REVIEW

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Nanoparticle-mediated delivery of small RNA molecules in tumor therapy

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In principle, RNA interference (RNAi) allows for the inhibition of any oncogene of choice, thus leading to novel concepts in tumor therapy. For their delivery, the RNAi-inducing small RNA molecules (small interfering RNAs, siRNAs) can be formulated in various nanoparticle systems, prior to testing them in preclinical animal models. The same is true for miRNAs that have more recently been explored in therapeutic miRNA replacement strategies. This puts high demands on the properties of the nanoparticles. This review article discusses various nanoparticulate systems for RNA delivery *in vivo* and gives an overview of preclinical studies on siRNA- or miRNA-based tumor therapy.

1. Introduction

Deeper insights into the mechanisms of tumorigenesis and into genetic alterations contribute to our increased understanding of the differences between normal and tumor cells, and allow for the identification of molecular markers and novel therapeutic target molecules. Certain oncogenes are upregulated (overexpressed) or mutated, which may lead to higher activities in signal transduction pathways and thus to increased tumor cell proliferation and survival. This has also led to the concept of „oncogene addiction", i.e., the hypothesis that, despite the multitude of genetic aberrations, certain tumors can be dependent just on one single oncogene in their tumorigenesis and progression (Jain et al. 2002; Weinstein 2002). Examples include the overactivation of EGFR in non-small cell lung carcinoma (NSCLC) or the constitutively active BCR-ABL fusion protein in chronic myeloic leukemia (CML). This offers avenues for novel therapeutic strategies relying on the targeted inhibition of such proteins, based for example on low molecular weight inhibitors such as Glivec® or vemurafenib, or therapeutic antibodies like Herceptin®, Erbitux® or Avastin® (for a comprehensive overview, see e.g. the National Cancer Institute website on Targeted Cancer Therapies (NCI 2014)). In contrast to classical chemotherapy, the therapeutic intervention is thus molecularly targeted. However, the development of suitable protein inhibitors is difficult, expensive and time-consuming, and not possible for all target molecules.

The approach of early interference already in the expression of the target gene, rather than the later inhibition of the protein, offers an attractive therapeutic alternative. This can be achieved by blockage or destruction of the corresponding mRNA; these strategies are called 'gene knockdown' or 'gene silencing' and in the broadest sense can be considered as gene therapy. Especially RNA interference (RNAi) has become an indispensable tool in the field of functional genomics, by allowing the targeted depletion of any therapeutically relevant gene product of choice. Beyond tissue culture, RNAi is also explored in preclinical animal models and in various clinical studies with regards to therapeutic applications in humans. The use of RNAi in animals and an outlook on future therapeutic applications is the focus of our review article.

Abbreviations: i.c., intracranial; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; miRNA; microRNA; KSP, Kinesin spindle protein; LNP, lipid nanoparticle; MRI, magnetic resonance imaging; NP, nanoparticle; PEI, poly(ethylene imine); RNAi, RNA interference; s.c., subcutaneous; SCC, squamous cell carcinoma; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

2. RNAi/siRNA-mediated gene knockdown; regulation via miRNAs

Comparable to classical approaches based on antisense technologies, RNAi offers the possibility for sequence-specific mRNA inhibition or cleavage. A major advantage for therapeutic applications lies in the fact that every cell already provides all necessary components of the RNAi machinery, thus 'only' requiring the delivery of the siRNA. In other words: the delivery of siRNAs is necessary and sufficient for the induction of a specific, RNAi-mediated gene knockdown. Since RNAi relies on a catalytical mechanism, i.e., one siRNA is able to cleave several mRNA molecules, low siRNA amounts are already sufficient for gene silencing. Nowadays, RNAi plays a key role in oncogene research with regard to either single inhibition of a selected target gene or the use of RNAi libraries for the identification and validation of novel oncogenes (see also the article by Adams et al. in this issue). Obviously, this also offers interesting therapeutic options.

Some years ago small double-stranded RNAs were discovered that are generated physiologically by the cell itself, which were termed microRNAs (miRNAs). In contrast to siRNAs, these miRNAs can inhibit the translation of many different mRNAs in parallel and thus interfere in the expression of several genes, again using the RNAi machinery (Friedman et al. 2009). Interestingly, certain miRNAs are pathologically downregulated in tumors; since they often inhibit oncogenes, these aberrantly low miRNA levels lead to oncogene activation. Consequently, restor-

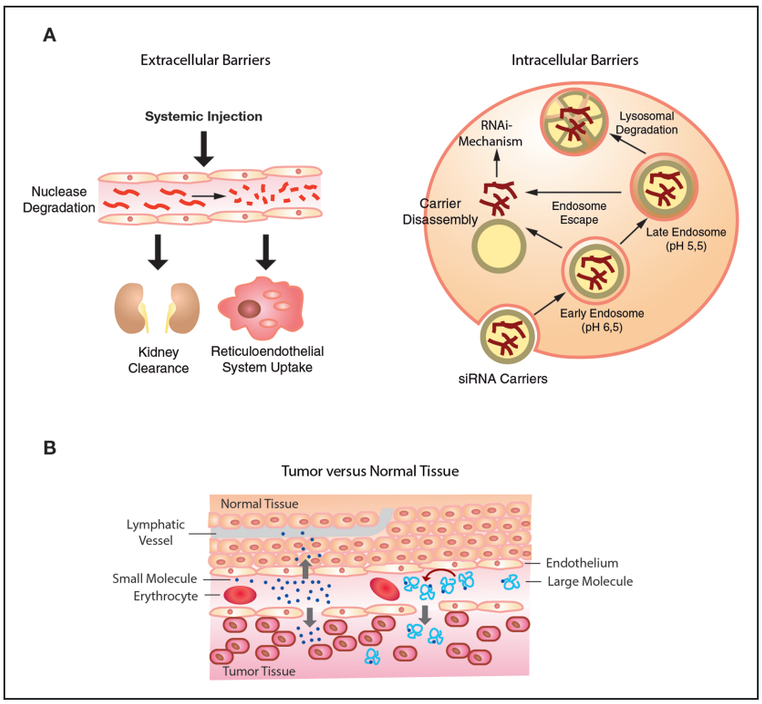

Fig. 1: Extra- and intracellular barriers for the efficient and safe transport of RNA and its delivery system in healthy tissue versus tumor tissue. (modified from Xu und Wang, Asian J. Pharm. Sci. (in press) (A) Naked siRNAs can be degraded in the blood or they can be removed from the blood stream by renal elimination or by macrophages (left). In addition, the cellular uptake of naked siRNAs is hampered by electrostatic repulsion between the phosphate groups of siRNAs and the cellular phospholipid membrane. By using siRNA nanoparticles (right) the cellular uptake can be improved. After endocytosis of nanoparticles the intracellular release of the siRNA from the endosomal-lysosomal system is required to achieve its interaction with the target mRNA in the cytoplasm and to prevent lysosomal degradation (right). (B) Facilitated transfer of siRNA from the blood flow into tumor tissue compared to normal tissue. Due to a fenestrated endothelium in tumors (endothelial cells can only form vessel walls with gaps) the passage - even of nanoparticles - in the adjacent tissue is facilitated (compare tumor tissue (down) to normal tissue (top)). This effect is called EPR (enhanced permeability and retention) and contributes to "passive tumor targeting".

ing normal levels in the case of pathologically low expressed miRNAs ('miRNA replacement') represents a novel potential therapeutic strategy in cancer (see e.g. Aigner 2011; Garofalo et al. 2014 for review).

3. Advantages and issues of RNA-based therapy

Small RNA molecules like siRNAs offer several advantages over other therapeutic drugs or compounds: they are synthetic in nature and, especially when compared to biologicals like antibodies or other protein-based therapeutics, relatively easy to manufacture. They can be directed against any target gene of choice and can be readily designed based on their complementarity to the target sequence, thus often being more specific than other cancer therapeutics. Their interference already on the level of protein expression offers the possibility of synergistic effects when combined with protein inhibitors. In the case of miRNAs, it is particularly noteworthy that they physiologically regulate several genes in parallel, often many of those being part of the same tumor-relevant signaling pathways. Thus, miRNA replacement interferes simultaneously with several molecular targets in cancer, often considered as 'pathway disease' (Bader et al. 2010).

Major hurdles for therapeutic applications still remain, like the safe and efficient *in vivo* delivery of small RNAs and, in the light of the transient nature of siRNA/miRNA action, effect durations as long as possible in order to prolong intervals between injections. This is even more problematic since small RNA molecules are drugs coming with several issues. They are relatively instable and prone to degradation in the body within seconds to minutes, and due to their charge and size, they are barely taken up by cells, which in turn is critically relevant for their subsequent intracellular effects. A solution to this problem is their formulation in nanoparticulate systems.

4. Nanoparticulate systems for the delivery of RNA

Nanoparticles for the transport of RNA-based drugs should (i) be resistant to degradation and protect the RNA, (ii) be non-immunogenic, (iii) inhibit non-specific interactions with serum proteins and non-target cells, (iv) reduce the rapid renal clearance of RNA in order to prolong the half-life in the blood circulation, (v) gain efficient and selective targeting, (vi) and mediate an efficient endosomal release. Additionally, safety and biocompatibility have to be accomplished for human use (Whitehead et al. 2009). The Figure 1 gives an overview of the challenges for RNA delivery systems after administration into the body and on their way to the site of pharmacological action. Undesired effects in the blood stream such as enzymatic degradation of RNA by nucleases or disintegration of the carriers by interactions with blood components have to be avoided as well as hemolysis, aggregation of blood cells followed by thrombosis and embolic events or the activation of the immune system (Schlenk et al. 2013). After reaching the target cell, the uptake of RNA has to be accomplished. This is seriously hampered by the high molar mass and the net negative charge of the phosphate groups, thus leading to electrostatic repulsion when interacting with the negatively charged cell membranes. Furthermore, the carrier should protect the RNA against degradation in the hostile endosomal/lysosomal intracellular environment and mediate its release into the cytoplasm, which is, with some exceptions, the site of pharmacological action (Scholz and Wagner 2012; Whitehead et al. 2009).

Table 1: Overview of nanoparticulate systems for the delivery of small RNAs, and their most important advantages and disadvantages (modified from Draz et al. 2014)

Nanoparticles	Advantages	Disadvantages
Liposomes	Biocomaptibility and biodegradation, efficient cellular uptake, numerous modifications	Complex production, instabilities, drug loss, rapid clearance
Polymers (dextrane, poly(ethylene imine), chitosan, poloxamers, etc.) and dendrimers	Easy and fast production of polymers and polyplexes, numerous modifications	Instability, toxicity of certain synthetic polymers
Cyclodextrins	Low toxicity, no immunogenicity, no degradation *in vivo*	High costs of production, low solubility
Silica-NP	High mechanical and physical stability, controllable porosity (allows multifunctional and sequential release), facile surface functionalisation	*In vivo* toxicity
Metallic and metal oxide NP (Au, Fe_xO_y, etc.)	Small particles sizes, extended circulation half lifes, multifunctional applications (theranostics, targeting, etc.)	Low colloidal stability, low or no biodegradation, low biocompatibility
Carbon based NP (graphen, carbon nano tubes)	High capacity for functionalization and loading, penetration through biological barriers, colloidal stability	Non-soluble, high toxicity, safety issues during production and use, high costs
Hydrogels	High porosity and loading capacity, biocompatibility and biodegradation, selective functionalization	High costs, instability
Quantum dots	Controllable emission, high photostability and chemical stability, potential as theranostics	High toxicity, agglomeration and instability

To meet these requirements, strategies successfully used for the transport of DNA were adapted to RNA delivery. Cationic lipids and polymers form tightly packed, stable nanosized lipoplexes and polyplexes, respectively, by electrostatic interaction with the polyanionic RNA. This results in enhanced cellular uptake and efficient protection against nucleolytic degradation (see e.g. Urban-Klein et al. 2005). The higher the molar mass of the carrier material and the number and density of charges, the more efficient is the formation of the nano-assemblies (Eltoukhy et al. 2012; Ochrimenko et al. 2014). However, these parameters correlate with intensive electrostatic interactions and detrimental effects on cell membranes. This so called "charge dilemma" highlights the necessity of developing RNA delivery systems with improved benefit-to-risk ratios. Although the development of RNA transfer systems might in general benefit from the know-how on DNA delivery, the requirements for RNA and DNA are different in many aspects. Due to the smaller size, lower number of charges and higher rigidity of the small double-stranded RNAs compared to DNA molecules, RNA undergoes weaker electrostatic interactions with cationic carriers and often forms smaller, less stable complexes (Scholz and Wagner 2012). The 2'-hydroxy group also makes RNA prone to its fast degradation by ribonucleases in body fluids.

Table 1 summarizes nanoparticulate carriers that are in preclinical use for the transport of RNA-based drugs in oncology. RNA delivery systems can be classified as synthetic or naturally derived materials. Lipid-based nanoparticles are the most advanced carriers, but often associated with limited stabilities and comparably complicated production processes. Polymers and dendrimers have gained increased importance due to their controllable production, numerous possibilities for structure modifications and custom-designable properties (Uchegbu et al. 2008). Inorganic nanoparticles offer advantages regarding stability, shelf-life, often low toxicity and non-sensitivity against enzymatic or microbial degradation. RNA can be incorporated into the core of solid inorganic nanoparticles, or used as electrostatic or adsorptive coating molecule on the nanoparticle surface. Depending on the type of material, these nanoparticles are often characterized by low or missing degradation behaviour (Draz et al. 2014).

Tumor tissue is usually abnormal in its form and architecture, with unique vascular characteristics (Fig. 1). Due to increased angiogenesis and hypervascularization, the newly formed blood vessels present large fenestrations and gaps, distinct vessel irregularities, increased permeability and the lack of a normal lymph drainage (Jang et al. 2003; Pecot et al. 2011). In pharmaceutical technology, these anatomical alterations can be systematically used for the design of nanoparticulate carriers that explore the so-called 'passive tumor targeting' by the EPR (enhanced permeability and retention) effect. Nanoparticles tend to accumulate in the tumor tissue after transfer through endothelial fenestrations (permeability) due to their nano-size, and are retained in the irregularly structured tissue characterized by reduced clearance via the lymphatic system (retention). The success of the passive targeting is greatly influenced by size, charge and hydrophilicity of the nanoparticles (Fang et al. 2011).

The second approach to selectively accumulate nanoparticulate carriers in cancer cells benefits from the pathological surface characteristics of tumor cells (so called 'active targeting'), aiming at a reduction of side effects in healthy cells (Ku et al. 2014). The technique is based on the conjugation of specific homing ligands, capable of being selectively recognized by the target cells, to the nanoparticles. Ligands from different groups such as antibodies (e.g. anti-VEGFR, anti-HER2) as well as non-antibody based ligands (e.g. transferrin, EGF, folic acid, carbohydrates such as mannose, lectins, integrins, etc.) have been used for active targeting of RNA delivery systems (Bae and Park 2011). Examples are discussed in the following section.

5. Examples for preclinical therapeutic applications

The wide variety of possibilities for therapeutically used nanoparticle-formulated siRNAs will be shown by discussing

Table 2: Selection of preclinical studies on the therapeutic application of siRNAs (i.v.: intravenous, i.p.: intraperitoneal, i.t.: intratumoral, i.c.: intracranial, s.c.: subcutaneous)

Nanocarrier	Application	Target	Reference	Observed effects / Aim of study
Liposomal Systems				
Cationic liposome LIC-101	i.v. / s.c.	BCL-2	(Yano et al. 2004)	Antitumor effect in liver metastasis model
Liposome	transurethral	PLK-1	(Nogawa et al. 2005)	Inhibition of bladder xenografts
Neutral liposome	i.p.	IL-8	(Merritt et al. 2008)	Inhibition of orthotopic ovarian carcinoma xenografts
Liposome	i.p.	NRP2	(Gray et al. 2008)	Inhibition of hepatic colorectal carcinoma xenografts
Liposome		STAT3	(Landen et al. 2007)	Inhibition of isoproterenol-induced ovarian carcinoma
Mixture of cationic and fusogenic lipids	i.v.	CD31	(Santel et al. 2006)	Inhibition of liver tumor xenografts, anti-angiogenesis
TfRscFv immunoliposome	i.v.	HER2	(Hogrefe et al. 2006)	Inhibition of mamma carcinoma xenografts
Polymers				
Atelocollagen	i.t.	VEGF	(Takei et al. 2004)	Inhibition of s.c. prostate carcinoma xenografts
Atelocollagen	i.t.	EGFR	(Nozawa et al. 2006)	Enhanced sensitivity of lung carcinoma xenografts towards chemotherapy
Atelocollagen	i.v.	EZH2	(Takeshita et al. 2005)	Inhibition of bone-metastasizing prostate carcinoma xenografts
Chitosan-coated polyisohexyl-cyanoacrylate	i.v.	RHOA	(Pille et al. 2006)	Inhibition of mamma carcinoma xenografts
Cyclodextrin-containing polycation-transferrin	i.v.	EWS-FLI	(Hu-Lieskovan et al. 2005)	Inhibition of metastatic Ewing's sarcoma xenografts
Cyclodextrin-containing polycation-transferrin	i.v.	–	(Heidel et al. 2007)	Safety/toxicity study in non-human primates (macaques)
Poly(ethylene imine) (PEI)	i.p.	HER2	(Urban-Klein et al. 2005)	Inhibition of s.c. ovarian carcinoma xenografts
Poly(ethylene imine) (PEI F25-LMW)	i.p., i.c.	Survivin	(Hendruschk et al. 2011)	Inhibition of s.c. / i.c. glioblastoma xenografts
Poly(ethylene imine) (PEI F25-LMW)	i.p.	VEGF	(Hobel et al. 2010)	Inhibition of s.c. xenografts, combination with Avastin
RGD-PEG-PEI	i.v.	VEGF R2	(Schiffelers et al. 2004)	Inhibition of s.c. neuroblastoma xenografts
Melittin-PEI25-PEG-EGF	i.v.	—	(Schaffert et al. 2011)	Inhibition of s.c. xenografts by cytotoxic poly(I:C) RNA
SS-PEI (bioreducible)	i.t., i.v.	hTERT	(Xia and Lin 2012)	Inhibition of s.c. liver xenografts
scAbGD2-PEG-g-PEI-SPION	i.v.	BCL-2	(Shen et al. 2012)	Inhibition of s.c. neuroblastoma xenografts + MRI
PEI-DA3 (PEI modified with deoxycholic acid)	i.t.	XIAP	(Jang et al. 2012)	Inhibition of s.c. colon carcinoma xenografts, combination with Taxol
Polyplex hydrogel	i.t.	Cyclin B1	(Kim et al. 2012)	Inhibition of s.c. prostate carcinoma xenografts / sustained delivery system

selected, representative examples of studies in preclinical animal models (Table 2).

Dependent on the locus and goal of the intervention, nanoparticles can be administered systemically (mostly by intravenous (i.v.) injection, sometimes injected intraperitoneally (i.p.) or subcutaneously (s.c.), or topically (local injection) into the tumor. Most studies are done on tumor xenografts, i.e. tumors established by injection of human tumor cells into immunodeficient mice, while tumor models relying on genetically or chemically induced tumor formation are less frequent.

Lipid/liposome-based systems are used most often and are most advanced with regard to their clinical use (see also the article by Kandil & Merkel in this issue). For example, the cationic liposome LIC-101 loaded with an siRNA for the specific knockdown of the oncogene BCL-2 led to growth inhibition of s.c. prostate carcinoma xenografts upon local injection into the tumor, and its i.v. injection resulted in antitumor effects in a liver metastasis model (Yano et al. 2004). Local injection was also explored in therapy studies in ovarian carcinoma xenografts. Intraperitoneal injection of neutral DOPC liposomes (1,2-dioleoyl-sn-glycero-3-phosphocholine) for the delivery of interleukin-8 (IL-8) specific siRNAs led to tumor inhibition, especially upon combination with the classic chemotherapeutic drug docetaxel (Merritt et al. 2008). When using the DOPC liposomes with STAT3 siRNAs in a spontaneous isoproterenol-induced ovarian carcinoma model, tumor inhibition was observed as well (Landen et al. 2007). The systemic (i.v.) application of liposomes may not only lead to their uptake into tumor cells, but also into the tumor endothelium, thus mediating anti-angiogenesis effects (Santel et al. 2006). Treating animals with liposomally formulated siRNAs directed against PECAM-1 (CD31) resulted in reduced growth of orthotopic prostate carcinoma xenografts. Hereby, the mixture of cationic and fusogenic lipids employed in this study mediated the preferential uptake into endothelial cells with subsequent inhibition of tumor angiogenesis as one central process in solid tumor growth (Santel et al. 2006).

As described above, coupling of ligands to the nanoparticle surface can lead to the selective binding to and internalization into the target cells of interest. Among others, natural

ligands to surface receptors (e.g., EGF, transferrin) are candidate molecules that have been explored. Alternatively, the covalent coupling of antibodies or, in order to decrease the size of the ligand component, of single-chain antibodies (scFv) to the nanoparticle surface has led to the development of so-called immunoliposomes. ScFv only comprise the variable regions of antibodies, binding with high affinity and specificity. Tumor-inhibitory effects in mamma carcinoma xenografts were for example observed with liposomes upon covalent coupling of scFv targeted against the transferrin receptor ('TfRscFv') which is highly expressed in many tumor cells (Hogrefe et al. 2006).

Besides liposomes, cationic polymers are broadly used for siRNA delivery upon systemic or local injection. Examples include nanoparticles based on atelocollagen, chitosan or cyclodextrin. In several studies, the intratumoral injection of atelocollagen/siRNA complexes led to the knockdown of the selected target gene and the growth inhibition of the respective subcutaneous tumor xenograft (e.g., of prostate carcinoma xenografts upon knockdown of the growth and angiogenesis factor VEGF (Takei et al. 2004)). Additionally, the atelocollagen/siRNA-mediated knockdown of the receptor tyrosine kinase EGFR was shown to lead to increased chemosensitivity towards chemotherapy in an s.c. squamous cell carcinoma (SCC) xenograft model. This study thus also demonstrates that knockdown strategies in combination with cytostatics may offer a therapeutic benefit (Nozawa et al. 2006). The i.v. application of the same carrier for the siRNA-mediated knockdown of the metastasis-relevant gene EZH2 also led to the inhibition of metastases, as shown in bone-metastasizing prostate carcinoma xenografts (Takeshita et al. 2005). Chitosan-based nanoparticles are suitable for siRNA delivery as well. The siRNA-mediated knockdown of the cell cycle relevant protein RhoA upon i.v. injection of chitosan-coated polyisohexacyanoacrylate/siRNA nanoparticles led to antitumor effects in mamma carcinoma xenografts (Pille et al. 2006). Likewise, cyclodextrin-containing polycations can be employed for delivering siRNA. Similar to the targeted liposomes described above, some studies relied on the specific nanoparticle uptake into TfR overexpressing (tumor-) cells. Here, however, the TfR ligand (transferrin) rather than an anti-TfR antibody was employed. Upon i.v. application in a mouse model, the growth inhibition of metastasizing Ewing's sarcoma was reported (Hu-Lieskovan et al. 2005). These systems were also tested early in safety and toxicity studies in non-human primates (macaques) (Heidel et al. 2007).

Other studies are based on the important group of synthetic cationic polymers, including poly(ethylene imine) (PEI). Upon complexation of siRNAs with low molecular weight, linear or branched PEI nanoplexes were obtained with systemic bioavailability upon i.p. injection. These allowed, for example, the observation of tumor inhibitory effects in an s.c. ovarian carcinoma xenograft model upon knockdown of the receptor tyrosine kinase HER2 (ErbB2) (Urban-Klein et al. 2005). Beyond systemic application, PEI complexes can also be injected locally (e.g., intracranially = into the skull), as shown for example in an orthotopic glioblastoma xenograft study (Hendruschk et al. 2011). Another study of the PEI/siRNA-mediated knockdown of VEGF also revealed that, dependent on the tumor model, in some cases the single VEGF knockdown can already result in profound growth inhibition while in others inhibitory effects are only moderate and enhanced in an additive way upon parallel inhibition of the target molecule on the protein level (Hobel et al. 2010). The same study also demonstrated that different modes of administration (here: i.v. and i.p.) led to marked differences in the biodistribution profile, despite systemic bioavailability in both cases. This also indicates some tissue specificity even in the absence of a targeted delivery approach mediated by ligands. Nevertheless, the coupling of ligands for increased cell specificity and uptake has been a goal in the case of PEI-based complexes as well. Usually, this is combined with the shielding of their positive surface charge, e.g. by the coupling of PEG, in order to prevent non-specific nanoparticle uptake. When exploring small peptides as ligands, for example the RGD peptide (Arg-Gly-Asp), the i.v. injection of RGD-PEG-PEI complexes for the delivery of siRNAs directed against the VEGF receptor VEGF-R2 led to growth inhibition of s.c. neuroblastoma xenografts (Schiffelers et al. 2004). The binding of melittin (a cationic polypeptide found in the bee venom) in addition to the ligand (here: the growth factor and EGFR ligand EGF) improved the otherwise often rate-limiting step of intracellular endosomal release (Schaffert et al. 2011). In this study, rather than using an siRNA, tumor growth inhibition was achieved by delivering a small double-stranded cytotoxic RNA molecule, poly(I:C).

More complex chemical polymer modifications are illustrated in the following examples. Branched PEIs with bioreducible disulfide bonds (SS-PEI, 0.8 kDa) were employed for the delivery of siRNAs inhibiting the human telomerase reverse transcriptase (hTert). Upon intratumoral or i.v. injection, growth inhibition of s.c. liver carcinoma xenografts was observed (Xia and Lin 2012). The constructs (scAb(GD2)-PEG-g-PEI-SPION) are based on PEG-PEI, functionalized with a single chain antibody and complexed with supraparamagnetic iron oxide nanoparticles. When used for delivering BCL-2 specific siRNAs, they not only mediated the target gene knockdown but also allowed for the simultaneous monitoring of the nanoparticles by magnetic resonance imaging (MRI) (Shen et al. 2012). In another study on intratumoral injection in s.c. colon carcinoma xenografts, a branched and deoxycholate-modified 1.8 kDa PEI, PEI-DA3, was shown to form a micelle-like structure that allowed the simultaneous delivery of siRNA (directed against the cell death inhibitor XIAP) and the cytostatic drug taxol (Jang et al. 2012). A polyplex hydrogel consisting of a linear 1.8 kDa PEI-poly(organophosphazene)-conjugate offered, upon intratumoral injection into prostate carcinoma xenografts, a sustained release profile and thus prolonged delivery of an siRNA directed against the cell cycle protein cyclin B1 (Kim et al. 2012).

Beyond therapeutic siRNA for the specific knockdown of a target gene or cytotoxic sequences like poly-IC, miRNA were explored in preclinical animal models as well, aiming at the restoration of normal levels in the case of pathologically low expressed miRNAs (miRNA replacement). Comparable to siRNAs, these miRNAs can be chemically modified (see the article by Grünweller & Hartmann in this issue) for increased stability, affinity and/or specificity towards their target mRNA, or to improve cellular uptake. Due to the chemical similarities between miRNAs and siRNAs, basically the same nanoparticulate carrier systems are employed. Table 3 summarizes studies in this emerging field.

6. Short overview of clinical studies

Some RNAi-based drugs are currently in clinical studies or have already completed early phases (I and II). Results outside oncology also revealed that the local application of naked siRNAs, even when possible from a medical viewpoint, is not necessarily sufficient. Upon closer examination, early reports on therapeutic efficacy of naked siRNAs often turned out to be non-specific effects rather than being based on the specific target gene knockdown.

The first data on siRNA efficiency in non-human primates were already published in 2006, based on their formulation in SNALPs (stable nucleic acid lipid particles) (Zimmermann et al. 2006). A proof-of-concept study on RNAi therapeutics

Table 3: Selection of preclinical studies on therapeutic miRNA replacement

Chemical composition / formulation	Application	miRNA	In vivo-model (mouse)	Therapeutic effect	Reference
Neutral lipid-emulsion / miRNA mimics	i.v.	let-7, miR-34a	Non-small cell lung carcinoma model	Reduced tumor burden	(Trang et al. 2011)
Lipid-based delivery system	local, systemic	miR-34a	Non-small cell lung carcinoma model	Inhibition of tumor growth	(Wiggins et al. 2010)
Liposome-polycation-hyaluronic acid nanoparticle with scFv for tumor *targeting*	systemic, i.v.	miR-34a	Lung metastasis model	Reduced tumor burden in the lung	(Chen et al. 2010)
Lipofectamin / miRNA mimics	i.t.	miR-29b	CML xenograft model	Inhibition of tumor growth	(Garzon et al. 2009)
Atelocollagen	i.v.	miR-16	Bone metastasis model (prostate carcinoma cells)	Inhibition of bone metastases	(Takeshita et al. 2010)
Poly(ethylene imine) complexation	i.p.	miR-33a, miR-145	S.c. colon carcinoma xenograft model	Inhibition of tumor growth	(Ibrahim et al. 2011)
in vivo-jetPEI / miRNA mimics	i.p.	miR-155	I.p. xenografts	Inhibition of tumor growth	(Cubillos-Ruiz et al. 2012)
Amphoteric liposomal formulation	systemic	miR-34	Orthotopic liver carcinoma model	Inhibition of tumor growth	(Daige et al. 2014)

in humans was done using LNP (lipid nanoparticle)-formulated siRNAs, directed against the oncogenes VEGF and KSP (kinesin spindle protein) in liver metastases (Tabernero et al. 2013). In the meantime, several drugs for use in different solid tumors are clinically treated (see e.g. Draz et al. 2014 for review). Beyond the SNALPs mentioned above, PEGylated lipopolyplexes, PEG-cyclodextrin with a covalently bound tumor-specific ligand, neutral liposomes or an implantable so-called 'local drug eluter', consisting of a biodegradable matrix, are explored (Draz et al. 2014).

7. Conclusions and Outlook

Beyond viral systems for nucleic acid delivery, which may be associated with issues including oncogenic potential, immunogenicity, difficulties in upscaling (i.e., the standardized production of larger amounts) and lack of tumor specificity, the nanoparticle-based formulation of small RNA molecules has emerged as a promising approach. While initially technologies were employed that had been developed for DNA delivery, it soon became clear that other requirements need to be met in the case of RNA transport systems. The past 15 years have witnessed substantial progress in this field. Beyond improving the still somewhat moderate efficiency, issues of biocompatibility, safety and quality assurance have entered the limelight. This applies to the formulation as well as to the RNA molecule itself: so-called 'off-target effects' which may be based on the non-specific binding to non-target mRNAs or the activation of intracellular defense mechanisms ('innate immune system') require a stringent selection of suitable siRNA sequences and their comprehensive testing with regard to specificity and absence of unwanted effects. Chemical modifications have been further developed in the last years and have added to enhancing efficacy and duration of the knockdown as well as to avoiding off-target effects (see the article by Grünweller and Hartmann in this issue). The more detailed knowledge on the cellular and molecular mechanisms that govern efficacy and specificity of RNA carrier systems or may lead to unwanted side effects allows for the even more precise development of suitable systems. In this context, it also becomes clear that cell culture experiments are very limited with regard to predicting effects in the living organism.

Since many years, RNA-based methods for the functional analysis of selected gene products or for the identification of novel candidate target molecules with oncogenic potential are indispensable and firmly established in preclinical tumor research. Unfortunately, this does not apply for therapeutic RNAi applications so far. After an initial 'hype' around RNAi in the early 2000's and the subsequent disillusionment when realizing early obstacles and problems, however, efforts have been increased in the last years. Various modified or non-modified RNA therapeutics, in part based on delivery systems, are in early phases of clinical trials (phase I – II). In the field of oncology, it becomes obvious once more that the ultimate goal cannot be *the* delivery system and *the* magic single siRNA: tumor heterogeneity with regard to localization, phenotype and molecular characteristics requires multiple different solutions. Rather, tailor-made RNA molecules which can basically be derived against any target gene and thus offer new possibilities in personalized tumor therapy, as well as a broad technology platform comprising different carrier systems should be able to lead to successful tumor-specific treatment approaches. Particularly promising are also combinations of different siRNAs for the simultaneous inhibition of more than one target gene or, as already exemplified in preclinical research, the combination with established chemotherapeutics, leading to higher cytostatic efficacy or lower dosage requirements.

Conflicts of interest: None

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