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Local siRNA delivery by non-viral vectors

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Since the discovery of the RNA interference (RNAi) phenomenon, RNAi-based therapies now present a huge potential for the treatment of many diseases, including inflammatory and infectious diseases and cancers. While numerous reports have described the development of small interfering RNA (siRNA) delivery systems for in-vivo applications, only a small number of siRNA-based therapies are currently under clinical development. This is essentially due to the lack of efficient and safe siRNA delivery systems for intravenous administration. However, the delivery of siRNA after local injection could represent an attractive route of administration to limit the issues of toxicity associated with systemic injection. We will describe here the different synthetic vectors which have been developed for the local delivery of siRNA in various organs.

Key words: siRNA – Delivery – Cationic lipid – Polymer – Conjugated siRNA – Local – Mucosal – Intracerebral – Intratumoral – Intraocular.

RNA interference (RNAi) is a very efficient process for post-transcriptional gene silencing in cells, induced by short interfering RNA (siRNA) consisting of double-stranded (ds) RNA of 21 to 25 nucleotides. SiRNA exert their gene-silencing activities by targeting mRNA in a highly specific manner via RNA-induced silencing complexes (RISC) which bind to the cognate mRNA and mediate its degradation.

The discovery of RNAi has generated much enthusiasm within the scientific community. In fact, siRNA molecules hold great promise as biological tools and novel therapeutic agents, particularly in the areas of oncology and infectious diseases. In several studies, siRNA has been shown to allow specific targeting of selected genes without the common adverse effects of less-specific drug-based therapies or the dangers of viral gene therapy. The development of efficient RNAi-based therapeutics faces some challenges, such as the design of optimized siRNA molecules and of suitable delivery systems able to deliver siRNA efficiently, safely and repeatedly for in-vivo applications. Concerning the design of optimized RNAi molecules, two strategies are usually described. The first consists of the chemical synthesis of small double-stranded RNA molecules of approximately 20 nucleotides with asymmetric 3' overhangs, which are directly taken up by the RISC complex in the cytoplasm. Conversely, short hairpin RNA (shRNA) is composed of a small RNA sequence in a tight hairpin-turn structure and is loaded into a plasmid vector which ensures the expression of the shRNA in the nucleus. ShRNA is then cleaved by DICER complexes to produce small interfering siRNA which is taken up by the RISC complex. Thus, depending on the type of RNAi molecule used, specific vectors have to be developed. These must be optimized depending on the route of administration, the tissue or cell type expressing the target gene and on whether short- or long-term effects are required. To achieve efficient siRNA delivery, these systems must ; a) condense siRNA in nanoparticles, b) protect siRNA from enzymatic degradation, c) facilitate cellular internalization, d) promote endosomal escape, and e) not stimulate innate immunity. In this report, we will focus on the delivery of chemically synthesized duplex siRNA and not on plasmid-encoding shRNA. Non-viral delivery systems represent a promising method of efficient siRNA delivery. Non viral delivery systems consist usually of lipidic, polymeric and peptides molecules. We will report different strategies which have been developed to efficiently deliver siRNA by local administration, using non viral vectors, including mucosal and non mucosal applications.

I. MUCOSAL siRNA DELIVERY

The choice of route of administration is an important determinant of the efficiency of siRNA delivery. Direct application onto mucosal surfaces such as respiratory, gastro intestinal or vaginal epithelium is an attractive alternative to systemic delivery. Indeed mucosal delivery is a non-invasive method allowing drug delivery directly to target sites which are the main points of entry of numerous pathogens. The mucosal route reduces nuclease degradation, nanoparticle hepatic clearance and serum-induced aggregation, and facilitates direct contact with target cells. Nevertheless, major hurdles to efficient mucosal delivery include the presence of mucus lining all mucosal surfaces and a tight epithelial cell arrangement sweeping away potential penetrative material using apical cilia. While some reports have described efficient mucosal delivery of naked siRNA in the lung [1], large volumes and significant amounts of siRNA were necessary to obtain a good deposition of siRNA on mucosal surfaces. Recent improvements have been made in mucosal siRNA delivery, leading to efficient inhibition of the targeted gene with a low dose of siRNA (*Table I*).

1. Lung

The development of efficient therapies using RNA interference for the treatment or prevention of lung diseases represents a promising approach. Due to a high exposure to pollution, the airways are susceptible to numerous diseases, such as cancers and inflammatory or infectious diseases. Many of these diseases could be treatable by local siRNA treatment, but some hurdles need to be considered when developing efficient siRNA delivery systems. Airways are relatively accessible for the local administration of drug therapies, but barriers need to be overcome for efficient siRNA delivery.

1.1. Challenges

The complex structure of the lung must be considered carefully. The majority of respiratory diseases, especially infectious diseases, are localized in the lower airways, thus siRNA delivery systems need to traverse the upper airways to reach their target in the alveolar surface of the lower airways. The upper airways present a highly branching structure with high mucocilliary clearance and the presence of mucus, preventing the entry of exogenous particles into the lower airways. To reach the lower airways, siRNA particles size and hydrodynamic behavior need to be optimized. The behavior of particles may be predicted by their aerodynamic diameter. Indeed, larger particles, with

Table I - Overview of siRNA delivery systems for mucosal targets.

Delivery system	Animal/route	Molecular target/model	Dosage/effect	Ref.
		Lipid		
Transit-KO	Mouse/intranasal	RSV-P/ Mouse model of RSV infection with Human RSV long strain HPIV-3/ Mouse model of PIV infection with PIV type 3 J5 strain	70 µg siRNA Reduction of 99 % of viral titer at 6 days post-viral-infection Protective and curative effect on RSV replication	[1]
Oligofectamine	Mouse/intranasal	NP and PA/Influenza infection	20 µg siRNA Combination of i.v. and i.n. administration of siRNA lead to inhibition of influenza virus replication	[15]
	Mouse/intravaginal	EGFP/transgenic EGFP mice UL27-29/Mouse model of HSV infection with HSV-2 wild type virus	~ 7.5 µg siRNA Reduction of EGFP expression at day 3 up to day 9 ~ 7.5 µg siRNA 80 % of survival in mice receiving both siRNA	[26] [26]
LIC-101	Mouse/intravesical	PLK1/bladder cancer model	600 nM/6 µM – 5 administrations Weak expression of PKL1, reduced tumor cells	[34]
Lipidoids	Mouse/intranasal	RSV/ Mouse model of RSV infection with RSV/A2 or RSV/B1 strain	~40 µg siRNA Two-log reduction in viral plaques numbers	[9]
PLAS	Mouse/intravaginal	Lamine A/C	8 µg siRNA 85 % of lamin A/C expression inhibition at 24 h	[32]
		Polymer		
PEI	Mouse/intranasal	AKt1/lung cancer model	80 % knockdown of AKt activity Suppression of tumor growth	[17]
	Mouse/intratracheal	EFGP/ transgenic EGFP mice model	35 µg siRNA More than 65 % of EGFP knockdown	[7]
Chitosan	Mouse/intratracheal	EFGP/transgenic EGFP mice model	0.6 µg siRNA 82 % of EGFP expression inhibition compared with non-treated group	[60]
	Mouse/intranasal	EFGP/transgenic EGFP mice model	3 µg siRNA 47 % inhibition of EGFP-expressing epithelial cells	[61]
PLGA	Mouse/intravaginal	EFGP/transgenic EGFP mice	~2 µg siRNA 50-60 % reduction of EGFP expression in vaginal tract at day 10	[31]
Thioketal nanoparticles	Mouse/oral	TNFα/ulcerative colitis mice model	~46 µg siRNA - five daily gavages Significant reduction of TNFα mRNA levels and reduced manifestations of ulcerative colitis	[24]
NiMos	Mouse/oral	TNFα/ulcerative colitis mice model	~ 24 µg siRNA – 3 gavages three-fold decrease of TNFα expression on day 14	[25]
		Conjugated siRNA		
PDT-DRBP	Mouse/intranasal	ROSA26 mice expressing luciferase	Extensive reduction of luciferase expression throughout the nasal and tracheal passages	[8]
TAT/cholesterol conjugated	Mouse/intratracheal	P38 MAP kinase	1~15 to 750 µg conjugated siRNA TAT or Cholesterol conjugation extend protein expression knockdown compared with naked siRNA	[19]
Cholesterol conjugated	Mouse/intravaginal	Nectin-1/UL29/Mouse model of HSV infection with HSV-2 strain 186	~15 µg siRNA Significant and durable protection against HSV infection	[33]
		Other		
Infasurf	Mouse/intratracheal	Codelivery with plasmind encoding Luciferase	30 µg siRNA Less-efficient luciferase expression inhibition than with D5W	[12]
D5W solution	Mouse/intratracheal Macaque/intratracheal	Codelivery with plasmind encoding Luciferase SC2 and SC5/Mouse model of SARS infection with PUMC01 SCV	30 µg siRNA More-efficient luciferase expression inhibition than with Infasurf Suppression of SARS pathogenesis in prophylactic or therapeutic protocol	[12]
GeRPs	Mice/oral	Mapk4 kinase	~ 400 ng siRNA 40 %, 50 % and 80 % of inhibition in the lung, spleen and liver, respectively	[21]

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an aerodynamic diameter greater than 5 μm are mainly deposed in the mouth, throat or upper lung tissues and rapidly eliminated. Similarly, particles smaller than 1 μm are directly exhaled and eliminated before deposition in the alveoli.

Clearance mechanisms also need to be considered carefully. In the lower airways, pulmonary macrophages ingest and degrade micro particles bigger than 260 nm, but smaller particles may escape macrophage clearance. The use of nanoparticles such as poly(lactic-co-glycolic acid) (PLGA) could represent a suitable delivery method [2].

The lung epithelium is covered with mucus, from the nasal cavity to the bronchioles, whereas the alveolar epithelium is lined with alveolar fluid composed of phospholipid surfactants. In the case of a siRNA target localized to the alveolar epithelium, surfactants could affect the efficiency of lipid-based delivery systems, thus it would be preferable to use other synthetic delivery systems such as polymers. Mucus secretion is modified by physiological conditions depending on the state of the disease. For example, inflammatory conditions lead to increased mucus secretion. The inhalation of mannitol, an osmotic agent, prior to siRNA delivery could reduce mucus secretion [3].

Once the siRNA reaches the cell of interest, it has to cross the cellular membrane to enter the cytoplasm. SiRNA are negatively charged molecules of approximately 13 kDa, hence they are not able, alone, to cross the cellular barriers. In the case of non-viral siRNA delivery systems, endocytosis is the major cellular uptake pathway involved in siRNA internalization, but particles need to be smaller than 150 nm. Several types of endocytic pathways are known, the most important in the lung being the caveolar-mediated endocytose. In this case, after internalization, particles are enclosed in caveosomes which are non acidic particles, and could directly reach the RISC complex in the cytoplasm.

Practically, siRNA delivery in the lung is usually achieved by inhalation or by intratracheal or intranasal administration. Due to the ease of application of intranasal administration, this method is the most-commonly used for siRNA delivery.

To achieve delivery by inhalation, siRNA need to be formulated in dry powder or liquid aerosol. In this case, formulation of the siRNA is a critical step, because lyophilization or aerosolization of siRNA could affect their efficiency and deposition in the lung. As reported by Lam *et al*. [4], different inhalation devices can be used, such as metered-dose inhalers (MDIs), dry-powder inhalers (DPIs) and nebulizers. MDIs, in which siRNA are dissolved or suspended in propellants, are commonly used but are not the ideal system for siRNA delivery because of the limited biostability of the siRNA formulation. DPIs present promising advantages for siRNA delivery: siRNA are inhaled as a cloud of particles allowing improved stability of siRNA formulations. An alternative to MDIs and DPIs is nebulization, which can be used for the delivery of large volumes. In all cases, it is very important to ensure the protection of the siRNA formulation. Indeed, siRNA could be damaged by the sheer force during the nebulization or drying process. Thus, formulation vectors need to protect siRNA from degradation. To date, no studies have reported on siRNA delivery by the inhalation method, due to the complexity of siRNA formulations required for inhalation [2].

Intratracheal and intranasal administration are widely used for lung siRNA delivery due to their suitability of injection. Intratracheal administration ensures optimal efficiency of siRNA delivery with minimal drug loss, but it remains an invasive and non-physiological procedure and cannot be considered for applications in clinical trials or human use. In experimental animal studies, pulmonary delivery is often achieved by the instillation of small volumes of suspensions into the tracheae of mice by endotracheal intubation using an aerosol device such as a Penn Century Microsprayer (in the case of liquids) or a Dry Powder Insuflator which enables precise quantification of the delivered dose [5]. The traditional procedure for intratracheal administration required micro-surgery in order to insert the catheter between the tracheal rings. Recently, an alternative method has been developed that does not require an incision in the tracheal tract, but rather the catheter is inserted in the trachea via the mouth, the trachea remains intact. This method is still, however, invasive and non physiological.

Intranasal delivery is an efficient alternative to intratracheal delivery as it is easy to perform and requires a low level of anesthesia. However, contrary to humans, mice are obligate nasal breathers, so extrapolation to humans of the potential efficiency of siRNA delivery by this method may be overestimated.

Several studies have used the inhibition of a reporter gene to optimize the development of non viral siRNA delivery systems for pulmonary administration. The majority of these studies have involved the inhibition of enhanced green fluorescent protein (EGFP) expression in endogenously EGFP-expressing mice [6]. Beyerle *et al*. reported the use of polyethylenimine (PEI) [7], Howard reported the use of chitosan and Eguchi reported the used of peptide transduction domain–double stranded RNA-binding domain (PTD-DRBD) peptides-based delivery systems [8]. The DRBDs bind to siRNA with high avidity, masking the siRNA's negative charge and allowing PTD-mediated cellular uptake (see details in *Table I*).

1.2. Pathologies

The lungs are subject to numerous diseases involving epithelial cell, such as cystic fibrosis and asthma, but also to infection by many viruses such as respiratory syncitial virus (RSV), SARS corona virus (SCV) and influenza virus. As RNAi-based therapies represent a promising approach to the treatment of these diseases, many siRNA delivery strategies have been developed, as described below.

1.2.1. Viral infections

1.2.1.1. Respiratory syncytial virus and parainfluenza virus

Respiratory syncytial virus (RSV) and parainfluenza virus (PIV) are the most common causes of serious respiratory infections in infants and young children. RSV infects the uppers and lower respiratory tract leading to croup, pneumonia and bronchiolitis. RSV and PIV infections could, notably, be reduced by the deletion of the P protein of RSV and PIV, which may be implicated in viral replication. Thus, Bitko *et al*. reported lung delivery of siRNA targeting the P protein of respiratory syncitial virus or parainfluenza virus [1]. Two siRNA against RSV and one against PIV were selected for an *in vivo* study. Mice were administered intranasally with approximately 70 μg siRNA complexed with TransIT-KO reagent, which is a polycation/liposomal formulation from Mirus Corporation (Madison, United States). Four hours later mice were intranasally infected with RSV or PIV. Evaluation of siRNA efficacy was assessed five to six days after viral infection, when the maximum RSV replication in the lung occurs. At this time, viral titers were reduced by approximately 99 % in mice treated with RSV or PIV siRNA compared with untreated mice. Notably, naked siRNA was also able to inhibit pulmonary viral titers. Furthermore, virus inhibition was highly specific, as RSV siRNA did not inhibit PIV and vice versa, and the TransIT-KO reagent did not cause discomfort in uninfected mice. When mice were co-administered with the three siRNA at 70 μg each and co-infected with RSV and PIV, both viral titers were significantly reduced. In addition, Bitko *et al*. also evaluated whether siRNA delivery was associated with curative effects once infection was well established. In this case, mice administered with siRNA at the same time as, or one day after, viral infection, showed stable, or increased body weights, compared with uninfected mice. The administration of siRNA later, two to four days after viral infection, led to less-effective protection against RSV resulting in a decreased mouse body weight, with a late increase in body weight. Altogether, these results suggest that RSV siRNA delivery in the lungs enables a protective or curative effect on RSV replication.

Using another strategy, Akinc *et al*. reported the use of lipidoids, a new class of lipid-like vectors, for the delivery of RSV-siRNA by intranasal administration. Lipidoids are obtained by the conjugated addition of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines. Mice were intranasally administered with lipidoids-RSV siRNA formulations at a dose of approximately 70 µg siRNA and four hours later infected with 10^6 plaque-forming units of RSV [9]. Virus titers were assessed at day 4 after infection. Akinc *et al*. observed that siRNA formulated with 98N12-5 lipidoids enabled more than two log-reductions in viral plaque numbers compared with naked siRNA which enabled only one log-reduction. These authors reported specific siRNA delivery in the lung with no gene silencing observed in the liver and kidney.

1.2.1.2. Severe acute respiratory syndrome corona-virus

Severe acute respiratory syndrome (SARS) is a newly emerging disease caused by SARS corona-virus [10], for which there is no efficient vaccine or treatment. Individuals infected by SARS virus present a high fever followed by severe clinical symptoms such as acute respiratory syndrome. Several studies have reported *in vitro* inhibition of SCV [11]. Li *et al*. reported the intranasal delivery of anti-SCV siRNA in a Rhesus macaque model of SARS [12]. In a first evaluation of the *in vivo* activity of anti SCV-siRNA, two siRNA, siSC2 and siSC5, targeting the SCV genome spike-protein-coding and ORF1b regions, respectively, were tested. SiRNA were co delivered with pCi-scLuc plasmid containing the corresponding siSC2 and siSC5 target sequences and the luciferase reporter gene. SiSC2, siSC5 (30 µg) and plasmid pCI-scLuc (30 µg) were formulated with D5W solution (dextrose 5 % in water) or Infasurf solution (Forest Laboratories Inc, New York, United States) and delivered by intratracheal administration in mice. Infasurf is a sterile organic solvent extract from calf lung lavage, composed of surfactant protein B and C [12, 13]. Twenty-four hours after siRNA delivery, co-delivery of siSC2, siSC5 siRNA and pCI-scLuc plasmid formulated in D5W solution resulted in a higher level of reporter gene expression and stronger RNAi silencing compared with siRNA and plasmid formulated in Infasurf solution. Further experiments in the Rhesus macaque model were carried out using D5W solution. SiSC2 and siSC5 siRNA were administered intratracheally at a dose of 30 µg formulated in D5W, following a prophylactic-, concurrent or post exposure protocol. Analysis of the macaque body temperature (an indicator of severity of SARS symptoms and of effectiveness of siRNA treatment) showed that all protocols led to a potent suppression of the SARS pathogenesis. Moreover, the authors observed diminished SCV viral levels and reduced acute diffuse alveolar damage, with no signs of siRNA-induced toxicity.

1.2.1.3. Influenza virus

Influenza virus is one of the most prevalent infections in humans. Current vaccines can prevent illness but need to be formulated every year because the viral antigens that elicit neutralizing antibodies change, rendering the previous year's vaccine ineffective against new subtypes. Many siRNA specific for the conserved regions of the influenza virus genome have been designed and tested for their abilities to inhibit influenza virus production *in vitro* [14]. Tompkins *et al*., have reported the efficiency of influenza virus inhibition *in vivo* using Oligofectamine-mediated siRNA delivery in the lung [15]. To assess the efficiency of siRNA delivery, mice were pretreated with 50 µg naked siRNA by intravenous injection, followed 16 to 24 h later by infection with H1N1 influenza virus. A second dose of 20 µg siRNA formulated with the lipidic carrier OligofectamineTM, at a charge ratio of 3:2, was administered intranasally. Two different siRNA against influenza virus protein NP and PA were tested, alone or in combination. At day 18 post challenge, 60 % of animals receiving the control siRNA had died, whereas animals receiving either anti-NP or anti-PA siRNA presented respectively, only 20 or 10 % of mortality. Of interest, animals receiving both siRNA showed 100 % survival. These authors also successfully evaluated the abilities of NP and PA siRNA to inhibit the replication of other influenza viruses such as H5, H7 and H9 influenza subtypes. This study demonstrated that the combination of hydrodynamic, intravenous delivery of siRNA with intranasal delivery leads to significant inhibition of virus replication. Tompkins *et al*. also observed that intravenous administration alone provided significant protection, but with less efficiency, suggesting that intranasal delivery contributed to survival.

These studies, involving different siRNA formulations and different siRNA target, have clearly shown that siRNA delivery to the lung is a promising and viable approach to the treatment of viral infections.

1.2.1.4. Lung cancer

Lung cancer is one of the most-frequent tumors worldwide with regards to incidence and mortality rates. Although knowledge in the field of lung cancer research has increased, the median survival time for primarily non-curable tumors is still less than 12 months [16]. Xu *et al*. assessed the potency of siRNA formulated with poly(ester amine) delivery for the treatment of lung cancer [17]. Protein kinase 1 (Akt1) is one of the most-frequently hyperactivated signaling pathways in cancer, notably in lung cancer. Poly(ester-amine) is a biodegradable polymer with a high transfection efficiency and low toxicity compared with polyethyleneimine (PEI). Anti-Akt1 siRNA was formulated with the polycation poly(ester amine) at a poly(ester amine)/siRNA charge ratio of 45, and administered in a mouse model of K-rasLa1 or urethane-induced lung cancer by a nose inhalation system. Mice were exposed twice a week, for total of four weeks, to aerosol in a nose-only exposure chamber, as described by Tehrani *et al*. [18]. Analyses of Akt1 expression and tumor growth were performed at the end of the protocol. This *in vivo* study clearly demonstrated the anticancer effect of Akt1 siRNA in the lung through aerosol inhalation and showed Akt1 protein expression knockdown of about 80 % in the lung. This was sufficient to suppress pulmonary tumor progression in K-rasLA1 mice and urethane-induced lung cancer mice. Moreover, aerosol delivery of Akt1 siRNA did not affect Akt1 protein expression in other organs.

1.2.1.5. Inflammatory disease

RNA interference is also a promising strategy to inhibit the expression of pathologically (over-)-expressed genes, notably in inflammatory pathologies such as chronic obstructive pulmonary disease. Moschos *et al*. investigated knockdown of p38 MAP kinase by conjugating siRNA with different non-viral delivery vectors such as cholesterol, TAT (48-60) peptide (derived from the transactivator of transcription (TAT) of human immunodeficiency virus) and penetratin peptide [19]. p38 MAP kinase is known to be implicated in the release of multiple pro-inflammatory mediators, including tumor necrosis factor (TNF-α) and interleukin (IL)-1. Inhibition of p38 MAP kinase could be a promising approach for the treatment of inflammatory diseases such as rheumatoid arthritis, Crohn's disease and psoriasis [20]. Intratracheal administration of approximately 15 to 750 µg siRNA conjugated either with TAT (48-60), penetratin or cholesterol in mice resulted in a 30-45 % knockdown of p38 MAP kinase expression at 6 h, as observed with naked siRNA. Nevertheless, increasing amount of siRNA did not lead to an improvement in mRNA expression knockdown, but rather to an improvement of the duration of knockdown, except when siRNA were conjugated with penetratin. TAT (48-60) peptide alone was also able to reduce p38 MAP kinase expression, suggesting that TAT (48-60) peptide is a modulator of p38 MAP kinase. It is interesting that TAT (48-60) peptide and cholesterol-conjugated siRNA did not elicit an innate immune response, contrary to penetratin-conjugated siRNA. This study suggests that conjugation of siRNA with CPP or cholesterol may extend, but not increase, siRNA knockdown in the lung, compared with naked siRNA. Moreover, some CPPs, such as

TAT peptides, are able to suppress unintended immunostimulation by recognition with the toll-like-receptor (TLRs) that are usually observed when siRNA is internalized in the endosome. Further studies are required to characterize the mechanisms of action of CPP on gene expression and immune activation.

2. Oral

Oral siRNA delivery is the most convenient and cost-effective means to deliver siRNA to diseased intestinal tissues. Nevertheless, the main challenges of oral siRNA delivery are to protect siRNA from degradation by gastrointestinal enzymes and to allow crossing of the intestinal mucosa and cellular barriers. Some studies have achieved successful oral siRNA delivery in animal models of inflammatory bowel diseases. SiRNA are usually encapsulated within biodegradable particles which protect them from degradation and target them to M cells in Peyer's patches or in inflamed tissues.

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are characterized by a chronic inflammation of the gastrointestinal tract. Many factors may be involved in the etiologies of theses phenotypes, including genetic predisposition, environmental and immune factors. Conventional treatments are based on anti-inflammatory and immune-suppressive drugs, but lack efficacy with high rates of recurrence. An alternative is surgical removal of the inflamed tissue, but this involves pain and numerous strains in everyday life for the patient. Thus, reduction of the inflammatory phenotype using an siRNA-based therapy could be a promising approach. Nevertheless, this requires the development of efficient vectors for oral siRNA delivery.

Aoudi *et al.* were the first to report successful oral siRNA delivery by encapsulating siRNA in biodegradable particles [21]. SiRNA, designed to silence Map4k4 (mitogen-activated protein kinase kinase kinase kinase 4) expression, were encapsulated in porous particles consisting of β-1.3-D-glucan shells derived from baker's yeast (GeRPs) by a series of chemical extractions. Particles were made of a central core of transfer RNA that was coated with layers of cationic PEI. SiRNA are placed between these layers of PEI, by electrostatic interactions. SiRNA-containing GeRPs were administered orally in mice, once daily, for eight days. The aim was to target the M cells present in Peyer's patches in the intestinal wall in order to reach macrophages where Map4k4 siRNA could modulate cytokine expression. By oral delivery of GeRPs loaded with approximately 400 ng siRNA, the authors reported the internalization of siRNA-GeRP particles in macrophages as expected, followed by a marked inhibition of Map4k4 mRNA levels of 40 % in the lung, 50 % in the spleen and 80 % in the liver, respectively, compared with controls. Moreover, the gene-silencing effects persisted for up to eight days after the last oral siRNA delivery, with no unexpected immune stimulation response. Of interest, oral delivery of GeRP particles loaded with only 400 ng siRNA lead to the efficient inhibition of mRNA expression: a dose five to 250 times less than the dosage usually used for systemic siRNA delivery [22, 23].

Similarly, in order to target inflamed intestinal tissues, Wilson *et al.* developed another oral siRNA delivery system based on the fact that diseased intestinal tissues are characterized by high levels of reactive oxygen species (ROS) produced at sites of inflammation, thus ROS could be used as a specific trigger of siRNA release [24]. Wilson *et al.* developed thioketal nanoparticles (TKNs) which release encapsulated agents in response to ROS. TKNs are derived from a polymer, poly-(1,4-phenyleneacetone dimethylene thioketal), composed of ROS-sensitive thioketal linkages. When delivered orally, siRNA-TKNs remain stable and intact in the harsh environment of the gastrointestinal tract, protecting siRNA and preventing its release into non inflamed tissues. At the site of intestinal inflammation, the presence of high levels of ROS enables the degradation of TKNs, leading to the release of siRNA at the target site of inflammation. Tumor necrosis factor α (TNF-α) plays an essential role in the persistence of intestinal inflammation. Thus, Wilson *et al.* chose to treat mice suffering from ulcerative colitis with TNF-α-siRNA. TNF-α-siRNA was first complexed with the cationic lipid dioleoyl trimethylammonium propane (DOTAP), and then co-encapsulated in TKNs. SiRNA-loaded TKNs have diameters of about 600 nm, in order to limit non specific uptake by enterocytes, and promote interaction with phagocytes. Mice were given, orally, TNF-α or scramble siRNA (approximately 46 µg siRNA) by five daily gavages. After seven days, the authors reported a marked, tenfold decrease in colonic TNF-α mRNA in treated mice compared with control mice and reduced clinical manifestations of ulcerative colitis. Moreover, formulation of TNF-α with another vector, such a PLGA or β-glucan particles, did not lead to a significant decrease in colonic TNF-α mRNA, suggesting that the specificity of TKNs for inflamed tissues is an important factor for an efficient TNF-α siRNA delivery. This study clearly emphases the importance of the vector design for efficient siRNA delivery to target cells. Indeed, TKNs have the chemical properties required for siRNA delivery in inflamed intestinal tissues.

At the same time, Kriegel *et al.* developed a polymeric microsphere-based delivery system, named NiMOS, for oral siRNA delivery in the same model of ulcerative colitis previously described by Wilson *et al.* [25]. The NiMOS delivery system comprises B-gelatin nanoparticles encapsulating siRNA, and further entrapped in a poly(epsilon-caprolactone) microsphere-forming nanoparticles-in-microsphere oral system. The composition of NiMOS promotes intestinal localization by delivery of gelatin particles to enterocytes and epithelial cells, after degradation of the poly(epsilon-caprolactone) matrix by lipases in the intestinal tract. To investigate the efficiency of the NiMOS system for oral delivery of siRNA, the authors developed a murine model of ulcerative colitis. These mice received orally anti TNF-α-siNRA-Nimos particles (approximately 24 µg siRNA) at days 3, 5 and 7 after the beginning of ulcerative colitis induction. At days 10 and 14, TNF-α mRNA and protein levels were lower in the mice treated with TNF-α-siRNA-NiMOS particles compared with control mice, with a three-fold decrease at day 14.

These studies show important advances which have been made in oral siRNA delivery, which may lead to significant developments in the therapeutics of the treatment of inflammatory and intestinal diseases in the clinic.

3. Vaginal

Vaginal siRNA delivery holds great potential for the prevention and treatment of various viral infections responsible for diseases such as genital herpes, acquired immune deficiency syndrome and cervical cancer. The advantages of vaginal administration are numerous: this method is non-invasive, bypasses hepatic clearance and provides local delivery directly to the target tissue, reducing the amount of therapeutic agent required for efficient gene silencing. Vaginal siRNA delivery has already been described in pre-clinical trials for the treatment of herpes simplex virus (HSV) [26], but the method remains challenging as siRNA molecules have to cross mucosal barriers and be taken up by cervicovaginal epithelial cells, while avoiding nuclease degradation.

3.1. Challenges

Considering this, intravaginal siRNA delivery systems need to be effective, safe and non-irritating to mucosal surfaces, as inflammation of vaginal tissues renders them more permissive to infection. The majority of the developed systems are based on liposomal carriers. Palliser *et al.* showed that FITC-labeled siRNA formulated with the commercial reagent Oligofectamine, and delivered intravaginally, is efficiently taken up by the vaginal and ectocervical epithelium [26]. Moreover, when siRNA-targeting EGFP were administered intravaginally with Oligofectamine to EGFP-expressing mice, GFP expression was reduced three days later and this effect persisted for at least nine days without systemic silencing in distant organs. Nevertheless, it has been reported that lipoplexes remain toxic and unstable at high ionic

conditions, leading to serious concerns regarding the use of liposomal carriers to deliver siRNA to mucosal surfaces [27-30]. Woodrow *et al.* have developed biodegradable polymer nanoparticles enabling efficient siRNA delivery in mucosal surfaces [31]. These authors reported that poly(lactic-co-glycolic acid) (PLGA) nanoparticles, with biodegradable and biocompatible properties, could be formulated with siRNA and delivered to the vaginal mucosa leading to efficient gene silencing. A single administration of siRNA/PLGA nanoparticles into vaginal mucosa produced effective and sustained gene silencing throughout the female reproductive tract for at least 14 days. In order to efficiently encapsulate siRNA, the authors chose to pre-encapsulate siRNA with spermidine which acts as a counterion, following which the siRNA-spermidine was encapsulates in PLGA nanoparticles at a charge ratio of 8:1 (N/P ratio), with a loading efficiency of 75 ng siRNA per milligram PLGA. After topical administration to the cervicovaginal tract, PLGA nanoparticles were detected in the vaginal canal and the uterine horns. Intravaginal delivery of siRNA targeting enhanced green fluorescent protein (EGFP) formulated with PLGA in transgenic GFP mice led to a reduction of GFP expression by 50-60 % in the reproductive tract up to ten days post administration using approximately 2 μg siRNA. Mice receiving siRNA formulated with PLGA showed earlier inhibition of EGFP expression without histological modification of the vaginal epithelium compared with mice receiving siRNA formulated with commercial liposomes (Lipofectamine RNAiMax from Invitrogen) which showed thickened vaginal epithelium with the presence of neutrophils. Thus, PLGA nanoparticles are attractive carriers for vaginal siRNA delivery as they are able to penetrate deeply into tissues and to silence gene expression efficiently using only a single dose of siRNA.

It is, nevertheless important to note that in these two studies, mice were pretreated with a progesterone derivative before siRNA delivery. Indeed, Wu *et al.* reported that conventional lipoplexes such as Oligofectamine, administered intravaginally, are unable to reach the vaginal epithelium under normal physiological conditions. Physiological changes in the vaginal tract throughout the estrus cycle have to be considered as they can affect the delivery of macromolecules into vaginal tissues. The outer layer of the epithelial cells becomes cornified and hyperkeratotic when mice are in estrus, as reported by Wu *et al.* [32].

Wu *et al.* recently investigated the challenges of delivering siRNA to vaginal tissues under normal physiological conditions without progesterone treatment, which mimics more accurately the human vaginal tract. They developed a novel alginate scaffold system containing muco-inert PEGylated lipoplexes based on DOTAP and cholesterol lipid, which provide a sustained vaginal presence of lipoplexes and facilitate the delivery of siRNA into the vaginal epithelium [32]. These PEGylated, lipoplex-entrapped alginate scaffolds (PLAS) were fabricated using a freeze-drying method. The use of PEGylated liposomes enabled better particle stability in the mucus, and the alginate scaffold facilitated the retention of these particles in the vaginal cavity following intravaginal administration. The authors compared the efficiency of intravaginal siRNA delivery of the PLAS system with that of cationic lipoplexes. The results showed a six-fold increase in the percentage of uptake of siRNA into vaginal tissues for the PLAS system compared with cationic lipoplexes. To assess the level of siRNA knockdown, mice were treated with 8 μg lamine A/C-siRNA formulated in PLAS system per dose, and two doses were administered intravaginally to each mouse on two consecutive days. Twenty-four hours later, the knockdown of lamine A/C in vaginal tissues was of 85 % in mice receiving lamine A/C siRNA treatments compared with control mice. This study was the first to demonstrate vaginal siRNA delivery using biodegradable scaffold and liposomes. This novel system is a promising approach for vaginal siRNA delivery and its ease of administration offers vital features for rapid clinical development.

3.2. Pathologies

The vaginal mucosa is one of the main entry points for numerous viral and bacterial pathogens which cause infectious and inflammatory diseases.

Herpes simplex virus 2 (HSV-2) infection causes significant morbidity and is an important cofactor in the transmission of HIV infection. SiRNA based-therapies could be promising treatment for these diseases as the durability of an RNAi-based virucide does not require an administration just before sexual intercourse, limiting the problems of compliance which are usually encountered with the use of classical microbicide. To investigate the ability of anti-HSV-2 siRNA to protect mice from HSV-2 virus, Palliser *et al.* pretreated mice with a progesterone derivative to arrest the mice in the diestrous phase of the estrous cycle, which may facilitate epithelial penetration [26]. The mice were then infected with a lethal dose of HSV-2 virus and treated with anti-HSV-2 siRNA formulated with the commercial lipid Oligofectamine®, two hours before and four hours after viral infection. Two siRNA were tested, targeting different essential viral protein, UL29 (a DNA-binding protein) and UL27 (envelope glycoprotein B). The results showed that only 25 % of mice treated with 500 pmol (approximately 7.5 μg) UL29 siRNA died compared with 75 % in control groups. On day 11 after viral infection, analysis of the disease severity showed robust protection against HSV-2 infection in UL29 siRNA-treated mice. Of interest, mice treated with UL27 siRNA were less protected, with only 60 % survival. Analysis of the vaginal mucosa of siRNA-treated mice on day 6 showed an intact epithelium with few apoptotic bodies compared with control mice, which showed a denuded mucosal epithelium and a high level of inflammatory cells. In a second study, these authors investigated the effect of an anti-HSV-2 siRNA treatment post infection. In this case, siRNA were administered alone or as a mixture, three and six hours after viral infection. Mice receiving only one siRNA present no survival advantages compared with control mice but, interestingly, mice receiving a mixture of siRNA had 80 % of survival, showing that post-exposure treatment could also be effective. It is also important to note that siRNA treatment does not elicit an immune response and inflammatory infiltrate. Nevertheless, Wu *et al.* showed that a lipidic reagent facilitated viral infection by eliciting a slight inflammation, which may be sufficient to enhance viral uptake [33]. Wu *et al.* investigated improving the durability of protection and to optimizing delivery systems in a mouse model of herpes simplex virus [33]. They developed cholesterol-conjugated siRNA with a single phosphothiorate linkage that protects against siRNA degradation by genital fluid RNAses. They identified the cell surface receptor nectin-1 which is a receptor for HSV-2, as a possible target for RNAi-based therapy in the case of HSV-2. To assess the efficiency of chol-siRNA conjugated for vaginal delivery, mice were pretreated with a progesterone derivative, as previously described, and challenged with 2 × LD50 HSV-2. Mice receiving two times approximately 30 μg nectin-1-siRNA conjugated with cholesterol were efficiently protected against HSV-2 infection only when the siRNA was administered prior to infection (up to seven days prior to infection). The authors reported that combining nectin-1 and UL29 siRNA led to uniform and significant protection against HSV-2, irrespective of the time of siRNA administration, before or after HSV infection. Moreover, intravaginal administration of chol-siRNA did not induce interferon or an inflammatory response. Thus, the use of cholesterol-conjugated siRNA seems to improve protection against HSV-2 viral infection as cholesterol does not elicit an immune response.

Altogether, the results of these studies present great promises for the prevention and/or treatment of sexually transmitted viral infections. Much work is required to optimize siRNA silencing efficiency, and to develop a delivery system suitable for an improved vaginal retention without pretreatment of the vaginal cavity, in order to be suitable for clinical application in humans.

4. Intravesical

Intravesical siRNA delivery is a promising approach compared

with systemic delivery. Indeed, intravesical siRNA delivery seems to achieve a similar effect to systemic delivery, with a 50- to 100-fold lower dose of siRNA complex. Moreover, the procedure of transurethral administration is quite simple and repeatable in the clinic, although administration into the murine bladder is rather difficult. As described by Nogawa *et al.*, in mice, the bladder was catheterized and the formulation was administered. A purse-string suture was then placed around the urethra to occlude it. After four hours, the sutures were removed and the mice recommenced their spontaneous voiding [34].

The majority of bladder cancer diagnoses are made in the early stage of the disease, but 30 % of cases recur at more advanced stages and form invasive cancers. In this case, the standard treatment is cystectomy, with numerous undesired side effects such as urinary dysfunction. The development of non-invasive treatments that preserve the bladder are, thus, desirable. In this way siRNA-based therapies are promising tools for the treatment of bladder cancer.

Nogawa *et al.* were the first to report intravesical delivery of siRNA from an investigation of the intravesical delivery of an anti-polo-like kinase-1 (PLKI-1) siRNA for the treatment of bladder cancer [34]. PLK-1 protein is a regulator of mitotic progression in mammalian cells. It has been reported that over expression of PLK-1 is usually associated with the development of numerous human tumors and in particular bladder cancer [35]. Nogawa *et al.* formulated anti-PLK-1 siRNA with the cationic liposome LIC-101 which contains 2-O-(2-diethylaminoethyl)-carbamoyl-1,3-O-dioleoylglycerol and egg phosphatidylcholine. The formulation was administered intravesically in an orthotopic bladder cancer mouse model. Mice were administered with UM-UC-3Luc tumor cells at day 0. Transurethral treatment with siRNAs was performed five times, once on each of days five to nine. Mice received 9 to 90 μg/ml PLK-1 siRNA. PLK-1 siRNA was successfully delivered into the tumors, leading to a weak expression of PLK-1 associated with fewer cancer cells observed in the siRNA-treated mice compared with control mice. Moreover, some treated mice showed a total eradication of the cancer cells without adverse effects, indicating PLK-1 siRNA as a promising tool for the treatment of bladder cancer. This study was the first demonstration of the inbibition of cancer growth in the murine bladder by intravesical siRNA/cationic liposomes delivery.

II. NON-MUCOSAL siRNA DELIVERY

We will described in this section local non viral siRNA delivery strategies that do not concern mucosal surfaces, including intracerebral, intraocular and intratumoral siRNA delivery (see *Table II*).

1. Central nervous system

Numerous genes expressed in the brain and central nervous system (CNS) have been identified as potential target for the treatment of neurological and psychiatric pathologies such as Huntington's or Alzheimer's disease. While siRNA have been widely used to investigate gene function in neuronal culture, the development of *in vivo* siRNA delivery systems to the brain has been impaired by the existence of the blood-brain barrier and the specific metabolism of the brain. Taking these factors into account, some reports have described the development of siRNA delivery systems optimized for the treatment of cerebral pathologies.

Hassani *et al.* were able to knockdown reporter gene expression in new born mouse brain using the JetSITM-lipid-based delivery system following intracerebroventricular injection [36]. Using only 7.5 pg siRNA formulated with JetSI TM, the expression of the luciferase reporter gene was reduced by 78 % at 24 h post-delivery in the brain. In further development, Cardoso *et al.* have developed a cationic-based siRNA delivery system that enhances siRNA delivery in neurons [37]. Intrastriatal injection of 3 μg siRNA formulated with DOTAP:cholesterol liposomes functionalized with Transferin (Tf-lipoplexes) in the ipsilateral hemisphere of mouse brain enabled a 40 % inhibition of luciferase expression in the striatum of endogenously-luciferase-expressing mice. Taken together, these studies suggest that local delivery of siRNA to the CNS is a feasible option for targeting CNS-based diseases.

Based on these preliminary studies, some authors have assessed the ability of RNAi-based therapies to treat neurological diseases such as Huntington's and Alzheimer's disease.

Huntington's disease (HD) is a neurodegenerative disease characterized by a loss of brain neurons, caused by expansion of a CAG repeat in the Huntingtin (Htt) gene. Inhibition of Hintingtin expression in brain neurons reduces the severity of the disease. Efficient siRNA targeting of the Htt protein has been identified *in vitro*. Wang *et al.* delivered Htt-siRNA to brain neurons of newborn HD mice to assess the benefits of siRNA on HD severity [38]. The formulation, comprising 0.2 μg Htt-siRNA with the cationic lipid Lipofectamine, was administered to newborn mice into the lateral ventricle, as reported by Shen *et al.* [39]. Ninety-six hours after siRNA delivery, the body weight loss of HD mice treated with Htt-siRNA was slowed as compared with control mice, and the life-span of treated mice was extended by two weeks. Moreover, siRNA treatment re-established motor function by delaying the onset and reducing the frequency of feet-clasping behavior of HD mice. By measuring Htt mRNA residual expression levels, the authors reported Htt mRNA was maintained for more than one week in the stratium but not in remote areas of the ventricle such as the cortex. This study was the first report of the successful use of an siRNA-based therapy in a HD mouse model using only one intraventricular injection, just after birth. However, further investigations are required to improve the biodistribution of the delivered siRNA and to develop a less-invasive delivery method.

As an alternative to the use of thecationic lipid Lipofectamine, which could be toxic for neuronal cells, DiFiglia *et al.* reported the use of a cholesterol-conjugated siRNA targeting htt in a new virus-mediated transgenic model of HD in adult mice [40]. A single administration of 0.2 μg cholesterol-conjugated-htt-siRNA in the right striatum of mice was sufficient to inhibit htt mRNA expression in the striatum for at least three days, leading to the amelioration of multiple neuropathological features and aberrant motor features for three weeks. Contrary to the findings of Wang *et al.*, DiFiglia demonstrated that siRNA treatment enabled therapeutical benefits in an adult mouse model of HD with a single pulse of siRNA delivery.

Similarly, in a mouse model of Alzheimer's diseases, Uno *et al.* reported efficient delivery of siRNA in the brain by combining high density lipoprotein (HDL) with an α-tocopherol-conjugated siRNA (Toc-siRNA) [41]. These authors identified a target gene encoding the b-site amyloid precursor protein-cleaving enzyme1 (BACE1), which is implied in Alzheimer's disease pathology. To assess the efficiency of Toc-siRNA/HDL *in vivo*, these particles were administered to the mouse brain by direct intracerebroventricular infusion with osmotic pumps, as described by Thakker *et al.* [42]. As compared with free Toc-siRNA, continuous injection of approximately 45 μg Toc-siRNA/HDL particles for seven days led to a broader and intense transduction of Toc-siRNA with a significant reduction in target BACE1 mRNA expression at the hippocampal and parietal cortex (up to 60 % with Toc-siRNA/HDL compared with free Toc-siRNA in the parietal cortex). Thus Toc-siRNA/HDL particles enabled efficient gene-silencing in the brain using a 1000-fold lower amount of siRNA than in a previous study. Improvements in gene-silencing efficiency could be explained by a better uptake of siRNA, as the Toc-siRNA/HDL vector system may utilize the physiological lipid metabolism. Indeed, α-tocopherol may be transferred from glial cells to neurons via the receptor-mediated pathway with HDL-like particles.

As reported by DiFiglia *et al.*, the next challenge is to improve the safe delivery of siRNA in the brain to maximize target specificity. Autosomal neurodegenerative diseases such as HD require treatment for years with adjustment or discontinuation when side effects arise.

Table II - Overview of siRNA delivery systems for non mucosal target.

Delivery system	Animal/route	Molecular target/model	Dosage/effect	Ref.
Lipid				
JetSI	Mouse/intracerebral	Luciferase/transgenic Luciferase mice	~7.5 ng siRNA 78 % reduction of Luciferase expression	[36]
Tf-lipoplexes	Mouse/intracerebral	Luciferase/transgenic Luciferase mice	3 µg siRNA 40 % of luciferase expression inhibition	[37]
Lipofectamine	Mouse/intracerebral	Htt/Newborn mice	0.2 µg siRNA Life span increased by 2 weeks	[38]
	Mouse/intraocular	VEGF- induced angiogenesis mouse model	1 µg siRNA Significant inhibition of VEGF expression	[46]
Cationic liposome	Mouse/intratumoral	Integrin alphaV/Human PC3 cancer cell model	1 µg siRNA Reduction of 75 % of tumor growth	[49]
Folate-linked nanoparticles	Mouse/intratumoral	Her-2/ KB tumor xenograft model	10 µg siRNA Significant inhibition of tumor growth at day 4 to 10 after siRNA administration	[50]
Polymer				
PEI	Rat/intrathecal	NR2B/ Rat	5 µg siRNA 83 % of NR2B mRNA expression reduction	[43]
	Mouse/intratumoral	PTN/glioblastoma mouse model	8 µg siRNA Inhibition of xenograft tumor growth	[51]
	Mouse/intratumoral	Ras1/subcutaneous prostate carcinoma model	20 µg siRNA – twice weekly sPEI/anti-ras1 diminished the growth of PC-7 xenograft significantly when compared with other groups	[53]
	Mouse/intratumoral	CUX1 /subcutaneous pancreatic cancer model	10 µg siRNA – 3 times a week over 3 weeks significantly decreased tumor volume accompanied by a marked reduction in CUX1 expression	[54]
Chitosan-based hydrogel	Mouse/intratumoral	TG2/melanoma and breast tumor mice model	~ 3 µg siRNA - twice weekly Significant inhibition of tumor growth (72 % reduction and 92 % reduction in melanoma and breast tumoral model, respectively)	[57]
Conjugated siRNA				
Cholesterol-conjugated	Mouse/intracerebral	Htt/mouse model of Huntington's disease	~ 75 µg siRNA Inhibition of Htt mRNA expression Improvement of neuropathological features for 3 weeks	[40]
Tocopherol-siRNA	Mouse/intracerebral	BACE-1/mice	~45 µg siRNA for 7 days 60 % reduction of BACE-1 mRNA expression	[41]
Chol-MPG	Mouse/intratumoral	Cyclin B1/human prostate carcinoma mice model	5 µg siRNA siRNA targeting of cyclin B1 compromises tumor cell proliferation	[58]
Other				
Atelocollagen	Mouse/intratumoral	HPV/cervical cancer mouse model	15 µg siRNA Suppression of tumor growth	[62]
	Mouse/intratumoral	E6, E7/cervical cancer mouse model	7.5 µg siRNA – once every 7 days for 30 days Significant reduction of tumor growth	[55]
	Mouse/intratumoral	VEGF/prostate cancer mouse model	750 ng to 7.5 µg siRNA Suppression of tumor angiogenesis and tumor growth	[56]

Hence the modularity of action of siRNA therapeutics is a promising tool for the treatment of these diseases.

Using different approach, Tan *et al*. used branched PEI for intrathecal siRNA delivery [43]. In a rat model, the delivery of 5 µg siRNA targeting the N-methyl-D-aspartate receptor subunit protein NR2B formulated with a branched PEI through an intrathecal injection (subarachnoid), led to an 83 % inhibition of NR2B protein expression and to the modulation of pain.

2. Ocular

The use of siRNA-based therapies is a very promising treatment for ocular diseases, such as cytomegalovirus infection or age-related macular degeneration (AMD). However, the administration method in the posterior segment of the eye is invasive and harmful if repeated. Indeed, in the most cases, the target site for siRNA treatment is on the posterior segment of the eye and siRNA is not able to penetrate alone into the cornea so it has to be delivered intravitreally. Moreover, siRNA have a short intravitreal half-life which implies repeated administration to obtain efficient modulation of targeted gene expression, with an increased risk of endophthalmitis and retinal detachment. Thus, optimized vectors have been developed to ensure cellular penetration, protection against degradation and long-term delivery.

SiRNA-based therapies are currently being tested in clinical trials, notably for the treatment of AMD [44]. VEGF is an endothelial

growth factor cell specific mitogen implicated in modulation of the angiogenic process, notably in AMD diseases. Many studies have shown successful intraocular delivery of siRNA targeting VEGF or the VEGF receptor, leading to the inhibition of neovascularization in several validated, preclinical models [45, 46]. Murata *et al.* reported that using a subconjunctival administration of 1 µg VEGF siRNA formulated with Lipofectamine, the expression of VEGF was significantly inhibited in an induced corneal model of angiogenesis without damage to the conjunctiva [46]. However, Kleinman *et al.* have recently shown that the inhibition of VEGF expression in these models using naked siRNA was not due to a sequence-specific RNA interference mechanism but rather to the intrinsic properties of siRNA molecules mediated by the TLR3 receptor [47]. Indeed, the authors demonstrated that 21-nucleotide or longer ds-RNAs displayed anti-angiogenic properties via TLR3 receptor activation.

3. Intratumoral

Many tumor models have been developed to study various cancers, including prostate, brain and pancreatic cancer. SiRNA-based therapeutics present a promising approach to the treatment of these diseases, as described in the report of numerous studies [48]. While most of these studies have shown systemic delivery of siRNA, local delivery is a promising alternative to increase target specificity and reduce off-targets effects. As reported below, various intratumoral delivery systems have been developed.

In the case of prostate cancer, which is the most frequently diagnosed cancer in men in the USA, tumor metastasis usually appears in bone, with skeletal metastases identified at autopsy in up to 90 % of patients who have died from the disease. Bisanz *et al.* reported intratumoral siRNA delivery in a model of human PC3 cancer prostate cells induced in tibia [49]. One microgram of anti-integrin alphaV siRNA was formulated with a cationic liposome-based formulation composed of dipalmitoylethylphosphocholine,dioleoylphosphoethan olamine, dipalmitoylphospho-ethanoamine, and polyethyleneglycol. The authors reported a reduction of integrin alphaV expression and of 75 % reduction in tumor growth. To improve the targeting of siRNA delivery and considering that some tumoral cells have high folate receptor expression, Yoshizawa reported intratumoral siRNA delivery using folate-linked lipid-based nanoparticles in a model of human oral epidermoid carcinoma cell (KB) xenograft [50]. Another strategy consisted of using PEI-based delivery systems. Based on a tumor model of brain cancer, specifically a mouse model of glioblastoma with U87 cells growing intracranially, Grzelinski *et al.* demonstrated that intratumoral administration of 8 µg siRNA targeting the growth factor pleiotrophin complexed with PEI, led to the inhibition of xenograft growth [51]. In the same way, PEI-based delivery systems have been widely used for intratumoral siRNA delivery, as reported by Kim *et al.* in a mouse model of subcutaneous prostate carcinoma xenograft [52], by Zhu *et al.* in a mouse model of subcutaneous pancreatic carcinoma[53] and by Ripka *et al.* in a model of subcutaneous pancreatic cancer [54]. Fujii *et al.* have also developed an original siRNA delivery system for intratumoral siRNA delivery in a model of cervical cancer [50]. SiRNA targeting human papillomavirus (HPV) protein was formulated with atelocollagen which is a highly purified type I collagen of calf dermis. The authors reported efficient suppression of tumor growth using only 15 µg siRNA. Similary, Yamato *et al.* efficiently inhibited tumor growth using siRNA formulated with atelocollagen in a similar model of cervical cancer [55] and Takei *et al.*, in a mouse model of prostate cancer [56]. Interestingly, Han *et al.* have developed a chitosan-based hydrogel which displays a temperature-dependant liquid-solid phase transition and could be directly injected into the tumor site and gradually degraded by enzymes at tumor sites after complete release of siRNA [57]. In another approach, Crombez *et al.* used an N-methylpurine-DNA glycosylase (MPG) peptide-based delivery vector for intratumoral siRNA delivery in different mouse tumor models [58]. Intratumoral administration of siRNA formulated with the peptide carrier MPG functionalized with cholesterol promoted efficient delivery of siRNA and reduced tumor growth in the different mouse tumor models.

III. CLINICAL STUDIES

While numerous studies have achieved the development of local siRNA delivery systems using non viral vectors, only a few delivery systems have been tested in clinical trials (*Table III*). This is notably the case for siRNA therapeutic siRNA-027 (termed AGN211745), which is a chemically modified siRNA targeting the VEGF receptor 1 and which has entered a phase II trial seemingly proving a clinically significant improvement in visual acuity with no serious adverse events or dose-limiting toxicity in a relevant subset of patients [44].

*

As reported here, various synthetic vectors for local siRNA delivery have been developed to improve efficiency of gene silencing. However, most of the synthetic vectors used for local siRNA delivery have been essentially optimized for *in vitro* applications, including Oligofectamine, TransIT-KO, chitosan or PLGA delivery systems. Moreover, some of them are commercial reagents without known composition, which would impair their optimization for further clinical development. Concerning mucosal siRNA delivery, lipid based delivery vectors are not the ideal vectors due to possible interaction with surfactants, notably in the lung. In the case of lung siRNA delivery, polymer-based delivery systems such as chitosan and conjugated delivery systems would be preferred. In the same way, vaginal administration of siRNA requires the use of polymer-based delivery systems, such as PLGA nanoparticles that are biodegradable and biocompatible. The use of PEGylated lipoplexes also seems to be efficient for vaginal siRNA delivery. Conversely, non-mucosal siRNA delivery does not require the development of sophisticated siRNA delivery systems. Indeed, efficient siRNA delivery in the central nervous systems is achieved using the commercially available vectors Lipofectamine and JetSI, or with conjugated siRNA as an alternative method. Nevertheless, intracerebral administration of siRNA is a highly invasive method that must be improved. In the same way, efficient intratumoral siRNA delivery is achieved using polymer-based delivery systems such as PEI or chitosan. All of these systems have previously been used for gene delivery but need to be optimized in order to reduce toxicity and improve efficiency. In this way, some studies have developed synthetic siRNA delivery especially designed for siRNA delivery such as aminoglycosides derivatives [59]. In the same way, GeRPS particles derived from baker's yeast or NiMos particles composed of b-gelatin nanoparticles have been specifically designed for oral siRNA delivery in order to avoid degradation of the siRNA by gastrointestinal enzymes, while promoting siRNA internaliza-

Table III - Non-viral siRNA delivery systems undergoing clinical trials.

Disease	Stage	Target	Delivery	Product name	Company/Institution
Age-related macular degeneration	Phase I/II	VEGF receptor 1	Intravitreal	AGN211745 (SiRNA-027)	siRNA/Merck
	Phase II		Intravitreal	PF-4523665	Quark Pharmaceutical
RSV infection	Phase II	RSV	Intranasal	ALN-RSV01	Alnylam Pharmaceuticals

tion into target cells. This review clearly shows that siRNA delivery systems must be optimized depending on the route of administration by considering the specificity of each system. The major challenge remains the improvement of safety, which currently clearly impairs further clinical development of siRNA delivery systems. Moreover, it remains difficult to translate the results obtained in animal models to the human, because of anatomical and physiological differences between humans and other animals. Routes of administration used in experimental animals are also generally not suitable for the future administration of the treatment in humans.

ABBREVIATIONS

Akt1, protein kinase 1; AMD, age-related macular degeneration; BACE1, b-site amyloid precursor protein cleaving enzyme 1; CNS, central nervous system; CPP, cell-penetrating peptide; D5W, dextrose 5 % in water; DPI, dry-powder inhalers; DOTAP, dioleoyl trimethylammonium propane; EGFP, enhanced green fluorescent protein; FITC, fluoresceine isothiocyanate; GeRPs, β1.3-D-glucan-encapsulated siRNA particles; GFP, green fluorescent protein; HD, Hungtington disease; HDL, high density lipoprotein; HSV, herpes simplex virus; IL, interleukin; MDI, metered-dose inhalers; MPG, N-methylpurine-DNA glycosylase; NiMOS, nanoparticles-in-microsphere oral system; NR2B, N-methyl-D-aspartate receptor 2B; PEG, polyethylene glycol; PIV, parainfluenza virus; PLAS, PEGylated lipoplex-entrapped alginate scaffolds; PEI, polyethylenimine; PLGA, poly(lactic-co-glycolic acid); PTD-DRBD, peptide transduction domain-double stranded RNA-binding domain; RNAi, RNA interference; ROS, reactive oxygen species; RSV, respiratory syncitial virus; TAT, transactivator of transcription; TNF, tumor necrosis factor; TLR, toll-like receptor; TKN, thioketal nanoparticles; SARS, severe acute respiratory syndrome; SCV, SARS corona virus; Toc, tocopherol; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

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