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## Review

# A review of the current status of siRNA nanomedicines in the treatment of cancer



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## ABSTRACT

RNA interference currently offers new opportunities for gene therapy by the specific extinction of targeted gene(s) in cancer diseases. However, the main challenge for nucleic acid delivery still remains its efficacy through intravenous administration. Over the last decade, many delivery systems have been developed and optimized to encapsulate siRNA and to specifically promote their delivery into tumor cells and improve their pharmacokinetics for anti-cancer purposes. This review aims to sum up the potential targets in numerous pathways and the properties of recently optimized siRNA synthetic nanomedicines with their preclinical applications and efficacy. Future perspectives in cancer treatment are discussed including promising concomitant treatment with chemotherapies or other siRNA. The outcomes in human clinical trials are also presented.

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## 1. Introduction

The RNA interference (RNAi) mechanism was first discovered in plants [1] and demonstrated in the roundworm *Caenorhabditis elegans* after the microinjection of double-stranded RNA (dsRNA) leading to a transitory high extinction of gene expression [2]. The RNAi concept is based on the interaction of endogen micro-RNA (miRNA) with messenger RNA (mRNA) thanks to the perfect sequence homology observed between the strands [3]. Over the last few years, many kinds of approaches have been created based on the copy or use of this endogenous gene expression regulation system including small hairpin RNA (shRNA; a single strand RNA with a hairpin loop structure), DNA plasmid coding to shRNA, and small-interference RNA (siRNA; a double strand RNA). These structures are all integrated in the miRNA pathway and produce effective gene expression inhibition. This paper will only focus on the RNA form as siRNA (for more information on miRNA, shRNA, see

review in the bibliography [4]). In the context of gene therapy applied to cancers, siRNA acts as a loss-of-function strategy that can inhibit virtually every single protein of interest expression regardless to its localization within the cells contrary to antibodies or tyrosine kinase inhibitors that respectively react only with surface antigens and tyrosine kinase protein. These small entities aim to modulate the expression of overexpressed or mutated genes identified as a key hurdle. Moreover, thanks to this non-integration in DNA, siRNA does not lead to genome modification, an important parameter for regulatory and safety considerations.

Major hindrance to their clinical development in oncology remains limited pharmacodynamic properties due notably to their physicochemical properties. For these reasons, the association of nucleic acid constructs with a multitude of delivery system [5]. These nanomedicines are necessary in order to protect and help them to cross these natural barriers (see our previous review [6]).

To date, numerous proteins have been targeted by siRNA embedded into non-viral delivery systems in cancer pathologies based on cell cycle, apoptosis, proliferation and angiogenesis pathway studies.

This review i) presents shortly the promising characteristics of nanocarriers to bypass the obstacles of intravenous injection and to

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target specifically cancer cells and ii) sums up the current status of preclinical and clinical studies conducted to date towards various molecular targets in this systemic context. The perspectives and opportunities to use siRNA delivery systems alone or in combination with other therapies are discussed.

## 2. siRNA delivery systems

siRNA interacts with cytoplasmic proteins, and in this way need to be taken up by the cell. This mechanism includes the first interaction with cell membrane, the penetration into classic endosomal vesicle or other mechanisms, and finally the escape of this vesicle to be delivered into the cytoplasm. Moreover, siRNA cannot be injected in systemic circulation. In fact, these nucleic acids are rapidly degraded by plasmatic nucleases. In consequence, the encapsulation of siRNA into a delivery system is expected to ameliorate their cell penetration, their delivery into cytoplasm after their protection in blood, their half-life time and finally their specific delivery on tumor site.

### 2.1. siRNA encapsulation

The encapsulation of hydrophilic drugs such as nucleic acids and proteins has been already developed for numerous formulation processes (Table 1). From the classic liposomes to new triblock micelles, various encapsulation efficiencies have been obtained. High yields (80%–95%) have been observed with various types of liposomes based on cationic lipids as 1,2-DiOleoyl-3-Trimethyl-Ammonium-Propane (DOTAP) [7,8], O,O'-ditetradecanoyl-N-( $\alpha$ -

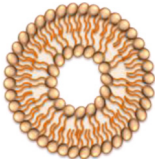



trimethyl ammonio acetyl) diethanolamine chloride (DC-6–14) [9], or cholesterol and their derivatives as 3 $\beta$ -[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol (DC-cholesterol) [10]. For nanoparticles, lower yield has been evaluated at 40% with calcium phosphate particle formulation developed by Li et al. [11], however, higher encapsulation efficiency has been observed for calcium phosphate nanoparticle process developed by Pittella and coworkers with 90% encapsulation yield [12]. For other nanoparticles, yields comprised between 65% to more than 95% are described with delivery system using natural lipids as lecithin [13,14], phosphatidylcholine [15], dextran [16], chitosan [17] or modified cholesterol [18] (Table 1). Contrary to liposomes and nanoparticles, complexes and micelles are usually formed with one kind of molecules and the encapsulation efficiency is depending in this case mainly on the lipid/siRNA charge ratio. Complete siRNA interaction was obtained with simple PEI complexes in association with polymer (glycol chitosan) [19] or protein (albumin) [20] and with copolymers as poly(2-(dimethylamino) ethyl methacrylate) (polyDMEAMA) [21], PEG-SS-poly(Pasp-DET) [22] or acetal-poly(ethylene oxide)-block-poly( $\epsilon$ -caprolactone) (acetal-PEO-b-PCL) [23].

However, if yields of encapsulation have been improved for each delivery system, important variations are observed on encapsulated doses of siRNA. While the majority of authors worked with around 200  $\mu$ g by formulation, large scales have been observed from 50  $\mu$ g [8,11,19] up to milligrams [15].

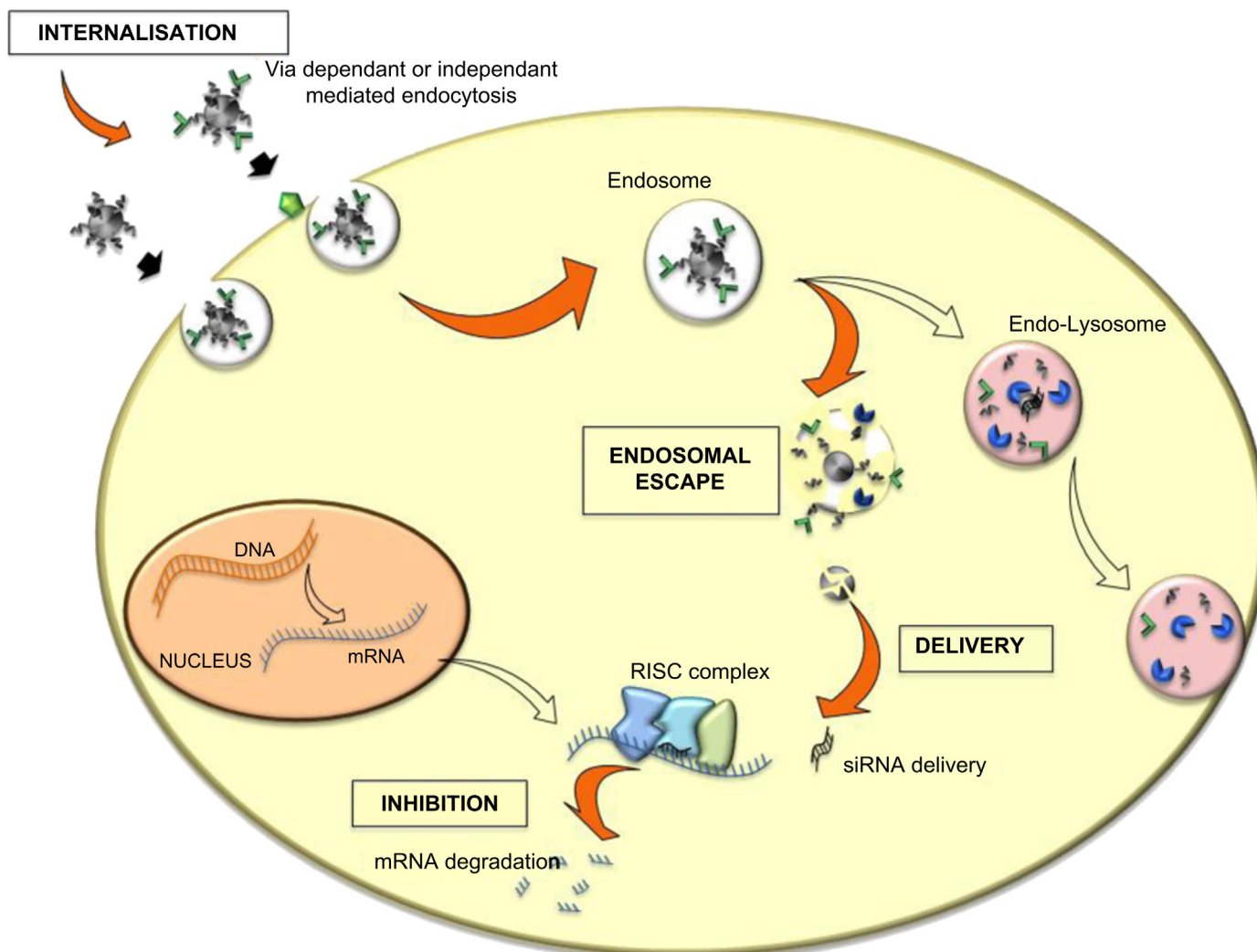
#### 2.1.1. Cellular uptake

Nanocarrier proximity with cells can allow their passive penetration into cytoplasm by different mechanisms (Fig. 1). Multitude of

**Table 1**  
Composition and encapsulation efficacy of siRNA delivery systems.

Type	Encapsulation	Composition	Encapsulation yield (%)	Refs.
 Liposome	Entrapment	DC-Chol, DOPE, DSPE-PEG, F'ab Her2	80	[10]
	Entrapment	DC-6–14, POPC, Chol, DOPE, DSPE-PEG	90	[9]
	Entrapment	DOTAP, DOPE, Chol, PEG-C16Ceramide	90	[7]
	Entrapment	DNA, protamine, DOTAP, Chol, DSPE-PEG	95	[8]
 Nanoparticle	Entrapment	PEG, lecithin, triglycerides, DOTAP, DOPE	65	[13]
	Entrapment/matrix	BHEM-Chol, mPEG-PLA	90	[18]
	Entrapment	Apolipoprotein A-I, Chol, CE, PC, Oligolysine	>90	[15]
	Entrapment	Dextran thiol, Dextran stearylamine, PEG-thiol	95	[16]
	Adsorption	Chitosan, Plurol, Ammonium nitrate, PIBCA	90	[17]
	Matrix	CaP, DOTAP, Chol and DSPE-PEG,	40	[11]
	Matrix	CaP, PEG-Pasp(DET-Aco)	90	[12]
Matrix	PEI, Tripalmitin, DSPE-PEG-folate, Chol, Lecithin	85	[14]	
 Complex	Matrix	PEI, Glycol chitosan polymer	100	[19]
	Matrix	PEI, HSA	100	[20]
 Micelle	Adsorption	poly(DMAEMA), mAB-SA	100	[21]
	Adsorption	PEG-SS-Pasp (DET)	100	[22]
	Adsorption	Acetal-PEO-b-PCCL	>90	[23]

Abbreviations, DC-Chol: 3 $\beta$ -[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol; DOPE: dioleoylphosphatidylethanolamine; DSPE-PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine methoxy (polyethyleneglycol)-2000; F'ab Her2: antibody fragment of human endothelium growth factor receptor; DC-6–14: O,O'-ditetradecanoyl-N-( $\alpha$ -trimethyl ammonio acetyl) diethanolamine chloride; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Chol: cholesterol; DOTAP: 1,2-DiOleoyl-3-TrimethylAmmonium-Propane; PEG-C16Ceramide: polyethylene glycol-C16-ceramide; PEG: polyethylene glycol; BHEM-Chol: N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide; mPEG-PLA: poly(ethylene-glycol)-b-poly(D,L-lactide); CE: cholesteryl oleate; PC: phosphatidylcholine; PEG-thiol: polyethylene glycol-thiol; PIBCA: poly(iso-butyl-cyanoacrylate); CaP: calcium phosphate; PEG-Pasp(DET): poly(ethylene glycol)-SS-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}; PEI: polyethyleneimine; HSA: human serum albumin; poly(DMAEMA): poly(2-(dimethylamino)ethyl methacrylate); mAB-SA: streptavidin-conjugated monoclonal antibody against CD22; acetal-PEO-b-PCCL: acetal-poly(ethylene oxide)-block-poly( $\epsilon$ -caprolactone).



**Fig. 1.** Intracellular trafficking of siRNA delivery systems. Internalization of delivery systems can be realized by several mechanisms as clathrin, caveole pathways or lipid rafts. Two modalities are observed in this phenomenon, a receptor mediated endocytosis and a non-receptor mediated endocytosis. In all these cases, delivery systems are entrapped into endosomal vesicles. These vesicles fuse with lysosome and inevitably lead to siRNA degradation. To avoid this, delivery systems need to escape the endosomal vesicles. Finally, siRNA is delivered into the cytoplasm and produces their inhibitor effect.

endocytosis pathways is possible for delivery systems: clathrin-mediated endocytosis, lipid raft or caveolae, phagocytosis and macropinocytosis [24]. For example, high-density lipoprotein nanoparticles developed by Shahzad et al., showed efficient cellular uptake with 80% favored by major lipid compounds [15]. However, the global negative charge created by PEG and required for the stealth properties of delivery systems for passive targeting can prevent cellular uptake by charge repulsion of negative cell membrane. This surface modification can consequently interfere with efficient cellular siRNA delivery. Nonetheless, various pegylated delivery systems have demonstrated their efficiency to entry into cells even with PEG recovery. For example, two kinds of pegylated nanoparticles developed respectively Guo et al. [25] and Yang et al. [18] demonstrated a great cellular uptake with 60% and up to 95% of penetrated cells thanks to cholic acid moiety and a cholesterol derivatives as N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxy carbonyl aminoethyl) ammonium bromide (BHEM-Chol) contained in shell structure. In addition, cationic lipid (DOTAP and oligolysinoyl lipid) constitution of liposomal forms led to a great cellular uptake with respectively more than 92% and 100% of siRNA labeled cells [26,27]. A complete cellular uptake was also obtained by poly(2-aminoethyl ethylene phosphate) (PPEEA)

cationic block of micelleplex on breast cancer cells [28]. A high *in vitro* inhibition was obtained by these micelleplex with 75% of mRNA and protein extinction [10,28]. Then, lipid composition or positive charge is essential point to favor the cellular uptake of delivery system.

The most obvious mechanism for internalization occurs via receptors associated with the ligands used for the active targeting. In this case, receptors mediate endocytosis induced by ligand fixation and this leads to a facilitated internalization of siRNA delivery system in cytoplasmic vesicles through clathrin pathway. For example, *in vitro* transferrin and integrin targeting on respectively polyplex and lipoplex surface resulted in 100% of cellular uptake on mouse neuroblastoma and human glioblastoma cells [29,30]. Two micelle formulations modified with RGD peptide recognizing the  $\alpha v \beta 3$  integrins showed a high cellular uptake in breast and ovarian cancer cells, respectively evaluated at 60% and 70% [23,31]. Finally, endogen molecules as folate can also be used to favor cellular uptake. Indeed, folate receptor expression was correlated with tumor localisation as described recently by Meier et al., in breast cancer [32]. Folate targeted siRNA nanoparticles penetrated at up to 90% in prostate cancer cells and produced a reduction of 80% mRNA and protein level *in vitro* [14].

### 2.1.2. Endosomal escape

After internalization, early endosomal vesicles are formed and mature in late endosomes characterized by an acidic pH and the presence of active enzymes to finally fuse with lysosome vesicles. In these endosomes, the degradation of nanocarriers can be initiated and nucleic acids destroyed by nucleases (Fig. 1). Accordingly, endosomal escape is a critical step for an efficient cytoplasmic siRNA delivery. Two principles have been already advanced to explain the mechanism of endosomal escape by delivery systems [33]. Firstly, proton sponge effect or pH buffering can be induced by the protonation of entrapped molecules in the acidic pH medium and produces an important inflow of water and ions as  $\text{Cl}^-$  and  $\text{H}^+$ , resulting in an osmotic swelling allowing the endosomal membrane rupture [34]. As PEG coating at the surface of delivery systems is known to limit the cellular uptake as well as the endosomal escape, new delivery systems have been developed with the view to break down the PEG linker in endosomal vesicle [34]. pH-sensitive linkers have been used in order to loss voluntary PEG chains and highlight the positive charges of polymers or lipids which can promote this proton sponge effect. Effective cytoplasmic localization was demonstrated by Wang et al., with nanoparticles containing N-(1-aminoethyl)iminobis [N-(olei-cylcysteinylhistinyl-1-aminoethyl)propionamide] (EHCO) [30]. This polymerizable surfactant EHCO is a pH-sensitive amphiphilic molecule and its use induced a high endosomal membrane disruption [35]. Poly-L-lysine nanocarriers have also been modified to ameliorate their endosomal escape capacity thanks to PEG molecules coupled with cholic acid and a pH sensitive benzoic imine linker. In acidic conditions, cleavage of this linker was induced. In this way, toxic positive charges of poly-L-lysine became directly in contact with endosomal membrane and conducted to efficient disruption and the release of siRNA into the cytoplasm [25]. PEG-b-poly(propyl methacrylate-co-methacrylic acid) (PEG-b-P(PrMA-co-MAA)) polymers associated with PAMAM dendrimer also proved their capacity to avoid the lysosome destruction, 3 h after incubation [36]. The pH-responsive element was due to protonation of carboxylate groups of MAA and induced the proton sponge effect [37]. This protonation also caused the displacement of PEG-b-P(PrMA-co-MAA) from the dendrimer complex involving a second mechanism of membrane destabilization. Indeed, cationic lipids, polymers or peptides can fuse with endosomal membrane and perturb the bilayer organization thus causing pore and disruption [38]. The use of DOPE, a zwitterionic co-lipid, usually had led to this phenomenon. As an example, DOTAP/DOPE/cholesterol liposomes induced a cytoplasmic localization of siRNA confirmed by confocal microscopy analysis [39]. Association of DOPE with DC-cholesterol also conducted to an efficient endosomal escape [40]. Intracellular fluorescence was associated to siRNA internalization of DC-cholesterol/DOPE immunoliposomes, 2 h after incubation on breast cancer cells and resulted in 95% of mRNA extinction [10]. Otherwise, cholesterol derivate, BHEM-Chol, also induced perturbation into cell/organelle membranes [41] and its use in polymer nanoparticles led to cytoplasmic accumulation and no colocalization with lysosomes evidenced by microscopy analysis [18]. Polymers as PAsp(DET) provided an excellent ability of endosomal destabilization through the conformational change of flanking 1,2-diaminoethane pH-sensitive moiety [42]. Indeed, calcium phosphate nanoparticles surrounded by PAsp(DET) efficiently escaped the endosomal vesicles after 3 h and showed 80% of *in vitro* mRNA extinction [12]. Finally, Varkouhi and coworkers highlighted the potential of peptides in the endosomal escape mechanism. Fusiogenic peptides penetrated into phospholipid bilayers and created a pore provoking swelling choc [33]. Only 2 h after incubation, sh-GALA peptides combined to liposomes were localized in cytoplasm

and confirmed their capacity to fuse with endosomal membrane and disrupt them [26]. *In vitro* mRNA extinction evaluated at 85% was induced after 24 h incubation with this sh-GALA liposomes [26]. Moreover, siRNA-peptide complexes developed by Canine et al., showed a perinuclear localization, demonstrating once again their efficient endosomal escape and *in vitro* protein extinction evaluated at 95% [43].

## 2.2. SiRNA protection

### 2.2.1. Enzymatic protection

To deliver intravenously an intact and efficient siRNA to the targeted cells *in vivo*, the protection of siRNA against plasmatic enzymes as nucleases is the first necessary step to overcome after systemic administration [44]. Naked siRNA is naturally completely degraded in 30 min into the bloodstream after recognition by plasmatic enzymes containing a double-stranded RNA binding domain [45]. To evaluate this degradation *in vitro*, incubations with serum or RNase A at physiological temperature have been commonly realized as an indicator of the protection level of siRNA and *in vivo* evaluation of siRNA protection was realized by half-life study after intravenous injections into rodents (Table 2).

PEI complexes based on PEI alone or on PEI coated with human serum albumin (HSA) or atelocollagen resulted in *in vitro* and *in vivo* protection evaluated at less than 1 h [20,46–48]. The commercial form of PEI (JetPEI) provided the best protection with 2 h of siRNA detection *in vitro* [49]. In fact, *in vitro* protein inhibition evaluated at 85% and 50% was provided by lipid substituted PEI complexes and PEI-F25 LMW [28,50] and atelocollagen complex with more than 95% [14,48]. However, *in vivo* analysis highlighted the difficulty of PEI complex to protect and carry siRNA with low transfection efficacy estimated less than 50% and similar results were found with atelocollagen complex [14,48]. An *in vitro* longer protection, up to 6 h, could be obtained with PEI when it was associated with glycol chitosan and produced 80% of mRNA extinction [19]. Interestingly,

**Table 2**  
Protection of siRNA by delivery systems.

Type	Delivery system	<i>In vitro</i> protection (h)	<i>In vivo</i> protection (h)	Refs.
Complex	Atelocollagen	1	No data	[48]
	PEI	<1	No data	[46,47]
	PEI/HSA	<1	No data	[20]
	JetPEI®	2	No data	[49]
	Glycol chitosan/PEI nanoparticle	6	No data	[19]
	MPG-8 Peptide based delivery	No data	5	[51]
Micelle	Polyion complex micelle (PCIM)	4	No data	[36]
	PLL nanocarrier	48	No data	[25]
	Micelleplex	No data	12	[28]
	Multifunctional micellar nanocarriers	No data	24	[23]
Liposome	Her2-PEG-immunoliposome	9	No data	[10]
	Multifunctional nano-device (MEND)	24	No data	[26]
	Lipidic particle	No data	>2	[7,54]
	Pegylated cationic liposome	No data	>9	[55]
	Wrapped liposome	>3	>6	[52]
	Nanoparticle system	24	24	[53]
Nanoparticle	Cyclodextrin nanoparticle	4	No data	[56]
	Dendrimer	PPI dendrimer	48	72

PEI: polyethyleneimine; HSA: Human serum albumin; PLL: Poly-L-Lysine; PPI: poly(propyleneimine).

peptide-based delivery with MPG-8 peptide was tested in mice and biofluorescence monitoring (BFI) of siRNA revealed a strong detection until 5 h after intravenous injection [51].

The micellar organization is in majority composed by hydrophobic block core and hydrophilic block extensions with positive charges siRNA can react with, being absorbed at this interface (Fig. 2). *In vitro* minimal time of protection induced by micellar systems estimated at 4 h, was obtained by polyion complex micelles (PCIM) and a maximal time, around 48 h, was reported by Guo et al., with PEG-SS-poly-L-lysine-cholic acid (PEG-SS-PLL-CA) nanocarriers [25,36]. Furthermore, *in vivo* validation was realized with two other micellar formulations. Micelleplex and multi-functional micellar nanocarriers demonstrated a long protection time of siRNA, respectively evaluated at 12 h and 24 h [23,28]. This long protection time allows an effective extinction *in vitro* and *in vivo*. In fact, dendrimers developed by Felber et al., inhibited 60% of the specific mRNA and 50% of protein *in vitro* [36,47] and analysis after intravenous injection into mice performed with siRNA poly-L-lysine micelles induced 60% of mRNA inhibition in tumor bearing mice [25].

Liposomes generally entrap siRNA into their aqueous core surrounded by a lipid bilayer shell (Fig. 2). In this case, degradation of siRNA depends on the delivery system stability into the bloodstream. Large variations have been observed between different liposome formulations. *In vitro* analyses showed a protection of 3 h and 9 h, respectively for wrapped liposomes [52], and multi-functional nano-devices (MEND) [26], and maximal time of 24 h have been obtained in some cases [10,53]. Moreover, with immunoliposomes, intravenous injections into mice were performed and blood analyses showed siRNA presence for at least 2 h and until 24 h. Lipidic particles [7,54], wrapped liposomes [52] and pegylated cationic liposomes [55] allowed an *in vivo* protection until 2 h, 6 h and 9 h respectively. Moreover, this protection is illustrated by extinction efficacy obtained with lipidic particle with extinction of mRNA at 70% *in vitro* and 60% *in vivo* [7]. As for *in vitro*, nanoparticle systems developed by Yagi et al., provided the longest protection time (24 h) [53]. Then, the first siRNA delivery system used in clinical test, i.e. cyclodextrin-containing polycations encapsulating siRNA, showed 4 h of protection after *in vitro* incubation with RNase A [56]. Finally, poly(propyleneimine) [57] dendrimers of siRNA were developed by Taratula et al. [58]. This system presented the best protection times *in vitro* (48 h) with more than 70% of mRNA extinction and *in vivo* (72 h).

### 2.2.2. Protection from immunity and natural clearance

Innate immune system is naturally developed to detect and degrade extrinsic unknown objects as foreign delivery systems. Specific organs as liver, spleen and kidneys play a crucial role in

their pharmacokinetic behavior as part of the reticulo-endothelial system (Fig. 3). In fact, the mononuclear phagocyte system (MPS) mainly situated in liver, represents a monocyte/macrophage rich zone that promotes filtration, capture and destruction of blood components as cellular fragments, bacteria, aggregates and also nano-objects [59]. Moreover, kidney by renal clearance mechanism controls the elimination of these delivery systems [59]. Important clearance appears when system diameter is less than 25 nm [60] (Fig. 3). Delivery systems whose size is comprised between 50 and 250 nm, can largely limit their degradation or elimination by these different mechanisms and increase their half-life in bloodstream. To illustrate this, a large majority of nanocarriers already developed for *in vivo* applications have their size comprised in this interval (Table 3).

The surface charge of the delivery systems is also a critical parameter to consider in order to obtain a suitable pharmacokinetic profile. Indeed, plasmatic proteins are negatively charged and can consequently form some aggregates with positive charges. These large complexes can be recognized by innate immune system (complement proteins or macrophages). The bricks of the complement are able to interact with plane surface and favor macrophage recruitment [60,61]. The endocytosis by macrophages can lead to the destruction of the delivery systems and thus siRNA. By presenting neutral or slightly negative charge at the surface, complement fixation and recognition by MPS can be avoided or largely limited (Fig. 3). To illustrate this, nanoparticles can present neutral or negative global charge, as mesoporous nanoparticles developed by Tanaka et al., or lipoprotein nanoparticles of Shahzad et al., (both with  $-3$  mV) and LNCs with  $+7$  mV of surface charge [13,15,57]. For other nanoparticles cited in Table 3, PEG has been added at the surface to create artificially a negative hydrophilic and flexible ring which can hide and limit the effect of positive charges of cationic lipids and/or polymers used for the complexation of siRNA. Indeed, PEG is able to modify zeta potential of nanoparticles thanks to dipole interactions with water [62]. In fact nanoparticles developed respectively by Yang et al., and Susa et al., exhibited negative or neutral global charge thanks to this PEG recovery [16,18]. PEI polymer provided the similar effect and led, for example, to a negative charge of  $-21$  mV for solid hybrid PEI nanoparticles [14].

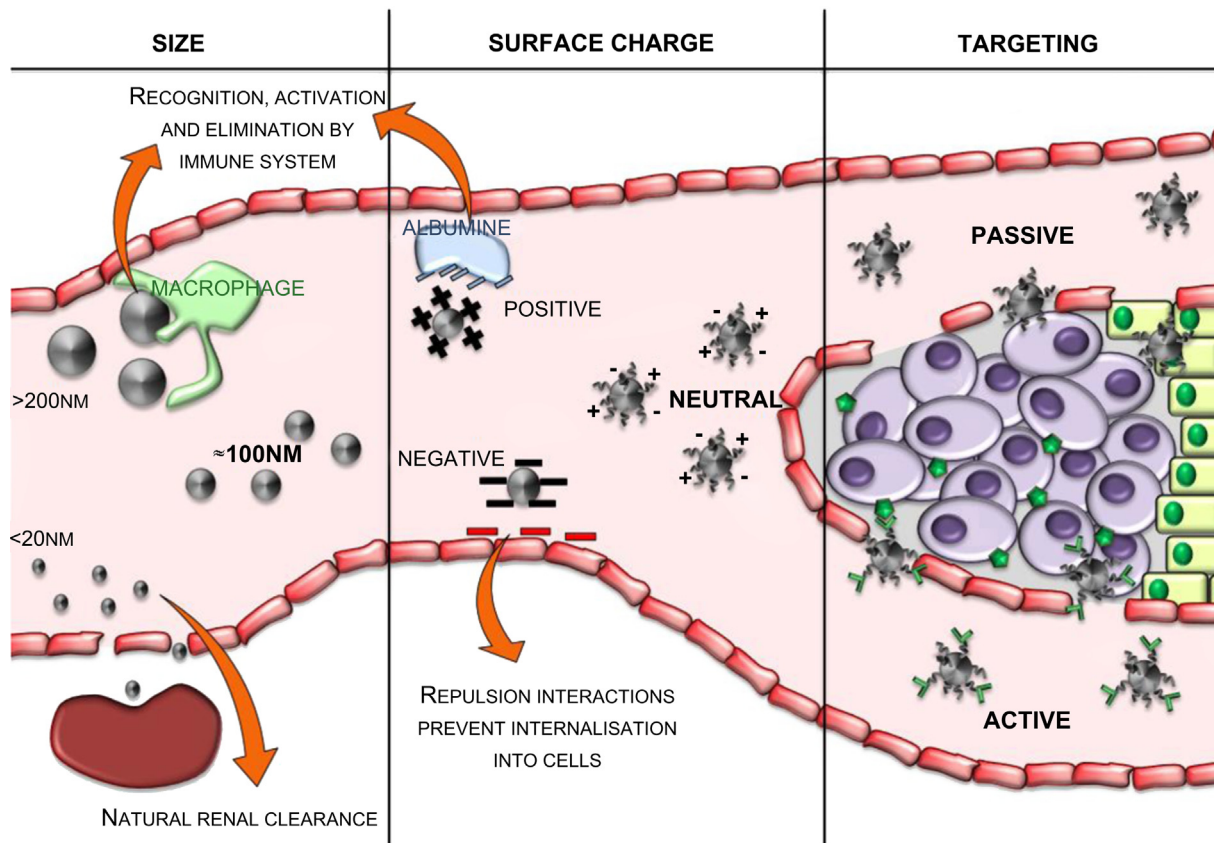
For micelles, copolymers like poly(DMAEMA) and PEO-b-PCL induce generally a neutral profile [21,23]. Furthermore, micelles can resort to pegylation in order to neutralize positive charge of block copolymers. For example, PCIM with PEG ring, were characterized by neutral zeta potential evaluated at  $+5$  mV [36], and micelleplex developed by Mao et al., had largely negative global charge with a zeta potential of  $-21$  mV [63].

Concerning peptide complexes, they present generally a slight positive charge as observed for peptide-based nanoparticles, and FDT nanocarriers with respectively  $+16$  and  $+10$  mV [43,51]. Same results were obtained with glycol chitosan/PEI complexes ( $+10$  mV) [19] and other polymer complexes based on oligoethyleneimine derivatives (OEI-HD) ( $-6$  mV) [29].

In the case of liposomes, pegylation is a necessary step to hide the high level of positive charges due to cationic headgroups. Even with PEG recovery, zeta potentials can stay highly positive as it is the case for liposomes of polycation hyaluronic acid (LPH) [64], liposomes polycation DNA (LPD) [8,65], lipidic particles [7] and pegylated cationic liposomes [55] characterized by a zeta potential comprised between  $+11$  and  $+48$  mV (Table 3). Despite these positive charges, lipidic particles and pegylated cationic liposomes developed respectively by Wu et al., and Sonoke et al., allowed an *in vivo* long time circulation and protection of siRNA explained by an efficient steric repulsion due to the high flexibility and hydrophilicity of PEG [62].



**Fig. 2.** Methods of siRNA complexation with delivery systems. The siRNA localization into nanocarriers differs according to structure and organization of the delivery system. siRNA can be incorporated into matrix structure and allows a global repartition in all volume of sphere (left). Second strategy consists to adsorb siRNA on surface of delivery systems in low depth (middle scheme). Last organization concerns shell/core structure observed for liposomes and some nanoparticles. In this case (right), siRNA is restrained in the core compartment of the delivery system.



**Fig. 3.** Optimal physicochemical parameters to bypass the hurdle of intravenous administration and obtain a tumor targeting. Physico-chemical parameters and surface modifications are crucial to obtain a suitable profile into blood and an efficient tumor targeting. Particle of size inferior to 20 nm and superior to 200 nm is rapidly eliminated by respectively renal clearance and innate immune system (MPS). Intermediate size particles with positive charge surface are also rapidly recognized by the immune system and eliminated. Negative surface allows a long circulating profile but repulsion interactions between them and cell membrane prevents their internalization and the efficient delivery of siRNA into the cytoplasm. Nanocarriers with neutral surface are used to develop passive and active targeting. Enhanced permeability and retention (EPR) effect thanks to leaky vasculature allows a passive targeting of tumor site. Finally, active strategy with specific ligand induces the internalization of delivery systems into cancer cells.

### 2.3. Tumor targeting

#### 2.3.1. Passive targeting

The advantage of delivery systems is to favor cellular uptake and target only tumor site and tumoral cells, in order to limit side effects and improve the therapeutic efficacy. Large fenestrations have been described on vessels situated around the tumor and they are associated with poor lymphatic drainage favoring the retention [66]. All of this provokes the Enhanced Permeability and Retention (EPR) effect demonstrated by Maeda et al., and leads to the development of passive targeting strategy consisting in natural accumulation in tumor site by long circulating or stealth delivery systems [66] (Fig. 3).

Numerous siRNA liposomes recently demonstrated this tumoral targeting. Neutral non-pegylated mesoporous silicon nanoparticles developed by Tanaka et al., and liposomes developed by Halder et al., have obtained a high tumor protein extinction superior at 80% after systemic injection into mice and consequently proved the accumulation of delivery system into tumor cells [57,67]. Passive accumulation was also achieved after single intravenous injection of fluorescent siRNA into pegylated LPD nanoparticles [68]. Cervical mouse cancer was successfully passively targeted by pegylated siRNA loaded lipid particles [7] and *in vivo* analysis highlighted an effective extinction estimated at more than 95% [65,69]. Recently, novel pegylated cationic liposomes proved their efficacy to target pulmonary metastatic melanoma [9]. Moreover, synthetic polymers as PEI complex used by Grzelinski et al., allowed an efficient

passive targeting of glioblastoma xenograft [46]. Then, new polymer, EHCO complexed with siRNA, was injected intravenously and demonstrated tumor site accumulation with 65% of protein extinction [29,30]. Micelleplex formed with triblock copolymer promoted breast cancer accumulation 4 h after intravenous injection [28]. Finally, Alexa555 siRNA lipoprotein nanoparticles were localized in ovarian tumor 48 h after a single intravenous administration [15].

Nonetheless, this strategy presents some limitations. EPR effect allows extravasation through inflammatory endothelium that possesses large fenestrations but does not lead to an exclusive cancerous cell targeting. Moreover, differential degree of vasculature, angiogenesis as well as tumor type, maturity and host environment, can play an important role in this EPR effect. Consequently, active targeting could be a way to enhance delivery system accumulation in desired tumor site and to help internalization into tumor cells.

#### 2.3.2. Active targeting

In parallel to a passive clustering, an active targeting is more and more envisaged to enhance the preferential tumor accumulation and avoid side toxic effect. This can be obtained by grafting at the surface of the delivery system markers or ligands only expressed or at least overexpressed in tumors (tumoral environment or tumoral cells) (Fig. 3). For this purpose, chemical modifications on polymers have allowed the fixation of many families of molecules as peptides, antibodies and/or glucides (see Ref. [6]).

**Table 3**  
Physico-chemical characteristics of siRNA delivery systems.

Type	Delivery system	Diameter [117]	Zeta potential (mV)	Refs.
Nanoparticle	Mesoporous silicon particle	26	-3	[57]
	Lipoprotein nanoparticle	10	-3	[15]
	Lipid nanocapsule (LNC)	60	-7	[13]
	Dextran nanoparticle	105	-0.2	[16]
	PEG-PLA nanoparticle	196	-17	[18]
	Lipid - PEI hybrid nanocarrier (LPN)	210	-21	[14]
Micelle	pH-responsive micelle	35	1	[21]
	Multifunctional micellar nanocarrier	103.4	4	[23]
	Polyion complex micelle (PCIM)	50-100	5	[36]
	Micelleplex	47.7	-21	[63]
Complexe	Peptide based nanoparticle	100-150	16	[51]
	FDT	78	10	[43]
	Glycol chitosan/PEI nanoparticle	350	10	[19]
	Polyplexes	155 ± 3	-6	[29]
	PPI dendrimer	101 ± 43	No data	[58]
Liposome	Liposome polycation	170	11	[64]
	hyaluronic acid (LPH)			
	Liposome polycation DNA (LPD)	114	25	[8]
	Liposome polycation DNA (LPD) + NGR	197	31	[65]
	Lipidic particle	179	41	[7]
	Pegylated cationic liposome	100	48	[55]

PEG-PLA: poly(ethylene-glycol)-b-poly(D,L-lactide); PEI: polyethyleneimine; FDT: fusogenic peptide - DNA condensing and endosomolytic motif - targeting motif; PPI: poly(propyleneimine).

Transferrin delivery system has demonstrated their efficacy to target tumor cell as transferring conjugated OEI-HD polyplexes with 85% of protein extinction in tumor cell after intravenous injection [29,30] and transferrin cyclodextrin-based nanoparticles with *in vivo* protein inhibition on murine model with a decrease estimated at 55% [70].

Tumor homing peptides are able to react with different receptors as, for example, integrin receptors with RGD peptide. In fact, Arg-Gly-Asp (RGD) motif is implicated in the fixation of alpha V integrin proteins on their receptor overexpressed on tumoral vessels [71]. Recently, two micelle formulations modified with RGD showed an efficient *in vivo* tumoral targeting in breast and ovarian cancer cells [23,31]. In fact, RGD chitosan nanoparticles showed *in vivo* mRNA and protein extinction respectively at 80% and 51% [31]. Moreover, *in vivo* experiments were also performed with RGD liposome delivery systems and conducted to a half-inhibition of protein expression in breast cancer cell bearing mice [39]. Another example has been prepared with the Asn-Gly-Arg (NGR) motif, discovered by phage display technology, that is able to interact with aminopeptidase N receptor (or CD13) [72]. The expression of this receptor was correlated with cancerous angiogenic property and cell mobility [73]. NGR liposomes were used by Chen et al., to target actively tumor vessels in sarcoma model and resulted in an efficient tumoral accumulation [65]. Ala-Pro-Arg-Pro-Gly (APRPG) peptide targets angiogenesis vessels as RGD and NGR [74,75] and was efficient on the surface of liposome formulation to induce an active *in vivo* tumor accumulation in colon tumor bearing mice [76]. Otherwise, peptide sequence isolated from releasing protein as hormone can be used to target specific cells or organs. For example, luteinizing hormone-releasing hormone (LHRH) plays an important role in multiple malignancies as cancer [77]. LHRH receptor has been described as a potential target for various cancers as hormone-dependent one [78]. A synthetic analog peptide was conjugated to siRNA

dendrimers and provided an efficient and selective tumoral accumulation in ovarian cancer model [58].

In parallel, other molecules including for example polyunsaturated fatty acids, folic or, hyaluronic acids, can be used for active targeting [79]. Hyaluronic acid participates in cell growth, differentiation and migration [80] and is overexpressed in various cancers [81,82]. Chen et al., used a strategy based on hyaluronic acid grafted on pegylated liposomes and showed a preferential tumor localisation in melanoma bearing mice [64].

Antibodies have also been used to target specific cell receptors. Transferrin plays a major role in cell growth and its expression has been correlated with cancer progression. Antibodies of transferrin receptors have been largely used to target actively cancer cells [83,84]. For example, Pirollo et al., grafted an anti-transferrin antibody fragment on liposome to induce an active targeting of prostate and/or pancreatic model, observed as soon as 20 min after intravenous injection [85].

### 3. Current targets of siRNA in cancer therapy development

Good candidates for gene silencing in cancer therapy are legion when considering *in vitro* demonstration and/or use that are associated with anti-cancer effects. Few of them are nevertheless in the pipelines of pharmaceutical companies. General concerns about the targets to be considered for further drug development could be 1) differential expression level between cancer vs non-cancerous cells (generally overexpression in cancer cells; cancer type specificity should thus be considered as well as personalized medicine), 2) genetic concerns, e.g. highlighting the target to be associated with spontaneous cancer development in animal models and viability of the knock out models to decrease risk of toxicity (targeted delivery systems can be used to overcome, at least partly this problem), 3) characterized and crucial roles in cancer development, progression, resistance..., 4) multiple functionalities and/or localizations (in particular intracellular proteins) that cannot be inhibited by chemicals or reached by other therapeutic tools such as antibodies, 5) cellular half-life: protein with very rapid turn-over will increase the difficulty to develop a vector that can be administered in a practical point of view to obtain appropriate knock down timelines.

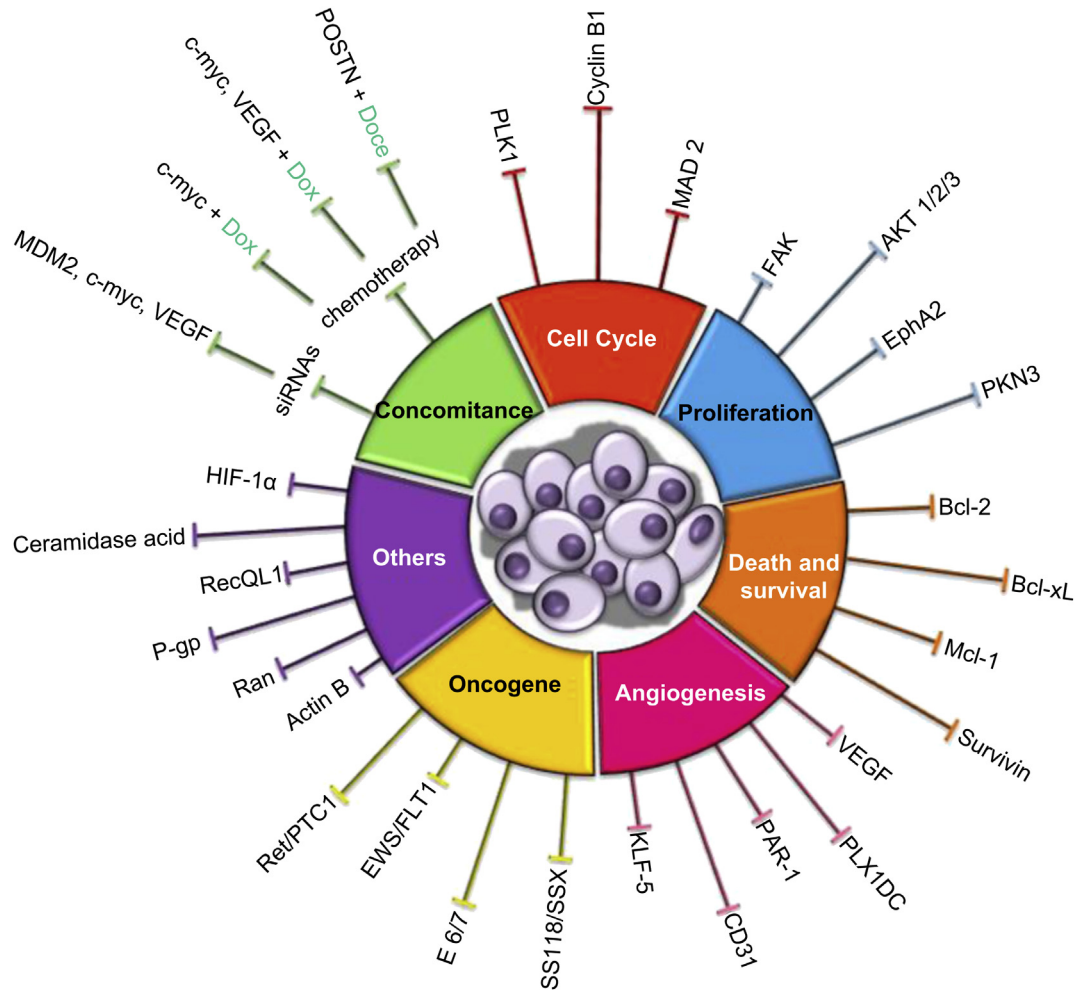
In this section we considered the different targets/pathways for which *in vivo* animal studies have been conducted successfully (Fig. 4). Table 4 summarizes these studies with detailed formulation, protocol and results.

#### 3.1. Cell cycle

Cell cycle progression is strictly controlled as demonstrated in 1989 by Hartwell and Weinert by numerous effectors and checkpoints [86]. Main effectors of cell cycle progression are cyclins and cyclin-dependant kinases (cdk) such as cyclin B1-cdk1 complex which is the main effector implicated in G2/M transition [87]. Numerous studies have proven the overexpression of cyclin B1 in various cancers as oesophageal squamous cancer [88], non-small lung cancer [89], renal cancer [90] and prostate adenocarcinoma and was recently associated with a poor survival prognosis in breast cancer [91]. Cyclin B1 siRNA inhibition strategy was employed in prostate and lung cancer *in vivo* [51]. Eight intravenous injections of 10 µg siRNA complexed with peptide-based delivery systems to mice produced a reduction of about 92% of the prostate tumoral volume (Table 4). In a sub-cutaneous lung cancer model, tumor reduction reached 70% and half of the animals treated presented longer survival periods [51].

Polo-like-kinase 1 (Plk1) plays key roles during mitosis notably in the activation of cyclin B1-cdk1 complex and anaphase-





**Fig. 4.** Pathway targeting by siRNA delivery systems in cancer. Representation of siRNA targeted molecules used in preclinical studies to develop an anti-cancer treatment. Dox: doxorubicin; Doce: docetaxel. Plk1: polo-like kinase1; MAD2: mitotic-arrest deficient 2; EphA2: receptor of ephrins; AKT 1/2/3: KLF isoforms of serin–threonin kinase; PKN3: protein kinase N3; FAK: focal adhesion kinase; Bcl: B-cell lymphoma; Mcl-1: myeloid cell leukemia; VEGF: vascular endothelium growth factor; PAR-1: protease activated receptor 1; KLF-5: Kruppel-like factor 5; PLX1DC: plexin domain containing 1; HIF-1 $\alpha$ : hypoxia inducible factor 1 $\alpha$ ; CA: ceramidase acid; MDM2: murine double minute 2; POSTN: periostin.

promoting complex/cyclosome (APC/C) for G2/M transition and spindle assembly checkpoint respectively [92]. Plk1 is overexpressed in multiple cancer types, including melanoma [93], non-small cell lung cancer [94], colorectal cancer [95], and breast cancer [96], with for some of them bad prognosis values. Inhibitions by siRNA or chemical agents validated Plk1 as an interesting target to combat cancer [97,98]. Two recent studies described Plk1 siRNA delivery systems. Sun et al., developed a micelleplex that was injected daily intravenously in sub-cutaneous breast tumor bearing mice from twelve days after tumor implantation to the end of the protocol at a siRNA concentration of 220  $\mu\text{g}/\text{kg}$ . This treatment led to 70% of tumoral volume reduction compared to control group [28]. This protocol also demonstrated the recovery of effective apoptotic pathways with activation of caspase 3. A second study was realized on a breast organotypic tumor model. Treatment with Plk1 siRNA in PEG–PLA nanoparticles led to a 75% tumor growth inhibition after seven intravenous injections of 20  $\mu\text{g}$  siRNA each [18].

Other checkpoint effectors' knock down could be useful to combat cancer, e.g. mitotic-arrest deficient 2 protein (MAD2) which is involved in the regulation of SAC [99]. MAD2, through sequestration of CDC20 into a complex, inhibits APC/C, maintains thereby the sisters' chromatids together till all chromosomes are bi-oriented to each spindle pole on metaphase plate, in order to prevent

mis-segregation [100]. Liver cancer [101], lymphoma [102], lung [103], colon carcinoma [104], express high levels of MAD2. This up-regulation of this SAC regulator is associated with mitotic delay and chromosome instability that finally contributes to hallmarks of cancer as aneuploidy and genetic instability [10]. Knock down of MAD2 led to pro-apoptotic effects in osteosarcoma [105]. Kaestner et al., administered PEI/anti MAD2 siRNA complex intravenously MAD2 siRNA to sub-cutaneous colon carcinoma bearing mice. Injections of 10  $\mu\text{g}$  of siRNA every two days during 14 days inhibited tumor growth of 35% [69].

### 3.2. Proliferation pathways

Cancer cells are characterized by sustained proliferation resulting from increased proliferative signals, decrease of negative feed-back signals and replicative immortality acquisition [10]. Cancer cells produce or force their environment to produce excessive growth factors as for example insulin growth factor (IGF), or epidermal growth factor (EGF) [106] but can become independent of these signals with mutations. Such external stimuli activate intracellular cascades like the MAPK pathways or the phosphoinositide-3-kinase (PI3K)/serin–threonin kinase AKT one, that promotes proliferation, division and survival and were clearly described in cancerogenesis mechanism [107]. EphA2 is a specific

**Table 4**

Protocol based on siRNA delivery in cancer therapy.

Pathway	siRNA target	Cancer model	Tumoral cell injection	Total dose (µg)	Number of injections	Day of injection	Inhibition of tumor volume	Other effects	Refs.		
Cell cycle	Cyclin B	Prostate	s.c	80	8	D0–21	70%–92%	50% long survival	[51]		
	Plk1	Breast	s.c	141	32	D12–44	70%	Caspase 3 activation	[28]		
Proliferation	MAD2	Colon	Orthotopic	140	7	D14–21	75%	/	[18]		
			s.c	70	7	D0–14	35%	Increased apoptosis	[69]		
	EphA2	Ovarian	i.p	5	1	D15	55%	/	[57]		
	AKT1	Prostate	s.c	600	6	D0–15	66%	/	[114]		
	AKT2	Prostate	s.c	600	7	D0–16	89%	/	[114]		
	AKT3	Prostate	s.c	600	8	D0–17	57%	/	[114]		
	PKN3	Prostate	Orthotopic	224	4	D1–4	60%	75% for metastasis	[117]		
Cell death and Survival	FAK	Ovarian	i.p	24–30	8–10	D7–31 or 38	60%	/	[31]		
	Bcl-2	Prostate	s.c	2000	10	D10–21	65%	/	[55]		
	Bcl-xL	Prostate	s.c	1200	12	D1–24	40%	No inflammatory response	[48]		
Angiogenesis	Mcl-1	Breast	s.c	140	7	D9–19	75%	Maximal dose tested 53 mg/kg = no toxicity	[27]		
	Survivin	Prostate	s.c	14	2	D2 and D7	30%	/	[14]		
		Prostate <sup>a</sup>	s.c	7000	7	50 mm – D0–18	53%	No inflammatory response	[25]		
	VEGF	Prostate	s.c	560	5	D0–28	87%	No inflammatory response	[144]		
		Melanoma	s.c	80	8	3–5 mm – D0–D28	77%	/	[168]		
	Oncogene protein	PAR-1	Melanoma meta	i.v	100	10	D0–35	/	Decreased metastase number (81%)	[168]	
			Prostate	Orthotopic	350	8	D35–49	65%	/	[117]	
		KLF-5	Lung carcinoma <sup>a</sup>	s.c	350	7	D2–8	60%	/	[53]	
		PLX1DC	Prostate	s.c	1500	10	D7–16	48%	Prolonged survival median	[53]	
	Other pathways	E6/7	Ovarian	i.p	24–30	8–10	D7–31 or 38	87%	Increased apoptose (35%),	[31]	
Renal <sup>a</sup>			s.c	120	3	D3–10	55%	No synergic effect with cisplatin	[7]		
Hypoxia		Metabolism	Ret/PTC1	Fibroblast	s.c	100	5	D0–9	90%	/	[17]
			EWS-FLI1	Ewing sarcoma	i.v	400	8	D1–24	80%	No inflammatory response	[70]
DNA repair		RecQL1	Synovial sarcoma	s.c	1000	10	D3–28	83%	/	[52]	
			Glioblastoma	s.c	300	6	D21–36	50%	/	[30]	
Nuclear transport	CA	BT474	s.c	240	12	D10–22	75%	Increased apoptosis, non-immunogenic	[63]		
		Colorectal meta	Spleen	500	10	D4–25	43%	/	[154]		
	Pancreatic meta	Spleen	250	10	D4–15	31%	/	[154]			
	Lung	s.c	400	8	D8–32	46%	/	[154]			
SiRNA concomitance	Resistance Migration	Neuroblastoma <sup>a</sup>	s.c	150	3	D7–13	49%	Increased apoptotic cell (12%), no hepatic toxicity	[29]		
		Breast <sup>a</sup>	s.c	160	4	D11–17	60%	/	[39]		
	c-myc, VEGF	HT-1080	s.c	320	4	D11–D14	65%	No inflammatory response	[26]		
		Melanoma <sup>a</sup>	i.v	18	2	D8 and 9	30%	No inflammatory response	[64]		
		Melanoma <sup>a</sup>	i.v	36	4	D10–18	/	Increased survival median + 6D, no inflammatory response	[8]		
Chemotherapy Concomitance	Doxorubicin	c-myc	Colon	s.c	72	3	D1–3	60%	Increased apoptosis	[65]	
		c-myc, VEGF	Ovarian	s.c	72	3	D1–3	57–60%	Increased apoptosis, no inflammatory response	[68]	
	Docetaxel	POSTN	Ovarian	i.p	24–30	8–10	D7–31 or 38	90%	Increased apoptose (45%), decreased proliferation (60%)	[31]	

Plk1: Polo-like kinase1; MAD2: mitotic-arrest deficient 2; EphA2: receptor of ephrins; AKT 1/2/3: KLF isoforms of serin–threonin kinase; PKN3: protein kinase N3; FAK: focal adhesion kinase; Bcl: B-cell lymphoma; Mcl-1: myeloid cell leukemia; VEGF: vascular endothelium growth factor; PAR-1: protease activated receptor 1; KLF-5: Kruppel-like factor 5; PLX1DC: plexin domain containing 1; HIF-1a: hypoxia inducible factor 1a; CA: ceramidase acid; MDM2: murine double minute 2; POSTN: periostin.

<sup>a</sup> Murine cell model, s.c: sub-cutaneous injection, i.p: intraperitoneal injection, i.v: intravenous injection.

receptor of ephrins that takes function in cell–cell contact and cell migration by activation of MAPK pathways promoting oncogenesis and increased expression has been detected in numerous cancers [108]. Inhibitions by RNAi technology proved the anti-cancer potential of *in vitro* targeting and local *in vivo* treatment respectively in malignant mesothelioma and squamous carcinoma [109,110]. One study exposed some results on intravenous injection of mineral nanoparticles loaded with EphA2 siRNA [57]. A single injection, two weeks after cell inoculation, was sufficient to lead to a 55% decrease of tumoral volume.

Numerous cancers, as for example skin cancer [111] and prostate cancer [112] have shown dysregulation of PI3K/AKT pathways. Many reviews have described oncogenic overexpression of AKT and its roles in chemoresistance [113]. siRNA knock down of three different AKT isoforms was explored by Sasaki et al., in prostate cancer model [114]. Atelocollagen was mixed with siRNA and injected every three days during two weeks by intravenous injections. All AKT isoforms siRNA conducted to an important regression of tumoral volume, i.e. 66%, 89% and 57% for respectively AKT1, AKT2 and AKT3 isoforms [114].

Then, the PKN3 protein, named also PKN $\beta$ , has been identified a few years ago [115]. Leenders et al., demonstrated their interaction with PI3K/AKT pathways and their antiproliferative effect on prostate cell by shRNA extinction [116]. *In vivo* inhibition strategy was realized with siRNA liposomal form [117]. Repetitive intravenous injections were realized on mice bearing organotypic prostate tumor and provided 60% of tumoral volume inhibition. Moreover, metastatic process was highly inhibited with 75% of metastasis number reduction [117].

Increased focal adhesion kinase [57] levels were reported in prostate [118], and breast [119] as well as in glioma [120]. FAK can be activated by integrins and is involved in multiple pathways controlling proliferation, survival, cell migration and anoikis resistance [121]. Its overexpression has been correlated with increased invasion and migration of several metastatic tumor types [122]. Inhibition of FAK protein has been obtained by different strategies: competitive molecule (FIP200); tyrosine kinase inhibitors (TKI) (PF-228) or RNAi (siRNA) [123]. Chemical inhibitors led to the regression of tumoral volume as demonstrated by Roberts et al. [124]. Delivery systems based on chitosan nanoparticles loaded with FAK siRNA, were developed for systemic treatment [31]. Twice weekly injections of 4  $\mu$ g siRNA allowed inhibiting 60% of human ovarian tumor growth in nude mice.

### 3.3. Cell death and survival

Pathways involved in cell death and survival have important role in cancer development [125]. Cancer cells display acquired or innate cell death resistance mechanisms (to apoptosis or other cell death pathways). Apoptosis regulation pathways form a very complex molecular network with the two intrinsic and extrinsic classically recognized triggering pathways [126]. Bcl-2 family proteins are major molecular regulators of mitochondrial pathway leading to apoptosis and clearly related with cancer progression [127]. Anti-apoptotic bcl-2 family members as Bcl-2, Bcl-xL and Mcl-1, have been shown to be overexpressed in cancer pathologies including leukemia [128] and myeloma [129]. Then, poor survival was also associated with these Bcl-2 members' up-regulation in hepatocarcinoma [130]. Moreover, chemotherapy resistance was early associated with Bcl-2 and Bcl-xL high level expression, respectively in gastric and breast cancer [131,132]. Intravenous injections of vectorized siRNA targeting these three molecules were evaluated in tumor bearing mice: Bcl-2 and Mcl-1 siRNA in pegylated cationic liposomes [27,55], and Bcl-xL siRNA complexed with atelocollagen [48]. In these studies, reduction of tumor growth reached 40%–65%. Abnormal expression or overexpression of inhibitor of apoptosis proteins (IAPs) as survivin that has been correlated with oncogenic progression was reported in hepatocellular carcinoma [133], lung cancer [134], and ovarian cancer [135]. In addition, numerous studies have evidenced higher survivin expression during metastasis development, such as in prostate cancer [136] and in poor prognosis in meningioma [137]. Different survivin inhibition strategies resulted in marked antiproliferative effects in endometrial and breast animal cancer models [138,139]. Solid lipid–PEI nanocarriers were developed by Xue et al., to carry survivin siRNA [14]. A low dose of siRNA, only 14  $\mu$ g delivered in twice injections, was sufficient to obtain 30% of tumoral volume regression.

### 3.4. Angiogenesis

Angiogenesis is an essential step for cancer development and has thus been recognized as a hallmark of cancers [125]. Without angiogenesis, cancer could not grow up to more than 2 mm of diameter as firstly emphasized by J Folkman. In this way, stopping neoangiogenic mechanism could constitute a key strategy to inhibit

tumor growth [140]. However, numerous inhibitors have already been developed to slow down angiogenesis such as antibodies and TKI (sunitinib<sup>®</sup>, sorafenib<sup>®</sup>, axitinib<sup>®</sup>, and pazopanib<sup>®</sup>) but with appearance of resistance siRNA could therefore provide an alternative strategy [141,142]. Vascular endothelium growth factors (VEGFs and VEGFA in particular) are the most described secreted molecules implicated in angiogenesis of human cancers [143]. Poly-L-lysine nanocarriers of VEGF siRNA administered intravenously reduced by 53% prostate sub-cutaneous tumors [25] while VEGF siRNA micelles led to 87% volume reduction [144].

As controversies on the benefits of such anti-angiogenic therapies arose recently, other angiogenic factors or markers could be interesting alternatives like protease activated receptor 1 (PAR-1) which is enrolled in thrombin regulation and whose expression is correlated with angiogenesis in many cancer types [145] or platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) [146]. Both have been targeted *in vivo* by siRNA in melanoma and prostate cancer models respectively. Reduction in tumor size and metastases has been obtained [147]. Finally, plexin domain containing 1 (PLXDC1) or TEM7 (tumor endothelial marker 7) is a protein showing an overexpression in cancerous endothelium [148] and a correlation with metastasis and poor prognosis [149]. Chitosan nanoparticles were used to carry PLXDC1 siRNA in orthotopic mice ovarian cancer model [31]. Low final dose of siRNA (maximal 30  $\mu$ g) injected in 10 times was sufficient to produce a strong blockage of tumoral growth with 87% of volume inhibition.

### 3.5. Other pathways

Hypoxia triggers angiogenesis via several cellular mediators including the well-known HIF-1 $\alpha$  transcription factor. Wang et al., developed multi-functional peptide-based carrier to deliver anti-HIF-1 $\alpha$  RNA that inhibited by 50% glioma tumor growth [30].

Kruppel-like factor 5 (KLF-5) is a transcriptional factor implicated in the epithelial cell proliferation and consequently in angiogenesis [150]. This protein was targeted by siRNA carried by wrapped liposomes in two sub-cutaneous cancerous models [53]. A total siRNA dose of 350  $\mu$ g was injected intravenously in murine lung carcinoma and a 60% of volume reduction was obtained. Prostate model was also assessed in a KLF-5 inhibition therapy [53]. Ten injections of 150  $\mu$ g siRNA were performed and decreased the tumoral volume to 48%. Moreover, this inhibition demonstrated their efficacy to prolong the survival median.

Furthermore, nucleo-cytoplasmic transport for signaling molecules and transcription factors is controlled by protein complexes situated at nucleus pore, including importin  $\alpha$  and importin  $\beta$ . Ran GTPase is also implicated in this transport regulation and can contribute to pathways of cell transformation facilitating tumor progression [151]. Inhibition of Ran was assessed in sub-cutaneous murine neuroblastoma and resulted in 49% of tumoral volume regression with a single low dose of siRNA (150  $\mu$ g) [29].

Considering the hallmarks of cancers as defined by Hanahan and Weinberg, bioenergetic metabolism is attractive for new therapies. Example is provided with the acid ceramidase (AC) that plays a key role in cancer development through the enhanced conversion of ceramide into the anti-apoptotic shingosine 1-phosphate [152]. A breast cancer mice model was developed to evaluate the effect of AC siRNA inhibition [63]. Results showed a strong inhibition with 75% of tumoral volume regression by using 240  $\mu$ g of siRNA.

RecQL1, a human helicase, participates in DNA repair and the maintenance of genome integrity. This protein is highly up-regulated in rapidly growing cells including various kinds of cancers [153]. Pancreatic, lung and colorectal cancer models in mouse have been treated respectively by intravenous injections of 250, 400, and 500  $\mu$ g of RecQL1 siRNA entrapped into non-viral delivery

systems [154]. These protocols induced alone 31%, 43% and 46% of tumor growth inhibition for pancreatic, colorectal and lung cancers. Finally, resistance mechanisms to chemotherapy include the over-expression of ABC efflux pumps such as P-glycoprotein (P-gp) that decrease intracellular chemotherapeutic agents like doxorubicin [155]. Jiang et al., worked on the effect of P-gp siRNA on doxorubicin resistant human breast cancer cells [39]. After treatment with P-gp siRNA liposomes, tumoral volumes were reduced to 63% vs doxorubicin alone (28%).

### 3.6. Virus and fusion oncogenes

As several cancer types can be induced by oncogenes from viruses, specific strategies can be developed to combat them. Examples are E6 and E7 oncogenes of human papilloma virus (HPV) that have been inhibited by siRNA strategy in murine sub-cutaneous renal carcinoma models [7] resulting in 55% of tumoral progression blockage and prolonged survival median in a metastatic model.

Genetic changes as chromosome rearrangement can cause the synthesis of abnormal fusion proteins. These later can turn out to be oncogenic and then favor the proliferation and progression of tumors. The ret/PTC1 fusion protein appears as a specific oncogene rearrangement presented in thyroid cancers [156]. de Martimprey et al., developed a siRNA strategy on ret/PTC1 protein [157]. These chitosan nanoparticles were able to strongly reduce the tumor size with 90% of volume reduction. EWS-FLI1 is another chimeric gene produced by abnormal chromosomal rearrangement, associated with Ewing sarcoma and targeted to develop a new therapeutic treatment [158,159]. The inhibition of EWS-FLI1 by siRNA was conducted by Hu-Lieskovan and coworkers [70]. Cyclodextrin based nanoparticles were injected 8 times until total siRNA dose of 400  $\mu\text{g}$  and proved an effective inhibition of tumoral progression with 80% of tumoral volume reduction. Finally, targeting of SS18–SSX, a fusion protein [160], described in synovial sarcoma by a consistent chromosome translocation (X; 18) was tested by Takenaka et al., in wrapped liposomes with repetitive intravenous injections. Their protocol allowed a strong volume diminution estimated at 83% [52].

## 4. Perspectives

### 4.1. RNAi in clinics: introduction

To date, very few nucleotide-based drugs reached the market. The first one was an antisense oligonucleotide from Isis Pharmaceuticals in 1998 to treat CMV retinitis (Formivirsen; Vitravene<sup>®</sup>) but the company stopped it because of its very limited market. This drug is the first proof that local delivery of RNAi-based treatment can be envisaged. The aptamer Pegaptanib (Macugen<sup>®</sup>) is the second example of nucleotide-based therapy approved by the FDA in 2004 for the acute macular degeneration (AMD) with local administration.

Despite more than 60 clinical trials conducted with antisense oligonucleotides to combat cancer ([clinicaltrials.gov](http://clinicaltrials.gov)) and 589 publications in pubmed database on “oligonucleotide and cancer” in “clinical trials”, (April 2013), no one reached the market. Among the most promising ones, anti-Bcl-2 antisense (Oblimersen<sup>®</sup>) did not get FDA approval after the phase III randomized trial for chronic lymphocytic leukemia [161] because of lack of efficacy in terms of intent-to-treat survival benefits. Great hope has been placed in siRNA that emerges now in clinical trials as detailed hereafter.

### 4.2. Clinical trials

Pubmed references 31 publications related to “siRNA and cancer” in “clinical trials” (April 2013) but only few clinical trials (all in

phase I clinical status) are listed on [clinicaltrials.gov](http://clinicaltrials.gov) for solid tumor treatment. CALAA-01 is an siRNA that reduced the expression of the M2 ribonucleotide reductase subunit (R2) and was shown to inhibit/reduce melanoma tumor growth *in vivo* in mice [162]. The CALAA-01 siRNA was protected from nuclease degradation within stabilized cyclodextrin-based nanoparticles targeted to tumor cells via transferrin [163]. These nanoparticles were injected intravenously in three patients and proved effective against tumor tissues [164]. Nowadays, the Phase I trial is in progress on the safety and the adapted dose of these siRNA nanoparticles.

A second non-viral siRNA delivery system, Atu027, is in its prospective phase of clinical trials. As described before, this nanoscale system has proved its *in vitro* and *in vivo* efficacy [117,147]. Moreover, these liposomes loaded with a PKN3 siRNA were injected intravenously for 4 h in a single dose in 24 human patients. The first results showed the good tolerance to Atu027 by patients [165]. Further studies will be conducted to determine the siRNA efficacy in terms of anti-cancer effects.

Phase I trial with a liposome-carried EphA2 siRNA associated with promising results in mice [57] has just started recruitment in summer 2012. A dose of 450  $\mu\text{g}/\text{m}^2$  of siRNA into nanomedicines will be injected intravenously twice weekly during 3 week cycles in patients diagnosed with ovarian cancer. The aim of Phase I will be the study of the safety of these siRNA–EphA2–DOPC and their maximal tolerated dose (MTD).

While these clinical trials were inconceivable ten years ago, they are nowadays ongoing and showing good tolerance levels with no evidence of side effects. Also encouraging results obtained with concomitant treatment in cancer have thus suggested the possibility to undertake personalized treatment in the future (early stage diagnosis, tumor screening, siRNA pool specific for the patient...).

### 4.3. Concomitant treatments

The transitory effects of siRNA are adapted to chronic disease [166] however, concomitant treatments allowed by the use of synthetic delivery systems (different siRNA, chemo and radiotherapy...) are necessary. The poor results obtained with single siRNA delivery have encouraged the development of concomitant strategies by combining several siRNA to target simultaneously different pathways or to use single siRNA with other therapeutic agents. For example, three siRNA were co-formulated in liposomes by Huang et al. [8,64]. These siRNA targeted MDM2, c-myc and VEGF proteins that were all implicated in the development of cancerous pathologies. Two intravenous injections of 9  $\mu\text{g}$  siRNA were able to produce 30% volume regression in a mouse melanoma metastasis model [64]. Moreover, double doses of siRNA increased the survival median by 6 days corresponding to 30% compared to the control group in the same cancer model [8].

Association and synergy between siRNA and other drugs could potentially improve their therapeutic effect. siRNA delivery systems have been already tested in association with classic chemotherapeutic agents. Multifunctional nanoparticles, transporting siRNA and doxorubicin, were developed by Chen et al., to demonstrate their effect [65,68]. A colon cancer model was used to test this concomitant treatment based on doxorubicin and c-myc siRNA. While siRNA or doxorubicin treatment alone led respectively to 35% and 8% of inhibition contrary to siRNA associated with doxorubicin that led to a 60% synergic volume regression [65]. Similar results were obtained in a second study conducted with different siRNA against c-myc and VEGF with or without chemotherapy [68].

Other chemotherapeutic agents have been tested in such combination, e.g. docetaxel with periostin (POSTN) siRNA entrapped into chitosan nanoparticles [31]. Periostin plays a significant role in cell invasion, survival, and angiogenesis, leading to metastasis

[167]. In an ovarian cancer model, injections of POSTN siRNA with docetaxel increased tumoral regression (90% reduction in comparison to 70% and 50% regression when siRNA or docetaxel was used alone) and decreased also significantly the number of tumoral nodules [31].

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