1 Introduction: Big potential for small interfering RNA in cancer therapy

1.1 Small interfering RNAs: A new class of cancer therapeutics

Significant progress has been made in the war on cancer, evidenced by the decline in cancer-related deaths in the US. According to the National Cancer Institute, approximately 65% of patients diagnosed with cancer will live longer than 5 years (1). These numbers are expected to improve even more with the advent of novel molecularly targeted cancer therapies. The traditional approach to cancer chemotherapy involves injecting highly toxic drugs to interfere with cell division, thereby killing the rapidly dividing cancer cells. However, this non-specific killing of rapidly dividing cells also destroys non-cancerous cells such as those in the bone marrow, leading to potentially serious side effects and providing a very narrow therapeutic index. As a result of an increased understanding of the molecular mechanisms underlying cancer development and progression, a new generation of cancer therapeutics are being developed to specifically interfere with molecules that are responsible for driving the growth and survival of the cancer cells (2). These molecularly targeted therapies have the potential to exert much more selective killing of cancer cells and may substantially reduce the often serious complications associated with current cancer treatments.

Small interfering RNA (siRNA) molecules are a promising new class of therapeutic agents that are perfectly suited for molecularly targeted cancer therapy. The siRNA molecules are double-stranded nucleic acids approximately 19-21 bp in length that act as the effectors of RNA interference (RNAi), a naturally occurring mechanism for post-transcriptional gene silencing (3). siRNAs interact with their cognate mRNAs through

Watson-Crick base pairing and subsequently trigger degradation of the target mRNAs in a sequence-specific fashion. The consequence of the mRNA degradation is a reduction in protein expression. This mechanism can be exploited therapeutically to inhibit the expression of a wide variety of disease-associated targets (4,5). Furthermore, because the RNAi mechanism results in sequence-specific mRNA degradation, it has the potential to help realize the goal of developing novel cancer therapies that specifically attack cancer cells while minimizing the effect on normal healthy cells.

1.2 Opportunities for siRNA in cancer therapy

Cancer is the number one cause of death for people under age 65 in the United States, accounting for nearly 25% of all deaths in 2001 (6). It is predicted that half of all men and a third of all women in the United States will develop cancer at some point in their lifetimes. According to the World Health Organization, death rates from cancer are expected to increase by 104% worldwide by 2020 (7). Needless to say, the war on cancer is still raging and the development of more-effective and less-toxic cancer therapeutics is urgently needed. siRNA molecules have the potential to revolutionize cancer therapy by providing highly potent and specific cancer cell killing with drastically reduced side effects. Some of the most promising targets for siRNA-based cancer therapy involve oncogenic fusion proteins resulting from chromosomal translocations, overexpressed or mutated oncogenes, or molecules controlling cell survival or division (8).

1.2.1 Targeting chromosomal translocations

In human cells, genetic information is stored in DNA molecules that are assembled into 23 pairs of protein:nucleic acid complexes called chromosomes. Typically, these chromosomal structures act to compact the DNA (which is nearly 3

meters long end to end), protect it from degradation or damage, help to control when particular genes are transcribed, and ensure proper replication and distribution of the genetic code during cell division. Sometimes, however, these chromosomal structures can be disrupted by rearrangements that cause part of one chromosome to break off and attach to a different chromosome, a process called translocation. Transcription factors and kinases involved in cell signaling, cycling, and death are common targets of the chromosomal translocations that can lead to cancer. This chromosomal rearrangement can result in the production of an oncogenic fusion protein or it can place a gene under the control of a new promoter; in either case, the product can cause a cell to become cancerous through processes such as overexpression of certain proteins and/or constitutive activation of cellular processes.

Several translocation breakpoints have been implicated in specific cancers including leukemias and lymphomas. One of the most notable cases of chromosomal translocation is the t(9:22) that leads to the altered Philadelphia chromosome 22 in chronic myelogenous leukemia (CML). This leads to production of the Bcr-Abl fusion protein that acts as an unregulated protein tyrosine kinase and is involved in neoplastic transformation. In 2001, Novartis received FDA approval for Gleevec (imatinib mesylate, STI 571), a small-molecular inhibitor of this Bcr-Abl fusion protein. Gleevec blocks the ATP-binding pocket of the tyrosine kinase domain of the fusion protein, thereby blocking its kinase activity. This method of specifically targeting the fusion product represents a powerful new technique for the treatment of cancers caused by chromosomal translocations.

RNA interference is a promising therapy for these cancers because it can specifically target and degrade the mRNA transcript corresponding to these aberrant fusion genes. Instead of blocking the action of the fusion protein itself, as performed by Gleevec, RNAi would degrade the transcript before translation could occur. Additionally, because RNAi is highly specific (even a few mismatches can abrogate function), it can target degradation of these unwanted fusion transcripts while leaving the normal versions untouched. This means that degradation will only occur in cancer cells where this fusion transcript is produced.

Another type of cancer characterized by a chromosomal translocation, t(11:22), is the Ewing's family of tumors (EFT), a poorly differentiated mesenchymal malignancy that arises in bone or soft tissue. It is the second most common primary osseous malignancy in childhood and adolescence (9). The translocation t(11;22) is commonly detected in EFT and produces the chimeric EWS-FLI1 fusion gene found in 85% of EFT patients(10). The EWS domain replaces the normal transcriptional activator domain in the 5' region of the FLI1 DNA-binding protein, leading to altered transcriptional activation that contributes to the tumorigenic phenotype (9). Reduction of the EWS-FLI1 protein in EFT cells in vitro or in subcutaneous xenograft tumors by antisense oligonucleotides complementary to EWS-FLI1 mRNA results in decreased proliferation (11-13), suggesting a potential therapeutic intervention directed at this tumor-specific chimeric gene. Small interfering RNAs (siRNAs) have recently been shown to silence the EWS-FLI1 gene and suppress proliferation of an EFT cell line in vitro (14-16).

1.2.2 Reducing the expression of overexpressed or mutated oncogenes

While fusion genes such as EWS-FLI1 represent ideal targets for RNAi because the sequence-specific degradation will only occur in cells expressing the fusion transcript, RNAi can also be used to treat cancers characterized by gene amplification and overexpression. Gene amplification can result when chromosomal replication goes awry, leading to the production of multiple copies of certain regions in the chromosomes. This can lead to the cancerous state if an oncogene such as K-ras, myc, or HER2/neu is included in this amplified region. Cancer cells may also have amplification of the multiple drug resistance (mdr) genes, causing them to develop resistance to many chemotherapeutic drugs. In 1998, Genentech received FDA approval for its drug Herceptin, a monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), for the treatment of advanced-stage breast cancer. While antibodies represent a promising therapy for targeting cancer-specific molecules like the HER2 protein, RNA interference also holds promise as an effective therapy because of its ability to specifically degrade the transcripts of these amplified oncogenes before the proteins are produced. A recent in vitro study by Faltos et al. demonstrated the use of RNAi for sequence-specific decrease in HER2/neu mRNA and protein levels, leading to antiproliferative and apoptotic responses in cells overexpressing HER2/neu (17). This indicates that RNAi therapy may be a feasible treatment for cancers that are characterized by overexpression of certain genes.

Another promising target for siRNA-based cancer therapy is the mutated K-ras gene found in over 85% of pancreatic cancers. Currently, the five-year survival rate of pancreatic cancer patients is only 4.4% (1). One of the reasons that this type of cancer is so deadly is that it has the ability to infiltrate nearby tissue and metastasize at an early

stage. New treatment modalities are required to treat the advanced stages of this disease. On a positive note, much progress has been made with respect to the molecular basis of pancreatic cancers, revealing the prevalence of the mutated K-ras gene. Ras proteins are GTPases that participate in signal transduction from growth factor receptors on the cell surface; a point mutation in this gene can lead to its constitutive activation that causes the cell to continuously receive a signal for proliferation. Recent studies have shown that reduction of K-ras levels in pancreatic cancer tumors leads to loss of anchorage-dependent growth and tumorigenesis (18,19).

1.2.3 Controlling cell survival and death

One of the hallmarks of cancer cells is their ability to avoid the normal regulatory signals that control cell growth and death (20). Although normal cell growth and division is characterized by a finely tuned balance between cell division and apoptotic cell death, the ability of cancer cells to perturb this steady-state allows them to accumulate and develop into invasive tumors. Anti-apoptotic proteins such as bcl-2 and survivin are overexpressed in many cancer cells. The Bcl-2 protein helps govern mitochondrial death signaling, a key step in the apoptotic mechanism. Inhibition of bcl-2 expression using siRNA can lead to apoptosis in vitro and slow the growth of tumor xenografts (21,22). Likewise, survivin has been show to help regulate cell death mechanisms by interacting with caspases and also helping control mitotic spindle formation. Survivin represents a promising target for molecularly targeted therapies since it is upregulated in many cancers but minimally expressed in normal tissues (8). Survivin inhibition by siRNAs can lead to cell arrest in the G2/M phase and inhibition of clonogenic survival of cancer

cells (23). In other cancer types, siRNA directed against survivin can induce apoptosis and significantly inhibit the growth of xenograft tumors in vivo (24).

Other possible targets are proteins that are necessary for processes involved in cell division. Although these molecular targets are not necessarily unique to cancer cells, they are often overexpressed in rapidly dividing cancerous cells with minimal expression in non-mitotic cells. Ribonucleotide reductase is an attractive target for cancer therapies since it catalyzes the reduction of ribonucleotides into deoxyribonucleotides necessary for DNA replication and repair. Several potent siRNA inhibitors of the M2 subunit of RNR (RRM2) have been identified, and these siRNAs have demonstrated the ability to inhibit the growth of tumor cell lines after transfection in vitro and transplantation into mice (25). A recent study by Avolio et al. also demonstrated the in vitro and in vivo efficacy of an siRNA targeting ribonucleotide reductase (26).

1.3 The challenge of systemic siRNA delivery

Despite the promises and hype surrounding siRNA therapeutics for cancer, the clinical realization of siRNA therapeutics faces several significant hurdles, foremost of which may be a safe and effective delivery method (27). Naked siRNA molecules are rapidly degraded by nucleases present in the bloodstream, and their small size leads to first-pass renal clearance (4). Chemically modified siRNAs can be designed to maintain functional efficacy while increasing their stability against nuclease degradation. Furthermore, attachment of specific targeting ligands can induce binding to protein carriers or uptake by the desired population of cells to be treated. For example, siRNA conjugated to targeting ligands such as cholesterol and antibodies have shown efficacy both in vitro and in vivo (28,29). While these methods for nuclease stabilization and

covalent attachment of targeting ligands are promising, nanoparticle-mediated delivery methods may provide functions not achievable with naked nucleic acids or direct attachment to targeting moieties.

The design of nanoparticle carriers for systemic siRNA delivery aptly highlights the challenges and intricacies associated with attempts to manipulate biological systems. Many times, therapeutic interventions thought to act through a certain mechanism may turn out to achieve the effect through an entirely different mechanism. Additionally, modifications designed to overcome a certain problem may only reveal a still more challenging barrier to success.

Surface decoration with hydrophilic polymers such as polyethylene glycol (PEG) has been used to minimize uptake by the reticuloendothelial system (RES) and stabilize nanoparticles against aggregation in physiological environments (30,31). Moreover, addition of targeting ligands to the surface helps to increase uptake of the injected nanoparticles by a specific cell type such as tumor cells (32). However, recent studies have demonstrated that these modifications do not necessarily achieve the expected results after systemic delivery. For example, polycationic nucleic acid carriers, even when coated with PEG for stabilization, exhibit extremely rapid clearance from the bloodstream after intravenous administration (33). Studies have also indicated that addition of tumor-specific targeting ligands to the surface of the nanoparticles does not increase the amount of the injected dose that reaches the tumor compared to non-targeted nanoparticles. Nevertheless, the targeted nanoparticles show significantly greater efficacy in terms of gene expression (plasmid DNA delivery) or target gene knockdown leading to tumor growth inhibition (siRNA delivery) (34,35). It is hypothesized that the

targeting ligands do not necessarily enhance the tumor localization of the injected nanoparticles, but instead act to enhance the internalization by the tumor cells once the nanoparticles achieve tumor localization.

There exists the potential that the short circulation times of these nanoparticle carriers could limit the potential differences that might arise between targeted and non-targeted forms if circulation times were extended. Longer circulation times have been achieved for nanoparticles that are cross-linked after formation, but irreversible cross-linking will inhibit the release of the payload after cell internalization. The use of reversible cross-linking systems that can respond to the reducing environment inside a cell represents a clever approach to the design of nanoparticle carriers that can be stable for prolonged circulation in the blood yet willingly release the payload when inside the cell (36).

Even if the nanoparticles do navigate the complex milieu of the bloodstream and begin to be internalized by the target cells of interest, a completely new set of barriers exist to potentially block therapeutic efficacy. Upon internalization, the nanoparticles must escape the vesicular compartment in which they were internalized. Smart polymer systems help address the barrier of endosomal escape by responding to changes in pH in the endosomes, leading to nucleic acid release and endosomal disruption (37,38). If the delivered therapeutic molecule exerts its effect in the cytosol (e.g., siRNA), then it has reached its site of action. However, many therapeutic molecules (e.g., plasmid DNA) must reach the nucleus to have their effect. This requires intracellular trafficking to the nuclear compartment, a process that can be severely diffusion-limited but may be aided

by the use of nuclear localization signals or peptides designed to harness the dynein motor complex (39,40).

1.4 An engineering approach for the design of siRNA therapeutics

An engineer desires to not only learn how a given system works but also how it can be manipulated to achieve a desired goal. Oftentimes, this is accomplished by dismantling the system and analyzing the component systems individually in a methodical and quantitative fashion. Once the nature and function of these component systems is sufficiently understood, they can be assembled into systems with higher levels of complexity that possess properties suitable for their intended application. In this way an engineer does not act as a passive observer, but instead actively seeks ways to apply new knowledge and improve existing technologies. This thesis describes an engineering approach to address the challenge of systemic delivery of small interfering RNA (siRNA) molecules for cancer therapy.

The analysis begins at the molecular level with an attempt to understand the properties and function of individual siRNA molecules. Chapters 2 and 3 focus on the behavior of siRNA molecules in vitro and in vivo, with a specific emphasis on understanding factors governing the magnitude and persistence of the inhibition after siRNA treatment. The results demonstrate that the rate of cell division is one of the most important factors governing the activity of siRNAs, and therapies targeting rapidly dividing cells will require different dosing schedules than therapies targeting non-mitotic cell populations.

Chapter 4 addresses the next level of complexity when these siRNA molecules are assembled into nanoparticles using cyclodextrin-containing polycations (CDP). The

siRNA and CDP self-assemble to yield macromolecular nanoparticles with distinct properties that emerge from the interactions between the individual components within the assembled system. Extensive physicochemical and biological characterization of these siRNA nanoparticles reveals how their properties can be tuned to make them suitable for systemic delivery of siRNA in vivo.

Chapters 5 through 7 examine the in vivo behavior and function of the siRNA nanoparticles. In Chapter 5, noninvasive live-animal imaging with positron emission tomography (PET) and bioluminescent imaging (BLI) is used to monitor the in vivo biodistribution and function of the siRNA nanoparticles in mice, providing important insights into the behavior of these nanoparticles inside a living organism. In Chapters 6 and 7, the nanoparticles are used to deliver therapeutic siRNAs to achieve tumor growth inhibition in disseminated and subcutaneous murine cancer models.

Finally, recommendations for future work in the area of systemic siRNA delivery for cancer therapy are offered in Chapter 8.

1.5 References

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