

# Targeting Cancer by siRNA; A Review on Current Strategies



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**Submission:** July 10, 2018; **Published:** September 14, 2018

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## Abstract

Recent development in RNA Interference (RNAi) brought enthusiasm in the scientific community and opened the new ways for many therapeutic applications. The RNAi is a phenomenon which is used to targets mRNA and chops them up. The most appealing work of RNAi is observed in cancer inhibition by silencing genome sequences. Ultimate level of specificity can be achieved through siRNA targeted therapeutics. Short interfering RNA(siRNA) belongs to dsRNA molecules that can be produced exogenously and endogenously. This review sums up the recent approaches for cancer inhibition by siRNA. The article also points out the current targets of siRNA in cancer therapy development including expression level of cancerous and non-cancerous cells, their genetic concerns and siRNA drug delivery system like lipid base Nano-vectors. Currently, many of RNA based drugs are in practice at clinical trials level to overcome this deadly disease. In view of the encouraging results of RNAi based therapy, it is obvious that in near future siRNA will prove itself as a major cancer inhibitor and can become a standard cancer treatment.

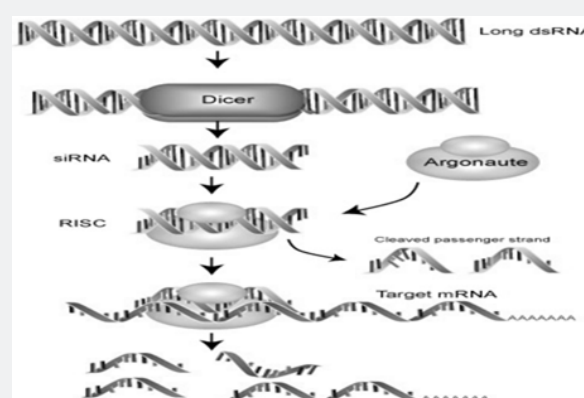
**Keywords:** siRNA; RNAi; Gene silencing; Cancer therapeutics

## Introduction

The accessibility to the human genome sequence revolutionized the strategy of utilizing nucleic acids with sequences complementary to specific target genes to improve drug discovery and target validation [1]. Development of sequence-specific DNA or RNA analogs that can block the activity of selected single-stranded genetic sequences offers the possibility of rational design with high specificity, lacking in many current drug treatments for various diseases including cancer, at relatively inexpensive costs [2]. RNA interference (RNAi) is one such example that is a simple and rapid method of targeted gene silencing in a range of organisms [3]. Gene silencing is done by degradation of RNA into short RNA strands that are involved in activation of certain ribonucleases, which target homologous mRNA [4]. The standard RNAi takes place at the post-transcriptional level and is initiated by the introduction of nucleic acidbased tools. It has been shown by several studies, conducted in-vivo and in-vitro, that RNAi- based therapy can be used for treating single-gene disorders and those with overexpression of proteins [5]. Currently, the three basic categories of RNAi tools used for this purpose are **siRNA** (small interfering RNA), **shRNA** (short hairpin RNA) and **miRNA** (microRNA) [6]. **siRNA** is the most commonly used RNAi tool for induction of short term gene silencing. It is a short, double-stranded RNA [dsRNA] duplex of 19-21 nucleotides in length, and is prepared from long dsRNA by the RNase III enzyme

Dicer (endogenously), or introduced into a cell exogenously [7]. The siRNA does not require genome integration and is easy to synthesize, that's why it is more suitable for drug use.

## Mechanism of Gene Silencing by siRNA



**Figure 1:** Mechanism of RNAi by siRNA: The DICER (enzyme) breaks double stranded RNA, to generate small siRNA/micro RNA. These chopped RNAs are then introduced into the RISC (RNA induced silencing complex), which targets specific mRNA for inhibition of their translation [2].

As illustrated in (Figure 1), on entering the cell, endogenously transcribed or exogenously introduced long dsRNA, triggers the

RNAi process. It is first processed by the RNase III enzyme Dicer in an ATP-dependent reaction. The Dicer processes long dsRNA into small [21-23 nucleotide] siRNA strands, with 2-nucleotide 3' overhangs [8]. siRNA can also be synthesized outside a cell and then be introduced into a cell through in-vitro delivery systems (transfection, electroporation etc) [9]. The siRNA is incorporated into the RISC RNAi effector complex, which consists of an Argonaute (Ago) protein as one of its main components [10]. Active RISC is formed when the passenger (sense) strand of the siRNA duplex is cleaved and discarded by Ago. The remaining (antisense) strand acts as the guide strand and escorts the RISC to its homologous mRNA, causing the endonucleolytic cleavage of the target mRNA [7].

Most cancers are caused by gene mutation or over expression of an oncogene, so it is possible to cure cancer by blocking disease-related gene in cancer cells with sequence specificity, through rational siRNA design [11]. Therefore, RNAi has rapidly established into one effective therapeutic technology for cancer, as it is an affordable technology for researchers [12]. Among the in-vivo and in-vitro clinical trials of RNAi based therapy of different

diseases, administration of most siRNAs was done by local delivery, typically via the intravitreal or intranasal routes. But, local delivery may not be appropriate for all diseases [13]. Under some conditions, systemic drug administration by intravenous (IV) injection is needed, and other delivery systems are also required to administer the siRNA [14, 15].

### siRNA in current Cancer Therapeutics

To target cancer, siRNA can be administered to whole organism or only to a specific tissue (eye, or brain tumor). In first case larger amount of siRNA is required to attain the desired down regulation while in second scenario, only small amount of siRNA is needed to target specific site with minimum side effects [14]. siRNA delivery, particularly with cholesterol conjugates, polymer-based nanoparticle strategies and liposomes, has also been extensively discussed with reasonable success. Targeting via peptides, aptamers and antibodies have also been published [16-42]. Different delivery methods to date are represented in Table 1. The table 1 reveals in very elaborative way the delivery methods, routes of administration, target gene and target organ for siRNA in different cancer types.

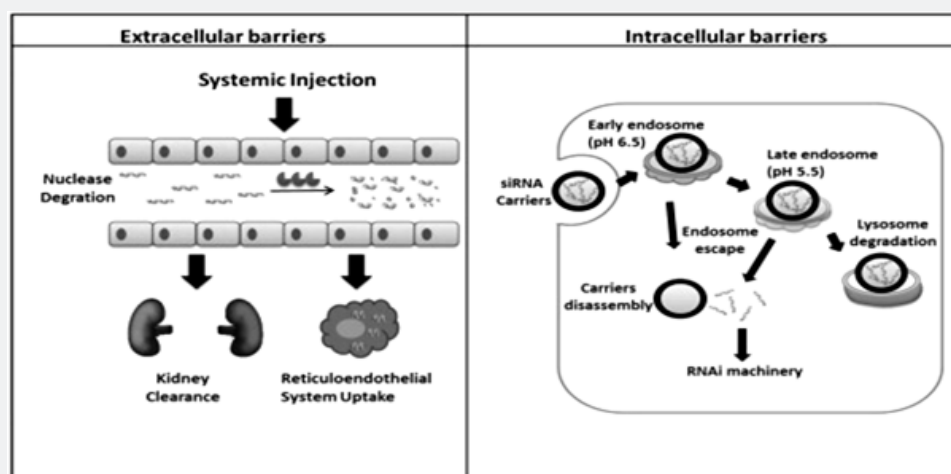
**Table 1:** *In vivo* siRNA for cancer therapy.

Sr.No	Cancer Types	Delivery System	Routes	Target Gene	Target Organ
1	Fibro sarcoma (JT8) [17]	Naked siRNA	intraperitoneal, intravenous, sub cutaneous., intra thecal	vascular endothelial growth factor	Subcutaneous
2	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3), [18]	Naked siRNA + gemcitabine	Intra venous.	Focal adhesion kinase	Orthotopic pancreas
3	Pancreatic adenocarcinoma (BxPC3), [19]	Naked siRNA	Intravenous.	Carcino embryonic Antigen Related Cell Adhesion Molecule 6	s.c., Orthotopic pancreas
4	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2), [20]	Naked siRNA	Intravenous.	Ephrin type-A receptor 2	s.c., Orthotopic pancreas, liver metastasis
5	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2), [21]	Naked siRNA + gemcitabine	Intravenous	Ribonucleoside-diphosphate reductase subunit M2	s.c., Liver metastasis
6	Breast cancer (MDA-MB-231), [22]	Naked siRNA	Intravenous	C-X-C chemokine receptor type 4	Lung metastasis
7	Colon cancer (HTC116), [23]	Liposome	Intraperitoneal	$\beta$ -Catenin	s.c., intraperitoneal
8	Liver metastatic spleen cancer (A549), [24]	Liposome	Intravenous	B cell lymphoma gene 2	Liver
9	Bladder cancer (UM-UC-3-LUC), [25]	Liposome	intra thecal	polo-like kinase 1	Bladder
10	Pancreatic carcinoma (Capan-1), [26]	Liposome	intra thecal	Somatostatin	
11	Prostate cancer (PC-3), [27]	CCLA (NeoPhectin-AT)	Intravenous	"Raf-1" (protooncogene)	Sub cutaneous
12	Breast cancer (MDA-MB-231), [28]	CCLA	Intravenous	"c-Raf"	Sub cutaneous
13	Glioma (U87), [29]	shRNA plasmid + pegylated immunoliposome	Intravenous	Endothelial Growth Factor Receptor	Brain
14	Ovarian carcinoma cells (SKOV-3), [30]	PEI	Intraperitoneal	Human epidermal growth factor receptor-2	Sub cutaneous

15	Ewing's sarcoma (TC71), [31]	shRNA plasmid + PEI	intra thecal	Vascular endothelial growth factor	Sub cutaneous
16	Cervical adenocarcinoma, colon cancer (HeLa, HTC116), [32]	Adenovirus vector	intra thecal	hypoxia-inducible factor-1	Sub cutaneous
17	Lung cancer (ACC-LC-172), [33]	Adenovirus vector	intra thecal	S-phase kinase-associated protein 2	Sub cutaneous
18	Meningioma IOMM-LEE, [34]	shRNA plasmid	intra thecal	urokinase plasminogen activator receptor+ matrix metalloproteinase 9	Brain osmotic pumps
19	Breast cancer (MDA MB 231), [35]	shRNA plasmid	intra thecal	urokinase plasminogen activator receptor+ matrix metalloproteinase 9	Orthotopic breast
20	Glioblastoma (SNB19), [36]	shRNA plasmid	Intraperitoneal	Urokinase-Type Plasminogen Activator Receptor + urokinase plasminogen activator	Brain
21	Glioblastoma (SNB19), [37]	shRNA plasmid	Intraperitoneal	urokinase plasminogen activator receptor+ matrix metalloproteinase 9	Brain
22	Glioblastoma (SNB19), [38]	shRNA plasmid	Intraperitoneal	urokinase plasminogen activator receptor+ matrix metalloproteinase 9+ urokinase-type plasminogen activator	Brain
23	Glioblastoma (SNB19), [39]	shRNA plasmid	intra thecal	matrix metalloproteases+ cathepsin B	Brain
24	Glioma (SNB19), [40]	shRNA plasmid	intra thecal	Cathepsin B, Urokinase-Type Plasminogen Activator Receptor	Brain
25	Cervical adenocarcinoma, lung cancer (HeLa S3, A549), [41]	shRNA plasmid + ATA	Intravenous	Polo-like kinase 1	Sub cutaneous
26	Neuroblastoma (N2A), [42]	PEI-PEG-RGD	Intravenous	Vascular Endothelial Growth Factor Receptor-2	Sub cutaneous
27	Ewing's sarcoma (TC71), [127]	CDP-AD-PEG-transferrin	Intravenous	"EWS-FLI1"	Multiple organ metastasis
28	Cervical adenocarcinoma (HeLa), [111]	HVJ envelope vector + cisplatin	intra thecal	"Rad51"	Intradermally
29	Melanoma (B16), [112]	ErbB2-protamine fusion protein	intra thecal., Intravenous	Avian myelocytomatosis virus oncogene cellular homolog, mouse double minute 2, Vascular endothelial growth factor	Sub cutaneous
30	Prostate cancer (PC-3), [113]	Atelocollagen	intra thecal	Vascular endothelial growth factor	Sub cutaneous
31	Germ-cell tumor (NEC8), [114]	Atelocollagen	intra thecal	Fibroblast Growth Factor 4	Testis
32	Prostate cancer (PC-3M-Luc), [115]	Atelocollagen	Intravenous	Enhancer of zeste homolog 2, "p110a"	Bone metastasis

In comparison to chemotherapeutic anti-cancer drugs, siRNA drugs have a lot of advantages. There are four significant benefits of siRNA as a potential cancer therapeutic strategy due to its special mechanism [16]. To start with, is its high level of safety. siRNA does not interact with DNA while acting on the post-translational stage of gene expression, and in this way maintains a strategic distance from the mutation and teratogenicity dangers of gene therapy. The second advantage of siRNA is its high efficacy. With just several copies of siRNA, striking suppression of gene expression can be caused in a single cancer cell. The best advantages of siRNA as compared to other drugs (small molecule

or antibody-based) are the unlimited targets and specificity, due to the complementary base pairing [43]. Basically, siRNA drugs are rationally designed to silence specific cancer-promoting genes to treat cancer, but of course they can also be designed to target any disease gene according to their mRNA sequence, effectively [44]. Despite these advantages, there are still several barriers hindering the clinical use of siRNA for cancer therapy (Figure 2). The first is the instability of siRNA under physiological conditions. It is readily digested by the serum nucleases, during its passage through the blood [45].



**Figure 2:** Barriers that hinder RNAi by siRNA: Extracellular barriers; include nuclease degradation intravenously and Intracellular barriers do degradation of RNA by endosomes in cell.

Among the biological barriers, firstly the siRNA, is encountered the nuclease activity in plasma and tissues, on administration [46]. Secondly, the role of kidney in siRNA clearance; as reported by the several studies in animals that the highest uptake of bio distribution of siRNA is shown by the kidney [47]. The third barrier is the effects of siRNA that are off-target and induce unexpected phenotypes that lead to complications in the interpretation of the therapeutic benefits offered by siRNA [48]. Lastly, the safety of siRNA is not up to the mark, and its high levels cause the activation of recipient's innate immune response and cytokines production

*in vitro* and *in vivo* [49,50]. Considering these barriers, certain chemical modifications and delivery methods must be required to transport siRNA to its target without detrimental effects. To accomplish that, variant materials are under exploration to confront the challenges of *in vivo* delivery. These materials include polymers, peptides, lipids, aptamers, antibodies, and small molecules [51]. With the help of rational designs and high-throughput screens successful systems have been developed or discovered. Table 2 shows the Merits and demerits of siRNA for cancer therapy [52].

**Table 2:** Merits and demerits of siRNA for cancer therapy [26].

Sr. No	Advantages	Disadvantages
1	siRNA Can be easily synthesized in laboratories	Costly, esp. at genome-wide level
2	Easy delivery to the cell via synthetic/natural polymers and lipids	Poor chemical stability and low half-life in circulation, without modification. High doses are required.
3	Post-transcriptional gene silencing(PTGS), does not require genome integration	Poses an 'off-target' effect(i.e the silencing of a gene whose expression should not be blocked)
4	Local administration and limited systemic injection	Some transcripts are difficult to silence e.g. those with high turnover
5	Higher level of safety does not degrade DNA	But Off-target effects cause high stimulation of immunity, inflammation, and cytotoxicity
6	Understandable mechanism of action at molecular level ( <i>In-vitro</i> ) Vast applications esp. in acute disease conditions; where high doses are tolerable	In contrast to <i>in vitro</i> , siRNA <i>in vivo</i> mechanism of action is not clearly understandable

### The Criteria for Designing siRNA Delivery System for Cancer Therapy

For systemic and safe administration of siRNA across physiological barriers to its site of action, such delivery systems should be synthesized that, dodge immune recognition, provide serum stability, prevent renal clearance, increase vascular permeability to reach cancer tissues, reduce interactions with serum proteins and non-cancer cells, contain low toxicity and allow cell entry and endosome escape to enter the RNAi

machinery [52,53]. To avoid the problems, a common strategy is to incorporate 2'-O-methyl and 2'-deoxy-2'-fluoro groups, nucleic acids (locked or unlocked), or phosphorothioate linkages [54]. Despite the solution of some problems by chemical modifications, encapsulation of siRNA by nanoparticles is better option for protecting it from degradation and immune system [55]. Unfavorable aggregation with erythrocytes can be caused due to the high positive charges of nanoparticles [56], but such interaction between serum proteins and nanoparticles can also help in uptake by cancer cells [57,58]. The typical strategy to

minimize this interaction is to shield the nanoparticle surface with polyethylene glycol (PEG) [59].

Ways by which siRNA leaves the bloodstream after systemic administration, are through the liver, spleen, kidney and lung. But the most common pathway is kidney clearance [60]. The glomeruli of kidney work as a natural filter which allows water and small molecules to pass into urine and larger molecules remain in the circulation [61]. The pore size of the glomerular filter is roughly 8nm [62], and typically kidney excretes molecules less than 50kDa in size [63]; the molecular weight of naked siRNA is about 13kDa [64]. That's why siRNA easily passes through glomeruli and drifts into the urine. The size of the delivery nanoparticle can be increased, by addition of synthetic materials to siRNA, to keep it for alternative organ targets rather than glomerular filtration through the kidneys [65]. Mostly highly effective delivery systems are larger than 20nm [66] such as dynamic polyconjugates (DPCs; 10nm) [67] and triantennary N-acetylgalactosamine [GalNAc] conjugates.

Most siRNA delivery systems include cellular internalization by endocytosis. The rate of cellular uptake is improved by siRNA delivery systems, as they induce receptor-mediated endocytosis by incorporating targeting ligands that bind specifically to receptors on target cells [68]. But this ligand-receptor interaction is hindered by adsorption of serum proteins on the nanoparticle surface [69]. Cell uptake, through endocytosis or non-endocytic mechanisms, of cell-penetrating peptides is used by other systems [70]. Additionally, the most important part of the siRNA delivery systems is low toxicity. If an unacceptable toxicity is provoked by siRNA delivery on either a cellular or systemic level, even the most effective siRNA delivery system will become useless. For

example, among the first vehicles to be studied for siRNA delivery, the viral vectors induce toxicity by activating immune responses [71,72]. Thus, as alternatives to viral vectors, synthetic lipids and polymers have been for nucleic acid delivery applications. Their formulation has been done carefully, to avoid activation of the immune response [73]. For clearance of larger molecular mass materials, they must be biodegradable. That's why high molecular mass polycations and polymers containing linkages that can be cleaved inside the cell are used. As they are biodegradable, they can help decrease cytotoxicity [74].

### siRNA Delivery Systems for Cancer Inhibition

Scientists have reported different strategies for cancer inhibition including siRNA [75]. The delivery of siRNA holds great importance. Different factors effect siRNA delivery including Biocompatibility, Serum stability, Biodegradability and Non-immunogenicity [76]. There are different categories of siRNA delivery systems including Lipid-based Nano-vectors, Polymer mediated delivery, Chemical modification and conjugate delivery systems [77]. Advantages and Disadvantages of Different siRNA Delivery approaches has been discussed in Table 3. Both 3' and 5' terminals of siRNA and the sugar backbone can be chemically modified [78]. The most important chemical modifications include the addition of 2'-deoxy-2'-fluoro and 2'-O-methyl [79]. Furthermore, at the 3'-terminal of RNA's sugar backbone, phosphodiester group can be replaced with phosphothioate group. 2'-O-alkyl can be combined with 4'-thiolate for chemical modification [80]. The chemical modification should not have negative effects on serum stability and gene silencing effect of siRNA. Different metabolites used in chemical modification can cause a safety issue [81-83].

**Table 3:** Advantages and Disadvantages of Different siRNA Delivery approaches [51].

S. No	Delivery Approaches	Advantage	Disadvantage
1	Virus-mediated	Successful in cells which are Transfection-resistant from dsRNA and plasmids, Integration produces stable RNAi even in the absence of a selection pressure'	Much laborious to produce, Possible biohazard
2	Synthesis by enzymes and Chemicals (in vitro)	speedy production and elevated purity	Transitory RNAi, Costly for more than a few siRNA
3	"DNA plasmid vector"	cost-effective for multiple sequences Stable RNA interference practicable via selection marker	Extra laborious and "Transfection-dependent"

### siRNA deliverance Using Lipid Based Nano Vectors

In order to improve the efficacy of plasmid DNA or siRNA transfection, a lipid formulation called as Lipofectamine 2000 is used [84]. Liposomes transfection mechanism includes the interaction between lipids, which are positively charged, and nucleic acids which are negatively charged [85], which forms a lipoplexes. DOPC (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) is an important component in siRNA delivery using lipids [86]. 1,2-dioleoyl-3-trimethylammonium-propane [DOTAP] and dioleoyl-phosphatidylethanolamine are among the other important

formulations for siRNA delivery [87]. SNALPs [stable nucleic acid-lipid particle], the lipids nanoparticles, are thought to be important vectors for siRNA delivery [88]. In SNALPs, diffusible polyethylene glycol surrounds the fusogenic and cationic lipids [89]. SNALPs gives high bioavailability, so there are more chances of siRNA delivery to cancer cells [90]. Lipoid nanoparticles which include the PEG-modified lipids and cholesterol [91], also proved to be better vehicles for siRNA delivery e.g. 98N12-5, which reduced the FVII or ApoB expression from 75 to 90 % in hepatocytes of mice [92].



## siRNA transfer by Polymers for Cancer Inhibition

Solid, biodegradable and colloidal systems; usually known as polymeric Nano particles can also be used for siRNA delivery e.g. poly (d, l-lactide) and poly (d, l-lactide-co-glycolide) [93]. Another type of polymer mediated siRNA delivery includes water soluble cationic polymers e.g. polyethyleneimine [PEI]. Cyclodextrin, first used in 1999 for siRNA delivery, proved to be better vehicle for siRNA delivery [94]. They are poly-cationic oligomers of di- amine bearing cyclodextrin monomers and di- methyl suberimide[95]. Efficacy of this siRNA delivery system can be improved by using Adamantane- PEG-transferrin and Adamantane- PEG are used for both in-vivo and in-vitro experiments Polyethylenimine proved important. Polycaprolactane are micelleplex Nano particles; during experiments they suppressed the expression of green fluorescent protein from forty to seventy percent [96]. Another component, m[PEG]-b-[PCL]-b[PPEEA] proved to be successful during trials on mice [97]. Other components including Poly [d,l-lactide] [PLA] and poly [d,l-lactide-co-glycolide] were successful with spermidine especially for gene silencing in vaginal mucosa during experiments by Saltzman in 2009 [98]. All these components have been successful during initial trials and have great potential to be used in siRNA delivery [99].

## Delivery Systems for siRNA Using Conjugates for Cancer Inhibition

Experiments on siRNA delivery using conjugates have shown positive results. Different types of conjugates are used including peptides, lipids and aptamers (modified oligonucleotides) [100]. The early experiments were performed using lipid-based conjugates such as cholesterol [101]. A pyrrolidone linkage, joined cholesterol on the 3-terminal of siRNA's sense strand. The pharmacokinetic behavior and transfection efficacy of siRNA was increased by cholesterol [102]. In the next step, high density lipoprotein was attached with the siRNA-cholesterol complex. It improved the gene silencing effect 8-15 times [103]. Transfection efficacy can also be improved by using cell-penetrated peptides such as trans-activator protein [TAT], which is taken from HIV-1[104]. Hetero bi-functional cross linker [Sulfosuccinimidyl – 4 – p-maleimidophenyl butyrate] was used to link TAT with siRNA's antisense strand [105]. Apart from its advantage of improving intracellular siRNA delivery, it can cause cyto-toxicity because of immunogenicity [106]. Insulin receptor substrate's expression was sixty percent reduced by using receptor-ligand mediatory delivery, in which a carboxylic group from D-[Cys-Ser-Lys-Cys] which is the peptide mimetic of IGF1, was bound to amine group at 5-terminal of siRNA's sense strand [107]. Better specificity and stability has made antibody-mediated delivery advantageous, in which a biotin-streptavidin linkage joined the siRNA with the monoclonal antibody; which targets the transferring receptor on blood-brain barrier [108]. During experiments on rat with intracranial transplanted brain tumors, the reporter gene expression was greatly reduced due to intravenous administration

of siRNA-Antibody complex [109]. As aptamers contains selective affinity for proteins, so streptavidin-biotin linkage was used to conjugate it to siRNA. It does not use transfection agents and still gives positive results. Gal-Nac& DPCs, the advanced delivery systems are currently in focus[110].

## Conclusion

siRNA drugs are one of the most promising drugs for cancer treatment due to their great advantages, such as high efficacy, excellent safety, specificity and unrestricted choice of targets. Many highly effective delivery systems have been developed for solution of the delivery problems of siRNA and are quite different in terms of size, structure and chemistry. But still for optimal delivery systems, some guidelines should be followed e.g. Nano particle size should be about 20-200nm, PEG should be used as a shielding agent, chemical modifications should be made to avoid renal filtration and phagocytic filtration, to prevent non-specific interactions and immune recognition in circulation, to avoid nuclease digestion respectively. Additionally, for siRNA uptake by cancer cells, endogenous or exogenous targeting ligands should be used. Although, the great potential of siRNA in cancer treatment has been demonstrated by a number of reports but there are still few remaining challenges to meet the full clinical potential of siRNA. Highs and lows are being experienced by siRNA drug development in recent years. Big pharmaceutical companies have shown over-optimistic attitude towards RNAi drugs. Hence, the key to siRNA drug development is a good delivery system. Significant breakthrough of research in siRNA drug delivery systems will make siRNA to occupy a strong position in the drug market, especially the anti-cancer drug market.

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DOI: [10.19080/JOJS.2018.01.555573](https://doi.org/10.19080/JOJS.2018.01.555573)

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