Delivery of gene silencing agents for breast cancer therapy

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Abstract
The discovery of RNA interference has opened the door for the development of a new class of cancer therapeutics. Small inhibitory RNA oligos are being designed to specifically suppress expression of proteins that are traditionally considered nondruggable, and microRNAs are being evaluated to exert broad control of gene expression for inhibition of tumor growth. Since most naked molecules are not optimized for in vivo applications, the gene silencing agents need to be packaged into delivery vehicles in order to reach the target tissues as their destinations. Thus, the selection of the right delivery vehicles serves as a crucial step in the development of cancer therapeutics. The current review summarizes the status of gene silencing agents in breast cancer and recent development of candidate cancer drugs in clinical trials. Nanotechnology-based delivery vectors for the formulation and packaging of gene silencing agents are also described.

Introduction: challenges in breast cancer treatment
Breast cancer is the most frequently diagnosed malignancy in women. In 2012, an estimated 229,060 new cases of invasive breast cancer and 39,920 cancer deaths were expected in women in the United States [1]. With the availability of modern diagnostic tools and increased use of adjuvant systemic therapies, significant progress has been made on early stage breast cancer treatment, and consequently the overall survival rates in breast cancer patients. However, only marginal improvements have been achieved in patients with relapsed metastatic cancer, making it an urgent medical need to develop new, effective therapeutics to treat late-stage breast cancer.

Small molecule inhibitors targeting selected protein kinases and monoclonal antibodies targeting cell-surface receptors have shown promising results in the fight against cancer, including breast cancer, in the past decade. However, the success stories have been limited to only a handful of drug targets. Many of the key cancer-causing genes are traditionally considered ‘nondruggable’ [2], and thus not enough effort has been dedicated to these genes. Moreover, tumor heterogeneity and genetic instability make it unlikely that a single target will suffice for long-term treatment of most solid tumors.

Ever since its discovery [3], RNA interference has been considered to be capable of rapidly and efficiently knocking down the expression of any gene in any cell type, thus opening a door to treat cancer by targeting every cancer-causing gene. Recent progress in research in gene silencing agents and their delivery systems has shed light on the potential of these therapeutic agents for cancer treatment.

In this review, we will summarize the current status of development of gene silencing agents as breast cancer therapeutics and describe the enabling systems for effective delivery of such therapeutics.

Gene silencing agents in breast cancer

Introduction
Two classes of gene silencing agents have been the focus of intense study in recent years: small interfering RNA (siRNA) and small non-coding microRNA. The siRNA molecule regulates expression of a specific protein via degradation of the mRNA molecule. It usually demands a perfect match between the siRNA oligo and the corresponding sequence in mRNA (Figure 1). On the other hand, microRNA molecules regulate gene expression via suppression of translation. One microRNA molecule often modulates the expression of a group of genes.

Small inhibitory RNA
Although originally discovered as long double-stranded RNA molecules, double-stranded siRNA constructs of 30 nucleotides or less have been the default choice to avoid interferon response from longer molecules [4]. Thousands of siRNA-related research articles have since...
been published that demonstrate the essential roles of the individual genes in cell growth and viability. Key cancer genes have also been identified through screening of siRNA/small hairpin RNA (shRNA) libraries for cell proliferation and survival [5]. These genes are involved in almost all the important signal transduction pathways that control tumor initiation, progression, metastasis, and tumor angiogenesis. Detailed information on the individual genes has been published elsewhere, and is not the scope of this article. It has been estimated that there are about 80 mutations in an individual breast tumor, of which a dozen are driving mutations [6]. Adding to the complexity, every cancer patient carries a unique spectrum of gene mutations, making the pool of mutant genes unimaginably large. While the current small molecule cancer drugs can only impact a very small portion of cancer-causing genes, the availability of the specially designed siRNAs targeting the large number of genes makes it possible for personalized treatment of breast cancer based on the genetic and epigenetic changes of every patient.

Genes and pathways that contribute to resistance to current cancer therapy have also been identified. Trastuzumab has been a key drug to treat Her2-positive breast cancer patients, but not everyone who is positive for Her2 responds to the treatment. In a large scale RNA interference screening with the Her2-overexpressing BT474 breast cancer cell line, Berns and colleagues [7] discovered that loss of PTEN expression caused resistance to trastuzumab treatment. Since PTEN is a negative regulator of the phosphoinositide 3-kinase (PI3K)/AKT pathway, it is speculated that activation of PI3K signaling confers therapy resistance to trastuzumab. To support this notion, it was found that overexpression of PI3K also caused therapy resistance [7]. Another application of siRNA is to sensitize chemotherapy by knocking down expression of multidrug resistant genes in breast cancer cells. Details will be described in the ‘Overcoming therapy resistance’ section below.

microRNA

The microRNAs can be arbitrarily divided into two groups based on their target genes in breast cancer. Table 1 lists microRNAs that play significant roles with known target genes in breast cancer. The group I microRNAs regulate key genes in cancer cell growth and
miR-708
miR-221/222
miR-206
miR-205
miR-200 family
miR-103/107

Dicer
Global microRNA downregulation
[10]

miR-21
PTEN/AKT pathway
Tumor progression and metastasis
[9]

miR-17/20

assays. The excitement and effort in this research field have not been successfully translated into Food and Drug Administration-approved drugs for treatment of human cancers. The fundamental problem with in vivo application of gene silencing agents is the lack of effective carriers for systemic delivery in order to overcome the multiple biological barriers [13]. Once inside the circulation, the therapeutic agent needs to survive attack from plasma ribonucleases. Without effective protection, most double-stranded RNA oligos will be digested within minutes. They will also need to escape elimination by the reticulo-endothelial system (the sinusoids of the liver, the spleen, and the alveolar beds of the lung). Upon reaching the tumor vasculature, the agents will have to fight against the unfavorable tumor interstitial pressure in order to cross the blood vessel wall. Once inside the tumor tissue, they still need to cross the extracellular matrix and bypass the connective tissues before reaching tumor cells. Since the unmodified double-stranded RNA oligos are negatively charged, they cannot pass the cytoplasmic membrane to reach the cytosol where they act. The default choice to facilitate cell entry of siRNA or microRNA is to package them into cationic nanoparticles. Nanotechnology has played a predominant role in the design of various forms of carriers for in vivo delivery of gene silencing agents so far.

### Nanotechnology in breast cancer therapy

Interestingly, nanotechnology has been used in breast cancer therapy for decades. Doxil, the liposomal formulation of doxorubicin, is the first nano-drug approved for breast cancer therapy; doxorubicin is a potent anticancer drug for breast cancer treatment. However, it tends to accumulate in the heart, thus causing severe cardiac side effects. Nano-formulation of doxorubicin dramatically reduced cardiac side effects [14] while preserving or even enhancing the therapeutic effect of the active drug [15]. Abraxane, the nanoparticle albumin-bound paclitaxel, is another success story of nanotechnology in breast cancer therapy. Paclitaxel has been widely used to treat multiple cancer types, including breast cancer. Traditionally, paclitaxel has been formulated in Cremophor® EL since the

### Systemic delivery of gene silencing agents for breast cancer therapy

**Challenges in delivery of therapeutic agents**

Most studies on the biological functions of gene silencing agents have so far been performed using cell-based assays. The excitement and effort in this research field have not been successfully translated into Food and Drug Administration-approved drugs for treatment of human cancers. The fundamental problem with in vivo application of gene silencing agents is the lack of effective carriers for systemic delivery in order to overcome the multiple biological barriers [13]. Once inside the circulation, the therapeutic agent needs to survive attack from plasma ribonucleases. Without effective protection, most double-stranded RNA oligos will be digested within minutes. They will also need to escape elimination by the reticulo-endothelial system (the sinusoids of the liver, the spleen, and the alveolar beds of the lung). Upon reaching the tumor vasculature, the agents will have to fight against the unfavorable tumor interstitial pressure in order to cross the blood vessel wall. Once inside the tumor tissue, they still need to cross the extracellular matrix and bypass the connective tissues before reaching tumor cells. Since the unmodified double-stranded RNA oligos are negatively charged, they cannot pass the cytoplasmic membrane to reach the cytosol where they act. The default choice to facilitate cell entry of siRNA or microRNA is to package them into cationic nanoparticles. Nanotechnology has played a predominant role in the design of various forms of carriers for in vivo delivery of gene silencing agents so far.

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active drug is hydrophobic. However, this solvent itself can cause severe side effects, such as allergic reactions and neutropenia. Packaging of paclitaxel into the 130 nm albumin-bound formulation allows for a 50% increase in drug dosage as a result of decreased overall toxicity compared to the solvent-based formulation [16].

**Nanotechnology-based delivery systems for gene silencing agents**

Multiple technology platforms have been developed to deliver gene silencing agents for cancer therapy. The most common approach is to package the double-stranded RNA into nanoparticles that are less than 200 nm in diameter. This approach takes advantage of the enhanced permeability and retention effect of the leaky tumor vasculature [17]. Since the tumor blood vessel endothelium is disorganized with gaps ranging from 100 to 500 nm at the cell junction [18], the nanoparticles can easily cross the fenestration to reach tumor interstitium (Figure 2). Based on the nature of the packaging material, this group of delivery carriers can be divided into lipid-based and non-lipid-based nanovectors. The lipid-based nanovectors include liposomes [19], stable nucleic acid lipid particles (SNALPs) [20], and lipidoid nanoparticles [21]. Non-lipid-based nanovectors contain chitosan [22], poly(amine amine) dendrimers [23], polyethyleneimines [24], or other polymeric materials as the building blocks. Conjugates composed of lipid-polymers or polymer-polymers have also been frequently used for siRNA delivery [25]. Another commonly used siRNA carrier is gold nanoparticles. In addition, nanoparticles free of any added materials except the siRNA building block itself have also been reported [26]. Depending on whether there are targeting moieties on the surface of the particles, these delivery carriers can also be divided into active targeting and passive targeting vectors (Figure 2). A few products that are currently in clinical trial are introduced below.

**RNA delivery by passive targeting**

siRNA oligos have taken the lead to reach clinical trials for cancer therapy. Most of the current candidate drugs are formulated into lipid-based nanovectors without active targeting moieties on the surface. Both TKM-PLK-1 from Tekmira Pharmaceuticals and ALN-VSP02 from Aplylam Pharmaceuticals use SNALPs as the delivery vector. The SNALPs consist of the ionizable cationic lipid 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) as the core lipid component. They have a high siRNA encapsulation capacity with a small and uniform size. TKM-PLK-1 consists of a polo-like kinase 1 (PLK1)-specific siRNA packaged in SNALPs. PLK1 is involved in cell cycle progression, and targeted delivery of PLK1 siRNA suppresses growth and metastasis of Her2+ breast cancer in orthotopic xenograft models [27]. ALN-VSP2 is the first product with dual targeting agents, consisting of siRNA oligos specific to the vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) [28]. It is hypothesized that knocking down both genes in the same cancer cells might have an additive or even synergistic effect on cell growth and viability. New cationic lipid molecules have recently been identified to prepare more effective SNALP for siRNA delivery [29]. It is anticipated that more gene silencing products based on SNALP will be developed in the coming years. Since SNALP tends to accumulate in the liver, the main indication of these drugs is to treat liver cancer or liver metastasis of other solid cancers. Atu027 from Silence Therapeutics is composed of a protein kinase N3 (PKN3)-specific siRNA in positively charged liposomes prepared with a mixture of cationic and fusogenic lipids [30]. PKN3 is a downstream effector of the PI3K pathway, one of the most important pathways in breast cancer biology. Knockdown of PKN3 impairs growth of primary breast and prostate tumor, and blocks tumor metastasis [31]. A phase I trial with advanced solid cancer has been completed with this agent [32].

siRNA-EphA2-DOPC is formulated by mixing the EphA2 siRNA with the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine in an excessive amount of t-butanol followed by lyophilization [33]. EphA2 encodes the ephrin receptor tyrosine kinase that is overexpressed in multiple cancer types, including breast cancer. Blocking EphA2 activity by antibody-drug conjugates or through knockdown of gene expression inhibits tumor growth [33]. Additionally, dual targeting of EphA2 and other cancer genes has shown enhanced tumor growth inhibition [34]. Although most of the initial studies have been focused on ovarian cancer, the disease indication for the current clinical trial includes all solid tumors [35].

**RNA delivery by active targeting**

CALAA-01 from Calando Pharmaceutics is not only the first siRNA therapeutic in a human cancer clinical trial but is also formulated with active targeting [36]. The siRNA specific for the M2 subunit of ribonucleotide reductase (RRM2) is encapsulated in a cyclodextrin nanoparticle. An affinity moiety of the human transferrin protein targeting ligand is decorated on the surface of the nanoparticle. It has been well documented that the transferring receptor protein is overexpressed on the surface of cancer cells, and can be used for effective tumor targeting [37]. The 70 nm nanoparticles are small enough to cross the fenestration following systemic administration. Once inside the tumor tissue, the targeting moiety would direct the nanoparticle to tumor cells with overexpressed transferring receptor. The first-in-human cancer trial has demonstrated accumulation of RRM2
siRNA in tumor tissues and gene-specific knockdown of expression [36]. Although the clinical trial was carried out with melanoma patients, there is no reason to believe that CALAA-01 cannot be used to treat other cancer types such as breast cancer given that RRM2 controls DNA synthesis and damage repair during the cell cycle.

**Multistage vector for RNA delivery**

The multistage vector (MSV) delivery system was designed to maximize tumor delivery of therapeutic agents through sequential negotiation with the biological barriers [13]. The system consists of a first stage nanoporous silicon microparticle and a second stage nanoparticle loaded into the nanopores of the first stage particle. For delivery of gene silencing agents, the double-stranded RNA molecules are packaged into 30 to 40 nm liposomes that are then loaded into the 60 to 80 nm pores of the porous silicon [38,39]. Once inside the bloodstream, the first stage particles travel with the blood flow and settle at tumor vasculature, where the liposomal siRNAs are released. The pace of siRNA release is determined by the diameter of the nanopore, the size of the liposome, and the rate of silicon degradation.

The first stage microparticles are designed based on size, shape, and surface chemical properties to achieve maximal tumor enrichment. The hemispherical and discoidal particles are more effective in adhesion to tumor vasculature than particles with other shapes such as spherical and cylindrical [40]. The size of the microparticle is a major determinant of particle accumulation. The 1 μm discoidal particles accumulate more than the sub-micrometer or the 3.2 μm particles with the same shape in melanoma tissues [40]. Interestingly, the size of the particle also affects the efficiency of affinity targeting. Surface conjugation of the RDG targeting moiety significantly enhances tumor accumulation of the sub-micrometer particles, but has minimum impact once the size exceeds 1 μm [40]. Surface chemical modification not only affects protein binding but also determines the loading efficiency of the second stage particles into the nanopores. Since the liposomal siRNA carries a negative Zeta potential, the surface of the nanopores is modified with polyamine to facilitate loading of nanoparticles [41].

This system has been successfully applied to deliver siRNA for cancer treatment with experimental tumor models [38,39,42]. Treatment of tumor mice with one dose of MSV/EphA2 siRNA resulted in knockdown of EphA2 expression for up to 3 weeks due to sustained release of liposomal siRNA [39]. It is suspected that the MSV/siRNA in tumor vasculature and other organs serves as a depot for constant supply of the gene-silencing agent [43]. In a recent study, Xu and colleagues [42] treated an orthotopic model of MDA-MB-231 primary tumor with siRNA targeting the *ATM* gene delivered in the MSV. Effective knockdown of *ATM* expression resulted in dramatic inhibition of tumor growth. Since efficacy and toxicity constitute the two major aspects of siRNA therapeutics, they also carried out studies to systematically evaluate toxicity that might have been caused by MSV/ATM. After careful evaluation, it was determined that no acute immunotoxicity or sub-acute toxicity was associated with MSV/ATM siRNA, paving the pathway for development of MSV/ATM siRNA as a therapeutic agent for breast cancer [42].
Overcoming therapy resistance

One area of siRNA therapeutics that has shown great promise is sensitization to chemotherapy. Overexpression of multidrug-resistant genes has been attributed to chemoresistance. MacDiarmid and colleagues [44] used a short hairpin RNA to knock down expression of the MDR1 gene in vivo, and subsequently treated murine models of human cancers with chemotherapy drugs. Navarro and colleagues [25] synthesized a DOPE-PEI (dioleoylphosphate-diyethanolamine-polyethylenimine) conjugate to enhance transfection capacity of the low molecular weight PEI. They used the conjugate to deliver MDR1-specific siRNA, and demonstrated sensitization to doxorubicin treatment of the otherwise resistant MCF-7 cells. We have recently demonstrated sensitization of docetaxel treatment by EphA2 siRNA in a murine model of human ovarian cancer [38].

Another area that has gained increasing interest in breast cancer therapy is the target of cancer stem cells. These cells are resistant to conventional chemotherapy, and are the lethal seeds for tumor recurrence and local and distant metastasis [45]. By comparing differential expression between the bulk of cancer cells and cancer stem cells, we have identified a group of candidate genes that might be essential for growth and survival of cancer stem cells [46]. Our recent experience with MSV delivery of cancer stem cell gene-specific siRNA oligos has resulted in the development of new breast cancer therapeutics. These agents are expected to play a significant role in the fight against breast cancer.

Conclusion and perspectives

Gene silencing agents will continue to contribute significantly to breast oncology treatment. Recent advances in RNA interference have resulted in the development of multiple candidate siRNA therapeutics being evaluated in the clinic. Non-coding microRNAs will follow suit to be added to the candidate drug list soon. On the other hand, development of delivery vectors for most solid tumor types has lagged. There are not many options available to deliver siRNA/microRNA to primary breast cancer. It is ever harder to deliver therapeutics to distant organs of breast cancer metastasis, such as the brain and bone. More effort should be spent on the design and development of tissue-specific and tumor type-specific delivery systems for siRNAs and microRNAs.

Abbreviations

DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; MSV, multistage vector; PEI, polyethylenimine; PI3K, phosphoinositide 3-kinase; PKN3, protein kinase N3; PLK, polo-like kinase; shRNA, small hairpin RNA; siRNA, small interfering RNA; SNALP, stable nucleic acid lipid particle.

Competing interests

MF is the founding scientist and a member of the Board of Directors of Leonardo Biosystems, and a member of the Board of Directors of ArrowHead Research Corporation, and hereby discloses potential financial interests in the companies. The other authors declare that they have no potential conflicts of interest.

Acknowledgements

The authors acknowledge financial support from the following sources: Department of Defense grants DODW81XWH-09-1-0212 and DODW81XWH-12-1-0414; National Institute of Health grants NIH RO1CA128797, NIH RO1CA138197, NIH U54CA143837, NIH U54CA149196, NIH U54CA151668; Breast Cancer Research Foundation; Cancer Prevention Research Institute of Texas RP121071; Kenneth Promise for the Cure KG081694; Golfer’s Against Cancer, Team Tiaa, Causes for a Cure.

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Published: 8 May 2013

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doi:10.1186/bcr3413
Cite this article as: Shen H, et al.: Delivery of gene silencing agents for breast cancer therapy. Breast Cancer Research 2013, 15:205.
Figure 1

A

Cytoplasm

aaa → mRNA degradation

B

aaa → suppression of protein synthesis

Target gene

siRNA

miRNA
Figure 2

(A) Nanotherapeutics
(B) Nanotherapeutics with affinity moiety