Review

Targeted Delivery Systems of Small Interfering RNA by Systemic Administration

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Summary: RNA interference (RNAi) is induced by 21–25 nucleotide, double-stranded small interfering RNA (siRNA), which is incorporated into the RNAi-induced silencing complex (RISC) and is a guide for cleavage of the complementary target mRNA in the cytoplasm. There are many obstacles to in vivo delivery of siRNAs, such as degradation by enzymes in blood, interaction with blood components and non-specific uptake by the cells, which govern biodistribution in the body. In order to achieve the knockdown by siRNAs in vivo, many delivery systems of siRNAs based on physical and pharmaceutical approaches have been proposed. In addition, the immune responses of siRNA must be taken into account when considering the application of siRNAs to in vivo therapy. This review focuses on recent reports about delivery systems and immune responses of siRNAs.

Key words: small interfering RNA (siRNA); RNA interference; liposomes; targeting; polymer; drug delivery system

Introduction

RNA interference (RNAi) is induced by 21–25 nucleotide, double-stranded small interfering RNA (siRNA), which is incorporated into the RNAi-induced silencing complex (RISC) and is a guide for cleavage of the complementary target mRNA in the cytoplasm (Fig. 1). Argonature 2 (Ago2) functions as a “slicer”, the RNA endonuclease cleaves a target RNA species as directed by the siRNAs guide (antisense) strand.1,2) Bertrand et al. compared the knockdown effects of antisense oligonucleotides and siRNA in cell culture and in vivo and concluded that the siRNAs is more efficient and its effect more.3) For therapeutic application, siRNA technology promises greater advantages over drugs currently on the market by offering new types of drugs that are easy to design and have a very high target selectivity, inhibiting a specific gene expression in the cytoplasm. In addition, they are expected to be low toxic due to their metabolism to natural nucleotide components by the endogenous cells systems.

There are many obstacles to in vivo delivery of siRNAs, such as degradation by enzymes in blood, interaction with blood components and non-specific uptake by the cells, which govern biodistribution in the body. In order to achieve the knockdown by siRNAs in vivo, many delivery systems of siRNAs based on physical and pharmaceutical approaches have been proposed. In addition, the immune responses of siRNA must be taken into account when considering the application of siRNAs to in vivo therapy. This review focuses on recent reports about delivery systems and immune responses of siRNAs.

Pharmacokinetics of Systemically Administered siRNAs

For effective drug therapy, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and unfavorable actions. In general, the lack of selectivity of most conventional drugs is closely related to their pharmacokinetic properties. The in vivo fate of a drug given by a particular administration route is determined by both the physicochemical properties of the drug and the anatomical and physiological characteristics of the body;4) therefore, the biodistribution characteristics of systemically administered siRNAs has attracted much attention. For the rational design of
Fig. 1. Schematic illustration of mechanisms of siRNAs action in the cells. RNAi is induced by 21–25 nucleotide, double-stranded siRNA, which is incorporated into the RNAi-induced RISC and is a guide for cleavage of the complementary target mRNA in the cytoplasm. Ago2 functions as a “slicer”, the RNA endonuclease cleaves a target RNA species as directed by the siRNAs guide (antisense) strand.

Targeted Delivery Systems of Small Interfering RNA

As far as the biodistribution characteristics of naked siRNAs are concerned, knockdown in the renal proximal tubules might be useful.\textsuperscript{11} For the broad applica-
tion of siRNAs in vivo, however, delivery systems for siRNAs need to be developed. The problems of systemic delivery of siRNA are shown in Fig. 2. To date, delivery has been studied by i) chemical modification approaches of siRNAs, ii) physical approaches, and iii) pharmaceutical approaches.

Chemical modification approaches: It has been suggested that natural siRNAs, which have a phosphodiester (PO) type backbone, have a number of disadvantages for clinical use, including their instability to nucleases and the low cellular uptake. Single strand 2'-OH siRNA was completely degraded after as little as 30 sec following incubation with 5% bovine serum at 37°C.\(^{12}\) However, duplex 2'-OH siRNA was completely degraded after as little as 30 sec following incubation with 5% bovine serum at 37°C.\(^{12}\) Additionally, more than 25\% of the duplex 2'-F siRNA remained intact after 48 h.\(^{16}\) Elmán et al. reported that degradation of LNA is suppressed for up to 48 h in 10\% mouse and human serum at 37°C.\(^{17}\) Although the plasma stability of 2'-OH siRNA is different, these observations suggest that (serum) stable siRNA could be achieved by chemical modification. As far as the in vivo applications of chemically modified siRNA are concerned, even although the modified siRNAs have greatly increased resistance to nuclease degradation in plasma,
this increase in stability does not translate into enhanced or prolonged inhibitory activity of target gene reduction in mice following tail vein hydrodynamic injection. They reported that the suppression of duplex PO/PO is prolonged at least for 7 days in vivo. Since duplex PO/PO siRNA is stable in serum to some extent, such chemical modifications of siRNA might not necessarily be more potent than unmodified siRNAs in animals.

Another type of chemical modification involves the conjugation of cholesterol to the 3’ end of the sense (anti-guide) strand of 2’-OH siRNA by means of a synthetic pyrrolidone linker (chol-siRNA). Presumably because of enhanced binding to serum proteins, chol-siRNA showed improved in vivo pharmacokinetic properties as compared with unconjugated siRNAs. In fact, the elimination half-life (t1/2) of chol-siRNA and siRNA is 95 and 6 min, respectively, after intravenous injection into mice at 50 mg/kg. Significant levels of chol-siRNA were detected in liver, heart, kidney, adipose, and lung tissues. These pharmacokinetic characteristics produced by protein interaction of chol-siRNA corresponds to previous reports that lipophilic prodrugs are distributed following interaction with proteins. Therefore, consideration of the hydrophilic/hydrophobic balance of siRNAs might be one of effective approach to enhance their in vivo use.

Recently, siRNAs was conjugated to poly(ethylene glycol) (PEG) via a disulfide linkage. The PEG-siRNAs conjugate forms polyelectrolyte complex micelles by interacting with polyethylenimine as a core-forming reagent. The VEGF siRNA-PEG/PEI PEC micelles showed greater stability than naked siRNA in the medium containing 50% FBS at 37°C and effectively silenced (up to 96.5%) VEGF gene expression in cultured prostate carcinoma cells.

**Hydrodynamics Approaches:** In 1999, Liu et al. and Zhang et al. reported that that high gene expression can be easily obtained in liver and other major organs by a simple intravenous injection of naked plasmid DNA via a tail vein if a high volume (about 1.6–2.0-ml/20 g-mice) of saline is delivered at a high velocity (about 3–5 s). The transfection activity in the liver is 104–105-fold higher than that in other organs (kidney, lung, spleen, and heart). As much as 45 µg luciferase per gram liver can be achieved by a single tail vein injection of 5 µg plasmid DNA into a mouse. Histochemical analysis using β-galactosidase gene as a reporter reveals that approximately 40% of hepatocytes express the transgene. Recently, Zhang et al. carried out electron microscopic observations which revealed that some fenestrae are enlarged and pores as large as a few µm in diameter are present. In addition, they reported that a hydrodynamically injected solution produces membrane defects and disturbs the cell interior. However, a transient increase of aminotransferase (ALT) and irregular heart function was observed in mice injected with saline or saline containing plasmid DNA.

The hydrodynamics approaches with siRNAs are listed in Table 1. In 2002, McCaffrey et al. and Lewis et al. demonstrated that hepatic RNA interference occurred in adult mice following intravenous injection of naked duplex siRNAs (40 µg) and shRNAs expressing naked plasmid DNA given in a high volume. The frequent hydrodynamic injection of naked duplex siRNAs or siRNA-expressing naked plasmid DNA dramatically reduced mRNA and protein levels of the targeted gene encoding the Fas receptor, caspase-3, caspase-8, hepatitis B virus, or mdr1a, respectively.

As far as suppression effects in organs produced by naked duplex siRNAs are concerned, Wesche-Soldato et al. investigated the suppression effects produced by hydrodynamic injection of naked duplex siRNAs (GFP) using GFP-transgenic mice. They observed that in all the GFP mouse tissues examined, including spleen and liver as well as heart, kidney, lung, muscle, brain, thymus, and Peyer patches, a decrease in GFP was evident. These observations suggest that suppression effects in organs produced by hydrodynamic injection of naked duplex siRNAs do not occur in specific organs. This discrepancy in the hydrodynamic approach between plasmid DNA and duplex siRNAs might be explained by the difference in physicochemical properties, especially their molecular sizes. However, further

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**Table 1. Hydrodynamics approaches for siRNAs after systemic administration**

<table>
<thead>
<tr>
<th>Route</th>
<th>Target gene</th>
<th>Disease model</th>
<th>Study organs</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>Luc</td>
<td>normal mice</td>
<td>liver</td>
<td>27</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Luc, GFP</td>
<td>normal mice</td>
<td>liver, kidney, spleen, Pancreas</td>
<td>28</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Fas</td>
<td>hepatitis mice</td>
<td>liver</td>
<td>29</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Caspase 8</td>
<td>hepatitis mice</td>
<td>liver</td>
<td>30</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Caspase 3, 8</td>
<td>hepatic ischemia/reperfusion mice</td>
<td>liver</td>
<td>31</td>
</tr>
<tr>
<td>Intravenous</td>
<td>HBV</td>
<td>hepatitis B mice</td>
<td>liver</td>
<td>32</td>
</tr>
<tr>
<td>Intravenous</td>
<td>MDR</td>
<td>normal mice</td>
<td>liver</td>
<td>33</td>
</tr>
<tr>
<td>Intravenous</td>
<td>GFP</td>
<td>GFP-transgenic mice</td>
<td>liver, kidney, spleen, thymus, lung, muscle, heart</td>
<td>34</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Caspase-8, Fas</td>
<td>hepatitis mice</td>
<td>liver, spleen</td>
<td>34</td>
</tr>
</tbody>
</table>
Intravenous delivery are listed in animals. The applications of carrier systems for ligands enables the cell-selective targeting of siRNAs in direct injection (intratumoral and intranasal) of been investigated in terms of the delivery of siRNAs. Among these polymers, atelocollagen and PEI have been used for plasmid DNA delivery and the important factors governing the transfection efficacy are i) the types of neutral or cationic lipid in liposomes, ii) the charge ratio of the complex, iii) the size of the complex, and iv) the dose of plasmid DNA. Therefore, these factors could also be important for the efficient delivery of siRNAs using PEI. In 2004, Ge et al. demonstrated that virus production in lung of infected mice is reduced by intravenous injection of siRNAs (60 μg) complexed with PEI at an N/P ratio of 5. Recently, Urban-Klein et al. examined intraperitoneal injection 2–3 times a week in a subcutaneous mouse tumor model, and found that the linear PEI/siRNA (0.6 nmol) complex, but not naked siRNA results in a marked reduction in tumor growth. They also found that the linear PEI/siRNA (HER-2) complex selectively accumulated in the solid tumor after intraperitoneal injection.

Atelocollagen is a highly purified pepsin-treated type I collagen from calf dermis. Intravenously injected human enhancer of zeste homolog 2 (EZH2) or human phosphoinositide 3'-hydroxykinase p110-α-subunit (P110-α) siRNA (50 μg)/atelocollagen complex inhibits the metastatic tumor growth in bone tissues in mice.

**Pharmaceutical approaches:** Since macromolecules with a molecular weight of less than 50,000 (approximately 6 nm in a diameter) undergo glomerular filtration and are excreted into the urine, duplex siRNAs (about M.W. 13,000) are expected to be eliminated by the kidney. Therefore, complex formation with cationic compounds or incorporation into liposomes are a promising approach for delivery of siRNAs because they are prevented from being excreted from the kidney due to their size. In addition, the positive charge of the complex of siRNAs and carriers could enhance cellular uptake through electrostatic interaction with the negative charge on the cell membrane. Moreover, surface modification of cationic compounds with PEG and/or ligands enables the cell-selective targeting of siRNAs in animals. The applications of carrier systems for systemic delivery are listed in Table 2.

**Cationic polymers:** Cationic polymers used for plasmid DNA delivery are of two types, natural polymers including chitosan and atelocollagen or synthetic polymers including poly(L-lysine), PEI, and dendrimers. Among these polymers, atelocollagen and PEI have been investigated in terms of the delivery of siRNAs in animals. Although some reports have involved the direct injection (intratumoral and intranasal) of siRNAs, with or without cationic carrier, attention focused on the systemic application because this is the ultimate goal for the therapeutic use of siRNAs.

Among the cationic polymers, PEI is considered to be the most effective vector for transfecting plasmid DNA into target cells in vivo. The transfection efficacy of the PEI/plasmid DNA complex has been determined based on i) the N/P ratio of the PEI complex, ii) the dose of plasmid DNA, and iii) the structure and molecular weight of PEI. Therefore, these factors could also be important for the efficient delivery of siRNAs using PEI. In 2004, Ge et al. demonstrated that virus production in lung of infected mice is reduced by intravenous injection of siRNAs (60 μg) complexed with PEI at an N/P ratio of 5.

**Liposomes, PEG liposomes, cationic liposomes, and galactosylated liposomes:** Cationic liposomes also used for plasmid DNA delivery and the important factors governing the *in vivo* transfection efficacy are i) the types of neutral or cationic lipid in liposomes, ii) the charge ratio of the complex, iii) the size of the complex, and iv) the dose of plasmid DNA. Therefore, these factors could also be important for the delivery of siRNAs using cationic liposomes. As far as the application of siRNAs are concerned, many groups have already reported the usefulness of cationic liposomes or modified liposomes for *in vivo* use, suggesting that liposomal systems are good candidates for siRNA delivery.

**Liposomes and PEG liposomes:** Regarding the application of neutral liposomes by siRNAs, Landen et al. reported effective tumor targeting using DOPC liposome-encapsulated siRNAs. In a mouse model of a solid tumor, the tumor accumulation of DOPC liposome-encapsulated Alexa 555 labeled siRNAs was about 10-fold and 30-fold higher than that of cationic liposomes (DOTAP) and naked siRNAs, respectively.

### Table 2. Pharmaceutical approaches for siRNAs after systemic administration

<table>
<thead>
<tr>
<th>Route</th>
<th>Target gene</th>
<th>Target organs/cells</th>
<th>Carrier</th>
<th>siRNAs condition</th>
<th>IFN study</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>Influenza virus</td>
<td>Lung</td>
<td>PEI</td>
<td>60 μg/mice</td>
<td>Little</td>
<td>48</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>Her-2</td>
<td>Tumor</td>
<td>JetPEI</td>
<td>0.6 nmol/mice, 2–3 times</td>
<td>—</td>
<td>49</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Ezh2, p110-α</td>
<td>Tumor</td>
<td>Atelocollagen</td>
<td>50 μg/mice, 3 times</td>
<td>Low</td>
<td>50</td>
</tr>
<tr>
<td>Intravenous</td>
<td>EphA2</td>
<td>Tumor</td>
<td>Neutral liposomes</td>
<td>150 μg/kg, 2-times</td>
<td>—</td>
<td>55</td>
</tr>
<tr>
<td>Intravenous</td>
<td>ApoB</td>
<td>Liver</td>
<td>Stable lipid particle</td>
<td>1 or 2.5 mg/kg</td>
<td>—</td>
<td>56</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>TNF-α</td>
<td>Peritoneal cells</td>
<td>Cationic liposomes</td>
<td>—</td>
<td>—</td>
<td>57</td>
</tr>
<tr>
<td>Intravenous</td>
<td>CD31</td>
<td>Tumor vasculature</td>
<td>Cationic liposomes</td>
<td>1.88 mg/kg, 6-times</td>
<td>Little</td>
<td>58</td>
</tr>
<tr>
<td>Intravenous</td>
<td>CD31, Tie2</td>
<td>Endothelial cells</td>
<td>Cationic liposomes</td>
<td>1.88 mg/kg</td>
<td>Little(IL-12)</td>
<td>10</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Bcl-2</td>
<td>Liver metastasis</td>
<td>Cationic liposomes</td>
<td>10 mg/kg, 10 times</td>
<td>—</td>
<td>59</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Raf-1</td>
<td>Tumor</td>
<td>Cationic liposomes</td>
<td>7.5 mg/kg, 5 times</td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Vaspessin V2 receptor</td>
<td>Kidney</td>
<td>Cationic liposomes</td>
<td>3.6 nmol/mice</td>
<td>—</td>
<td>61</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Ubc-13</td>
<td>Hepatocytes</td>
<td>Galactosylated liposomes</td>
<td>0.29 nmol/g</td>
<td>Low</td>
<td>68</td>
</tr>
</tbody>
</table>

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Three weeks of treatment with DOPC liposome-encapsulated siRNAs (tyrosine kinase receptor, EphA2) at an siRNA dose of 150 µg/kg reduced tumor growth. Recently, Zimmermann et al. demonstrated that a systemic liposomal formulation can silence the disease target apolipoprotein B (ApoB). ApoB-specific siRNAs have been encapsulated in liposomes (PEG-CDMA: DlinDMA: DSPC: cholesterol) and administered by intravenous injection to monkeys at doses of 1 or 2.5 mg/kg. A single siRNA injection resulted in dose-dependent silencing of ApoB messenger RNA expression in the liver 48 h after administration, with maximal silencing of >90%.

Cationic liposomes: In 2003, Sørensen et al. demonstrated that intraperitoneal injection of cationic liposome (DOTAP)/siRNAs (TNF-α) complex inhibited lipopolysaccharide (LPS)-induced TNF-α gene expression; consequently, the survival rates of mice in LPS-induced septic shock was much improved. As far as the distribution of cationic liposomes/siRNAs is concerned, intravenously injected cationic liposome (AtuFECT01:DphPE, 14.5 mg/kg lipid)/naked Cy3-fluorescence siRNA (1.88 mg/kg) complex was taken up by the vascular endothelium in different organs (heart, lung, liver, and spleen) with a markedly delayed clearance rate compared with naked siRNAs in mice. In heart and lung, fluorescence declined gradually within 2 h. In liver and spleen, on the other hand, fluorescence was retained up to 20 h. In a mouse tumor model, an intravenous cationic liposome (DOTAP)/Alexa 555 labeled siRNA (10 µg) complex showed sporadic presence within tumor tissues, and immunofluorescence studies suggested siRNA accumulation in CD31-positive endothelial cells. Cationic liposome (AtuFECT01:DphPE)/2′-O-methyl siRNAs complex is also predominantly taken up by the endothelial cells of blood vessels in the liver and tumor after intravenous injection into tumor-bearing mice. In antiangiogenic cancer therapy, intravenously injected cationic liposome/2′-O-methyl siRNA (CD31) complex at an siRNA dose of 1.88 mg/kg (injection for 6 consecutive days) produced inhibition of tumor growth in xenograft mouse models.

In a mouse model of liver metastasis, antitumor activity was observed following the intravenous administration of cationic liposomes (LIC-101; 2-O-(2-diethylaminoethyl)-carbamoyl-1, 3-O-dioleylglycerol and egg phosphatidylcholine)/siRNA (Bcl-2) complex at an siRNA dose of 10 mg/kg following two 5-day cycles of daily injections. In a mouse solid tumor model, the growth of subcutaneous inoculated human prostate cancer was inhibited by the intravenous injection of cationic cardiolipin liposome/siRNA (Raf-1) complex at an siRNAs dose of 7.5 mg/kg in two 5-day cycles of daily injections.

In the mouse kidney, Hassen et al. reported that intravenously injected cationic liposome (DOTAP)/siRNA (vasopressin V2 receptors) at an siRNA dose of 3.6 nmol (~50 µg) could reduce functional expression of the vasopressin V2 receptors. Western blot analysis revealed the presence of vasopressin V2 receptors in the mouse inner medulla.

Galactosylated liposomes: Hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them in the cell interior. In order to achieve liver parenchymal cell-specific gene transfection, the galactose moiety is introduced into either cationic polymers or cationic liposomes. Recently, Sato et al. reported that endogenous gene (Ubc13 gene) expression in the liver was inhibited by 80% when siRNAs (Ubc-13) complexed with galactosylated liposomes (Gal-C4-Chol: DOPE) were administered to mice intravenously at a dose of 0.29 nmol/g. The schemes of targeted siRNA delivery to hepatocytes by galactosylated liposomes are shown in Fig. 4.

Immune Responses of siRNAs

It is important to evaluate the immune responses by the application of siRNAs and/or their carrier complex. The activation mechanisms of the mammalian immune system by siRNA are well reviewed by Marques and Williams. Cytokine responses might cause side effects while, in contrast, activation of the immune system by siRNAs (its carrier complex) might actually be useful for the treatment of viral infections and tumors.

Naked duplex siRNAs: In general, siRNA duplexes (generally 19–21 base pairs) are thought to be short enough to bypass dsRNA-induced non-specific immune responses in vertebrate cells. Heidel et al. reported that the lack of IL-12 and IFN-expression in mice suggests that the synthetic siRNAs used in this study do not elicit an observable immune response when naked duplex siRNAs were administered by conventional, hydrodynamic or intraportal methods at an siRNA dose of 2.5 mg/kg.

Cationic liposomes and polymers: However, the development of delivery systems of siRNAs causes activation of the immune system and induces the production of cytokines in vivo. In 2002, Sioud et al. reported that intravenously injected siRNA/cationic liposome (DOTAP) complex (charge ratio (~1:1) varied from 1:2 to 2:1) at an siRNA dose of 5 nmol could activate the immune systems in mice. This observation agrees with the fact that STAT1 in lung and spleen was activated after intravenous injection of an siRNA/cationic liposome (DOTAP) complex (charge ratio (~:+) of 1:4) at an siRNA dose of 50 µg in mice. Cationic polymer, PEI and poly-L-lysine, com-
plexed with siRNAs also activate the immune system in cultured human peripheral blood mononuclear cells.\textsuperscript{74} In contrast, Ge et al. reported that PEI complexed with siRNA (120 \mu g/mouse) at N/P ratio of 5 are not induced the interferon-\alpha in mice.\textsuperscript{48} Further studies might needed the activation of immune systems related to the condition of the formulation, i.e., siRNA dose, N/P ratio of complex, and types of polymer etc.

Sioud et al. reported that siRNA sequence-dependent activation of the immune system by siRNAs/cationic liposomes (DOTAP) complex in cultured adherent peripheral blood mononuclear cells.\textsuperscript{75} As far as the sequences of siRNAs are concerned, the GU-rich sequence of siRNAs might account for the activation of immune systems in mice.\textsuperscript{76} Morrissey et al. synthesized chemically stabilized siRNAs in which all 2'-OH residues were substituted with 2'F, 2'-O-Me, or 2'H residues and suggested that the immune system was activated by siRNAs/lipid particles, and not by chemically modified siRNAs/lipid particles after intravenous injection into mice.\textsuperscript{76} More recently, Judge et al. showed that the immune stimulation by siRNAs can be completely abrogated by selective incorporation of 2'-O-Me G or U into one strand of the siRNA duplex.\textsuperscript{77} These observations strongly suggest that activation of the immune response could be controlled by optimization of the sequence and/or chemical modification of siRNAs.

**Conclusions**

Successful gene therapy by siRNAs depends on the development of efficient delivery systems. Here, we have described recent advances in systemic delivery systems for siRNAs. The immune responses and its controlling factors are also discussed. Since in vivo effects produced by these delivery systems are markedly advanced, efficient gene therapy of a number of refractory diseases using siRNAs could be achieved in the near future.

**References**


1000–1004 (2002).


55) Landen, C. N. Jr., Chavez-Reyes, A., Bucana, C., Schmandt, R., Deavers, M. T., Lopez-Berestein, G. and...


