNA and is confirmed by the recent approval

[4] https://www.alnylam.com/wp-content/uploads/2017/11/Lumasiran-ASN_Capella-Slides_FINAL11032017.pdf., accessed 30 December 2019

[5] https://www.alnylam.com/wp-content/uploads/2019/04/Lumasiran-Phase-2-OLE-ISN-2019.pdf, accessed 30 December 2019

[6] https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-givosiran-acute-hepatic-porphyria, accessed 11 February 2020

[7] https://www.alnylam.com/wp-content/uploads/2019/04/Milstein-CSHL-Oligosv6.pdf, accessed 30 December 2019

of the Givlaari®.

Next years will be crucial. First, to see the very long-term efficiency and safety of siRNA drug and secondly, to develop new safe nanocarriers able to reach other non-liver or non-tumor tissues. RNAi has not yet revealed its full therapeutic potential and new siRNA-based drugs will be soon marketed by the pharmaceutical industry.

Funding

This work was supported by a public grant overseen by the French National Research Agency (ANR) as part of the "Investissements d'Avenir" program (Labex NanoSaclay, reference: ANR-10-LABX-0035). Mévidette El Madani is grateful for the Financial support of the National Research Center, Cairo Egypt during her postdoctoral followship.

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